Characterization of an Isoflavonoid-Specific Prenyltransferase from *Lupinus albus*[1][W][OA]

Guoan Shen, David Huhman, Zhentian Lei, John Snyder, Lloyd W. Sumner, and Richard A. Dixon*

Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401

Prenylated flavonoids and isoflavonoids possess antimicrobial activity against fungal pathogens of plants. However, only a few plant flavonoid and isoflavonoid prenyltransferase genes have been identified to date. In this study, an isoflavonoid prenyltransferase gene, designated as *LaPT1*, was identified from white lupin (*Lupinus albus*). The deduced protein sequence of LaPT1 shared high homologies with known flavonoid and isoflavonoid prenyltransferases. The *LaPT1* gene was mainly expressed in roots, a major site for constitutive accumulation of prenylated isoflavones in white lupin. LaPT1 is predicted to be a membrane-bound protein with nine transmembrane regions and conserved functional domains similar to other flavonoid and isoflavonoid prenyltransferases; it has a predicted chloroplast transit peptide and is plastid localized. A microsomal fraction containing recombinant LaPT1 prenylated the isoflavone genistein at the B-ring 3′ position to produce isowighteone. The enzyme is also active with 2′-hydroxygenistein but has no activity with other flavonoid substrates. The apparent K_m of recombinant LaPT1 for the dimethylallyl diphosphate prenyl donor is in a similar range to that of other flavonoid prenyltransferases, but the apparent catalytic efficiency with genistein is considerably higher. Removal of the transit peptide increased the apparent overall activity but also increased the K_m. *Medicago truncatula* hairy roots expressing *LaPT1* accumulated isowighteone, a compound that is not naturally produced in this species, indicating a strategy for metabolic engineering of novel antimicrobial compounds in legumes.

Prenylated flavonoids and isoflavonoids have long been known to possess unique bioactivities relative to their unmodified parent compounds, particularly potent antimicrobial activity against fungal pathogens (Harborne et al., 1976). This enhanced bioactivity likely results from the presence of the lipophilic prenyl side chain, which facilitates the transmembrane transport of these compounds (Harborne et al., 1976).

The prenylation of flavonoids and isoflavonoids is catalyzed by plant membrane proteins located in plastids (Laflamme et al., 1993; Sasaki et al., 2008; Akashi et al., 2009). However, only a few flavonoid prenyltransferase genes have been identified to date, due to difficulties associated with the study of membrane proteins. These newly identified genes include naringenin 8-prenyltransferase (*SfN8DT-1*), genistein 6-prenyltransferase (*SfG6DT-1*), and isoliquiritigenin dimethylallyltransferase (*SfiLDT*) from *Sophora flavescens* (Sasaki et al., 2008, 2011) and a pterocarpan 4-dimethylallyltransferase (*G4DT*) from soybean (*Glycine max*; Akashi et al., 2009).

In addition to *S. flavescens* and soybean, prenylated isoflavonoids are commonly found in several legume species, for example white lupin (*Lupinus albus*) and other *Lupinus* species (Harborne et al., 1976; Schröder et al., 1979; Tahara et al., 1984, 1989; Gagnon et al., 1992; Katagiri et al., 2000; Bednarek et al., 2001). In contrast to the typical microbially induced synthesis of isoflavonoid defense compounds in many plant species, *Lupinus* species constitutively produce various monoprenylated and diprenylated isoflavonoids, with the major components being genistein derivatives such as wighteone (6-prenylgenistein), isowighteone (3′-prenylgenistein), and lupiwighteone (8-prenylgenistein; Fig. 1), in addition to minor amounts of cyclized pyrano derivatives (Harborne et al., 1976; Schröder et al., 1979; Tahara et al., 1984, 1989; Gagnon et al., 1992; Katagiri et al., 2000; Bednarek et al., 2001). *Lupinus* species, therefore, are good model plants for studying the biosynthesis of prenylated aromatic compounds. The accumulation of such compounds in *Lupinus* species is further increased by exposure to either biotic or abiotic elicitors (Schröder et al., 1979; Shibuya et al., 1992; Gagnon and Ibrahim, 1997). Enzymes responsible for the prenylation reactions have been shown to be membrane bound (Schröder et al., 1979; Laflamme et al., 1993). However, genes encoding lupin isoflavone prenyltransferases have not yet been identified.

We here report the characterization of an isoflavone prenyltransferase from white lupin named LaPT1.

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* Corresponding author; e-mail radixon@noble.org.

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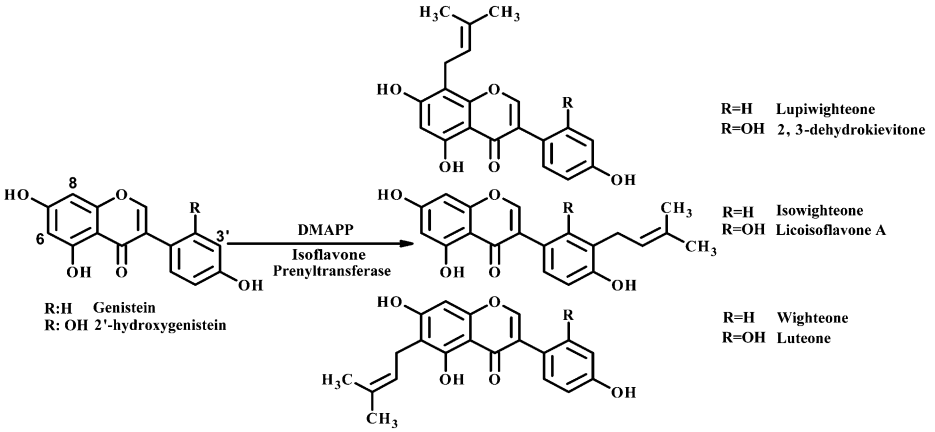

Figure 1. Scheme of major reactions of isoflavone prenylation occurring in white lupin. The compounds shown are the major monoprenylated derivatives of genistein (R = H) and 2′-hydroxygenistein (R = OH).

LaPT1 exhibited close amino acid sequence similarity to other known flavonoid prenyltransferase proteins, and its transcript expression positively correlated with the accumulation of prenylated compounds. A yeast microsomal fraction containing recombinant LaPT1 protein catalyzed the prenylation of genistein at the 3′ position in vitro. The production of the same 3′-prenyl genistein in *Medicago truncatula* hairy roots expressing the *LaPT1* gene provides confirmation of the in vivo activity of the corresponding enzyme and proof of concept for the metabolic engineering of prenylated isoflavonoids.

RESULTS

Identification of a Candidate cDNA Encoding an Isoflavone Prenyltransferase

From a total of about 8,000 white lupin EST sequences obtained from our own EST collection (Tian et al., 2009) and the National Center for Biotechnology Information database, two candidate ESTs were selected based on their sequence similarities with known flavonoid and isoflavonoid prenyltransferases. One EST (FG092841), obtained from developing roots of white lupin 'Netherland' (Tian et al., 2009), shared 35.7% and 35.7% sequence identities with SfN8DT-1 and G4DT, respectively, but higher sequence identity (96.2%) with VET2-2 (soybean homogentisate phytyltransferase). However, the expression profile of this gene did not show any obvious association with the accumulation of prenylated compounds in lupin; therefore, the gene is more likely related to the biosynthesis of vitamin E or plastoquinone that mainly occurs in the aerial parts of plants (Venkatesh et al., 2006).

Another candidate EST (FF836984) originated from a phosphate-depleted preemergent cluster root library of white lupin 'Ultra.' This EST showed 50.4% and 40.8% identities with SfN8DT-1 and G4DT, respectively, but lower identity (17.8%) with VET2-2. The complete sequence was obtained by 3′ RACE using oligo(dT)-primed cDNA. The 1,637-bp full-length cDNA (GenBank accession no. JN228254) contained a 64-bp 5′ untranslated region, a 346-bp 3′ untranslated region with a poly(A) tail, and an open reading frame of 1,227 nucleotides encoding a putative polypeptide of 408 amino acids, which shared 57.5% and 45.9% identities with SfN8DT-1 and G4DT at the amino acid level, respectively (Fig. 2A). We designated this gene as *LaPT1*.

Prediction of transmembrane domains using TMHMM 2.0 (http://www.cbs.dtu.dk) indicated that LaPT1, like SfN8DT-1 and G4DT, has nine putative transmembrane domains with two long extracellular loops between domains 2 and 3 and domains 6 and 7 (Fig. 2B). Two characteristic sequence motifs were present in these two large loops, the conserved prenyltransferase motifs NQxxDxxxD and KD(I/L)xDx(E/D)GD common to both flavonoid and homogentisate prenyltransferases (Sasaki et al., 2008; Akashi et al., 2009; Fig. 2A). The LaPT1 polypeptide possesses a putative transit peptide sequence for targeting to the chloroplast, as predicted by ChloroP 1.1 (www.cbs.dtu.dk/services/ChloroP/), TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP), and SignalP-HMM (http://www.cbs.dtu.dk/services/SignalP-2.0/). The predicted transit peptide is 17 amino acids in length, which is shorter than that of G4DT (44 amino acids; Sasaki et al., 2008). LaPT1 was predicted to be a basic protein with a predicted theoretical pI of 9.40 (http://www.expasy.org/tools/pi_tool.html), typical of chloroplast envelope proteins, which often have unusually high pI values of more than 8.8 (Ferro et al., 2002). These data suggest that LaPT1 is a membrane-bound protein localized in plastids.

Phylogenetic Analysis of LaPT1

A neighbor-joining phylogenetic tree was constructed to analyze the evolutionary relationship of LaPT1 with other prenyltransferases using the deduced amino acid sequences of *LaPT1*, flavonoid and

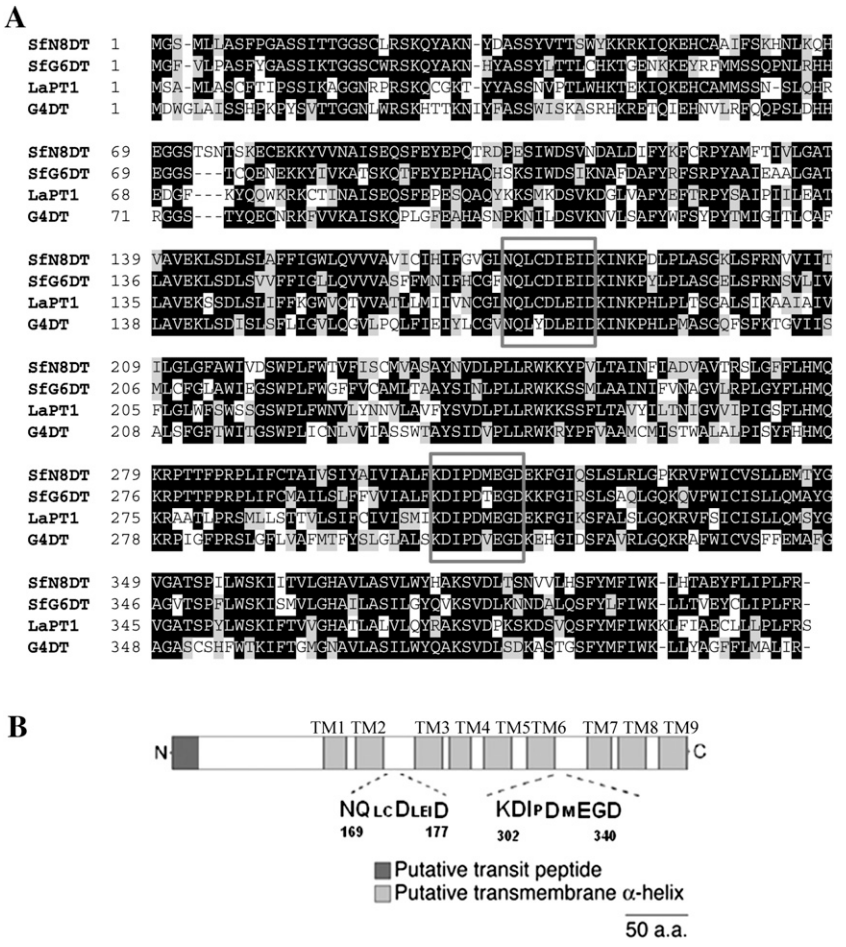

Figure 2. Primary structures of flavonoid prenyltransferases. A, Alignment of LaPT1 with *S. flavescens* SfN8DT-1 (GenBank accession no. AB325579), *S. flavescens* SfN6DT (GenBank accession no. BAK52291), and soybean G4DT (GenBank accession no. AB434690) sequences. The two conserved NQxxDxxID and KDI/LxDxE/DGD motifs are boxed. Identical amino acids are shown in white on a black background, and similar residues are marked in white on a gray background. B, Organization of the transmembrane domains in LaPT1. a.a., Amino acids.

isoflavonoid prenyltransferases from *S. flavescens* and soybean, and other related prenyltransferases involved in vitamin E and plastoquinone biosynthesis in plants (Fig. 3). LaPT1 was grouped into the same clade as SfN8DT-1, SfG6DT, and G4DT and was clearly separated from the prenyltransferases involved in vitamin E and plastoquinone biosynthesis.

Expression Profiling of *LaPT1* in White Lupin

Because the *LaPT1* EST sequence was initially obtained from phosphate-deficient roots, we determined whether the expression of this gene is root specific and inducible under phosphate deficiency. Quantitative real time (qRT)-PCR analysis indicated that *LaPT1* transcripts are expressed in young shoots and pod walls, but the transcript levels are extremely low (Fig. 4A). The level of *LaPT1* transcripts was 3 orders of magnitude higher in roots than in aerial parts (Fig. 4A). *LaPT1* expression was almost undetectable in immature seeds (Fig. 4A).

As expected, phosphate deficiency significantly changed white lupin root morphology and induced large numbers of cluster roots, but qRT-RCR analysis did not show significant induction of *LaPT1* expression by phosphate deficiency (Fig. 4B).

Functional Characterization of Recombinant LaPT1 Protein in Vitro

A chloroplast transit peptide of 17 amino acid residues was predicted in LaPT1, but as the actual length of the transit peptide has not been experimentally determined, we generated two truncated forms, LaPT1$\Delta_{1\text{-}17}$ and LaPT1$\Delta_{1\text{-}44}$ (with 17 and 44 amino acid deletions from the N terminus, based on the predictions of the transit peptides for LaPT1 and G4DT, respectively), together with the full-length cDNA, for expression in the yeast W303A1 strain in order to determine whether the transit peptide region inhibits the enzymatic activity of LaPT1. Microsomal pellets containing recombinant protein were isolated by ultracentrifugation and tested for prenyltransferase activity using genistein as prenyl acceptor and dimethylallyl diphosphate (DMAPP) as prenyl donor. Genistein is the major precursor of prenylated isoflavones in white lupin (Fig. 1). Products were extracted with ethyl acetate and analyzed using reverse-phase HPLC.

All three recombinant proteins efficiently utilized genistein as a prenyl acceptor (Fig. 5A; Supplemental Fig. S1). Interestingly, microsomal extracts containing LaPT1$\Delta_{1\text{-}17}$ showed similar activity levels to microsomal extracts containing the full-length protein, whereas microsomal extracts containing LaPT1$\Delta_{1\text{-}44}$

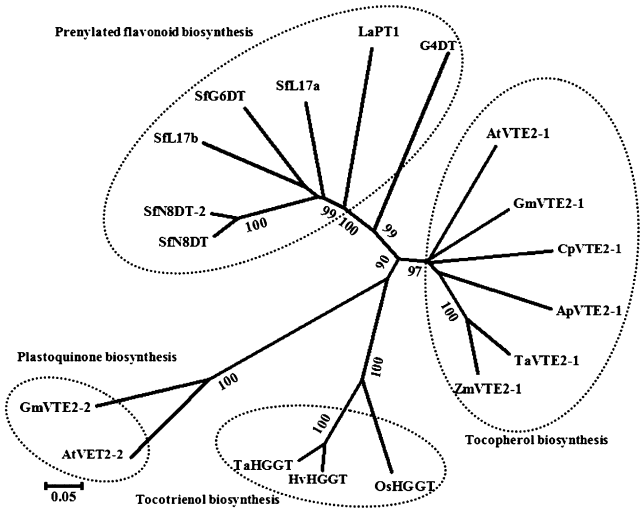

Figure 3. Phylogenetic tree for the putative protein sequence of LaPT1 and related prenyltransferase proteins of plants. Protein sequences were aligned using ClustalX. The neighbor-joining phylogenetic tree was drawn using MEGA4. Bootstrap values greater than 90 are shown, and the branch lengths represent relative genetic distances. The scale bar indicates 0.05 amino acid substitutions per site. The protein sequences used for comparison and their accession numbers are as follows: LaPT1 (white lupin; JN228254); AtVTE2-2 (Arabidopsis (Arabidopsis thaliana) DQ231060); GmVTE2-2 (soybean; DQ231061); OsHGGT (*Oryza sativa*; AY222862); HvHGGT (*Hordeum vulgare*; AY222860); TaHGGT (*Triticum aestivum*; AY222861); SfN8DT-1 (*S. flavescens*; AB325579); SfN8DT-2 (*S. flavescens*; AB370330); SfL17b (*S. flavescens*; AB370329); SfL17a (*S. flavescens*; AB371287); SfN6DT (*S. flavescens*; BAK52291); CpVTE2-1 (*Cuphea pulcherrima*; DQ231058); AtVTE2-1 (Arabidopsis; AY089963); ApVTE2-1 (*Allium porrum*; DQ231057); GmVTE2-1 (soybean; DQ231059); TaVTE2-1 (*T. aestivum*; DQ231056); and ZmVTE2-1 (*Zea mays*; DQ231055).

exhibited about 6-fold higher activity than that of the full-length protein (Fig. 5A). No activity was observed using microsomes from yeast cells transformed with an empty vector (Fig. 5D). No prenyltransferase activity could be detected if active protein extract was omitted or boiled, and no activity was observed when genistein, DMAPP, or Mg^{2+} was absent from the enzyme reaction.

The enzymatic product of LaPT1 was characterized using a number of separation and spectroscopic approaches. The product had the same retention time as an authentic standard of isowighteone (3′-prenylgenistein) on reverse-phase HPLC and appeared distinct from the other two isomers, lupiwighteone (8-prenygenistein) and wighteone (6-prenylgenistein; Fig. 5, B and C). Ultra-performance liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry (UPLC-ESI-qTOF-MS) analyses further confirmed that the product of intact and truncated LaPT1 was a monoprenylated genistein with mass-to-charge ratio (*m/z*) 337.11, with the same retention time as the authentic standard of isowighteone (Supplemental Fig. S2). No evidence for the formation of diprenylated or multiprenylated genistein derivatives was observed.

The product of recombinant LaPT1 protein exhibited a maximum absorption at 262.3 nm; this is the same as that of the isowighteone standard and close to that of genistein (262.5 nm). The maximum absorption bands of lupiwighteone and wighteone are at 265.8 nm and 267.0 nm, respectively (Supplemental Fig. S3). Isoflavonoids have a characteristic band II in the UV absorption spectrum ranging from 220 to 260 nm, due to absorption of the A-ring benzoyl system. The band II of lupiwighteone (265.8 nm) and wighteone (267.0 nm) shifts toward longer wavelengths compared with the parent compound genistein (262.5 nm); this is because the prenyl group is located on the A-ring in lupiwighteone and wighteone and extends the conjugated system of the A-ring. In contrast, the prenyl group of isowighteone attached to the 3′-position of the B-ring does not significantly affect the A-ring benzoyl system. The similarity between the UV spectra of the LaPT1 product and the parent compound genistein (262.5 nm), therefore, suggests that LaPT1 most likely catalyzes the prenylation of genistein on the B-ring.

The LaPT1 enzymatic product and the three authentic prenyl genistein isomer standards were further analyzed based upon their distinct fragmentation patterns by tandem mass spectrometry (MS/MS) analysis. This provided the clearest authentication of the enzymatic product of LaPT1, as its fragmentation pattern was identical to that of the isowighteone standard (Supplemental Fig. S4, A and B) but was distinctly different from those of wighteone or lupiwighteone (Supplemental Fig. S4, C and D). The differences in the MS/MS spectra were indicative of the different attachments of prenyl groups on the A-ring or B-ring. Both the product of LaPT1 and the isowighteone standard had a characteristic ion of *m/z* 107 that was absent from the spectra of wighteone and lupiwighteone. The ion of *m/z* 107 is typically derived from the A-ring without a prenyl group by Retro-Diels-Alder reaction, implying that the prenyl group is

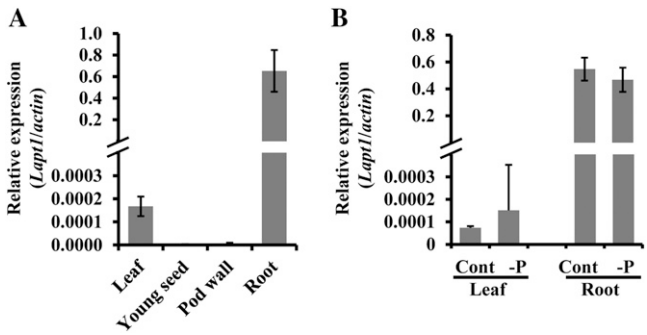

Figure 4. qRT-PCR analysis of *LaPT1* transcript levels (relative to *Actin*) in white lupin 'Ultra.' Data are averages and SD from three independent biological replicates with three technical replicates. A, Transcript levels in various tissues of mature plants. B, Transcript levels in young seedlings under control (Cont) and phosphate-deficient (−P) conditions.

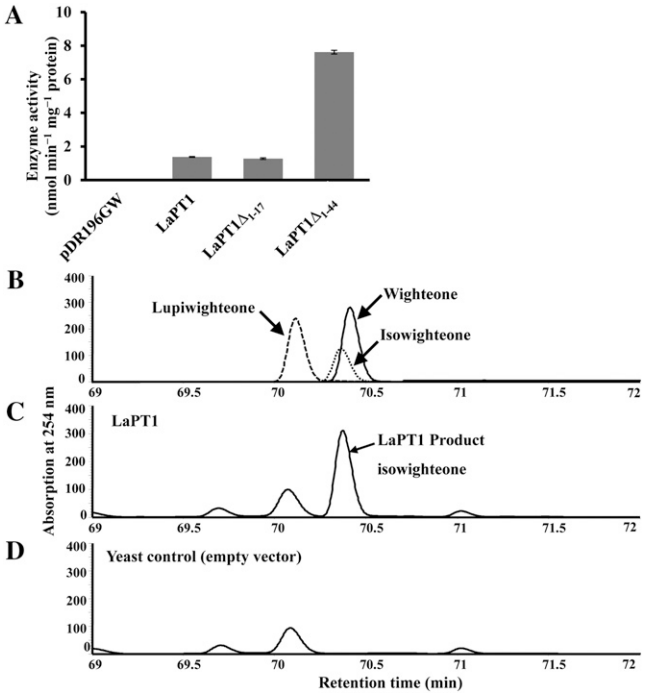

Figure 5. Identification of the enzyme products of LaPT1 protein with genistein as substrate by reverse-phase HPLC. A, Quantitative comparison of the enzyme activity of full-length LaPT1 protein and two truncated variants, LaPT1$\Delta_{1\text{-}17}$ and LaPT1$\Delta_{1\text{-}44}$. B, Overlap of HPLC traces for three authentic standards. C, HPLC trace for the product of the LaPT1 enzymatic reaction. D, HPLC trace from the control reaction using microsomal extract from yeast transformed with empty vector.

located on the B-ring of the LaPT1 enzyme product. In contrast, wighteone and lupiwighteone present distinctive ions of *m/z* 133 derived from the B-ring without the prenyl group. Taken together, our data are consistent with the LaPT1 enzymatic product being isowighteone (3′-prenylgenistein).

The activities of microsomal extracts containing full-length and truncated LaPT1 variants were strongly affected by changes in pH and temperature (Supplemental Fig. S5). Highest activities were at around 50°C (Supplemental Fig. S5A); rapid denaturation likely took place at higher temperatures. The enzymes exhibited highest catalytic activities under basic conditions (Supplemental Fig. S5B). As a basic protein with a theoretical pI of 9.40 (http://www.expasy.org/tools/pi_tool.html), LaPT1 is possibly more stable under basic conditions.

LaPT1 activity was absolutely dependent on the presence of divalent cation cofactors such as Mg^{2+} or Mn^{2+}, with a significant preference for Mg^{2+} (Supplemental Fig. S6). LaPT1 activity was found to decrease in the order $Mg^{2+} > Mn^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Ca^{2+}$, but Cu^{2+} and Fe^{2+} failed to allow the production of isowighteone (Supplemental Fig. S6). No activity was observed in buffer without the addition of metal ions.

The allylic donor preference of LaPT1 was determined using genistein as prenyl acceptor and the following prenyl donors: DMAPP, geranyl diphosphate, farnesyl diphosphate, or isopentenyl diphosphate. Reverse-phase HPLC analysis showed that only DMAPP could act as prenyl donor, indicating that the donor specificity of LaPT1 is the same as those of SfN8DT-1 and G4DT (Sasaki et al., 2008; Akashi et al., 2009). The apparent K_m of full-length and truncated LaPT1 variants for DMAPP as prenyl donor was calculated to be 54.75, 46.75, and 121.7 μM, respectively (Supplemental Fig. S7), compared with 106 μM for SfN8DT-1 and 150 μM for G4DT. The K_m of full-length and truncated LaPT1 variants for genistein was calculated to be 0.3, 0.5, and 38.8 μM, respectively (Supplemental Fig. S7), compared with 55 μM for SfN8DT-1 and 68 μM for G4DT for their preferred flavanone and pterocarpan prenyl acceptor substrates, respectively.

To address the prenyl acceptor specificity of LaPT1, flavanones (naringenin, liquiritigenin, hesperetin), flavone (apigenin), chalcones (2-hydroxychalcone, 2′-hydroxychalcone, isoliquiritigenin), isoflavones (daidzein, formononetin, genistein, 2′-hydroxygenistein, biochanin A, 7-hydroxyisoflavone), and flavonols (kaempferol, quercetin, taxifolin) were incubated with the yeast microsomal membrane fraction containing recombinant LaPT1 protein, with DMAPP as prenyl donor, and products were analyzed by reverse-phase HPLC. The results showed that the substrate specificity of LaPT1 was rather narrow; in fact, the enzyme only exhibited high activity toward genistein and 2′-hydroxygenistein (with 20% more activity than with genistein as substrate) but showed no or unquantifiable activities with any other flavonoids or isoflavonoids tested. The product of the reaction with 2′-hydroxygenistein did not cochromatograph with the previously characterized compound luteone (6-prenyl 2′-hydroxygenistein; Supplemental Fig. S8) and, therefore, is likely the 3′-prenyl derivative, as observed with genistein.

By using degenerate primers based on the conserved domains of prenyltransferases for 3′ RACE, we obtained two further sequences with high identity to, but distinct from, LaPT1; these may represent the enzymes responsible for the formation of other prenylated flavonoids in white lupin.

Subcellular Localization of LaPT1

LaPT1 had a predicted chloroplast transit peptide at the N terminus, similar to both SfN8DT-1 and G4DT, which appear to be localized in chloroplasts (Sasaki et al., 2008; Akashi et al., 2009). To investigate whether LaPT1 is localized in chloroplasts or other plastids in roots where both *LaPT1* transcripts and prenylated compounds accumulate, a binary vector harboring a LaPT1-GFP fusion protein was used to transform *M. truncatula* 'Jemalong A17' seedlings for the generation of hairy roots. *M. truncatula* was chosen because it is a model legume species that does not naturally produce

prenylated isoflavones. There are abundant plastids showing red chlorophyll autofluorescence in *M. truncatula* roots cultured under light, and root extracts appear green (Supplemental Fig. S9). The transgenic hairy roots were further observed with a laser confocal scanning microscope. The cells located in the pith region of the root were enriched in chloroplasts with red autofluorescence (Fig. 6B), whereas the root hair cells and epidermal cells only contained plastids without chlorophyll. The GFP signals overlapped the red autofluorescence from chloroplasts, indicating a chloroplast localization of the LaPT1-GFP fusion protein (Fig. 6, A–C). Other circular structures of GFP fluorescence were not associated with chlorophyll fluorescence in root hairs (Fig. 6D) and epidermal cells (Fig. 6E), suggesting the localization of LaPT1 to other types of plastids without chlorophyll. GFP signals were distributed around the red chlorophyll signal in the superimposed images, forming a bright green circle with relatively weak fluorescence in the center of the chloroplast (Fig. 6C), typical of the localization of a chloroplast envelope membrane protein (Ferro et al., 2002). Removal of the plastid transit peptide resulted in a truncated LaPT1 protein that appeared to direct GFP fluorescence to the vacuole (Fig. 6F).

In Vivo Functional Characterization of *LaPT1*

To investigate whether LaPT1 has the same catalytic activity in vivo as observed in vitro, the open reading frame of LaPT1 was cloned into the plant expression vector pB7WG2D.1 with a GFP marker for convenient visual selection (Karimi et al., 2002) for generating transgenic hairy roots from *M. truncatula*. qRT-PCR analysis showed that the *LaPT1* open reading frame was expressed in GFP-positive lines (Fig. 7A). Methanolic extracts from transgenic hairy roots were then analyzed by reverse-phase HPLC. Compared with transgenic control lines expressing empty vector, a new product appeared at the same retention time as isowighteone in 37 out of 50 *35S*::*LaPT1* transgenic lines analyzed (Fig. 7, B and C). Because the retention times of isowighteone and wighteone are so close, the product was further confirmed by supplementing the methanolic extract of a *35S*::*LaPT1* transgenic line with authentic standards of wighteone and isowighteone. This analysis clearly showed that the new product comigrated with isowighteone rather than wighteone (Fig. 7D). In addition, UPLC-ESI-qTOF-MS analysis confirmed that the product (retention time = 18.43, *m/z* 337.11) was a complete match to authentic isowighteone. The low level of isowighteone in the roots (up to 1–4 ng 10 mg^{-1} dry weight) was consistent in multiple batches of root cultures (Fig. 7B). Thus, LaPT1 can convert endogenous genistein to isowighteone in planta, providing proof of concept for the metabolic engineering of antimicrobial prenylated isoflavone derivatives in transgenic plants.

DISCUSSION

LapT1 Is a Plastid-Localized 5-Hydroxyisoflavone 3′-Prenyltransferase

The prenylation of flavonoid derivatives increases their biological activity, presumably by increasing lipophilicity and thereby affinity for membrane targets, and prenylated isoflavonoids are commonly found in the Leguminosae, where they serve as antimicrobial compounds (Tahara and Ibrahim, 1995). Therefore, transfer of a prenyl group from an allylic

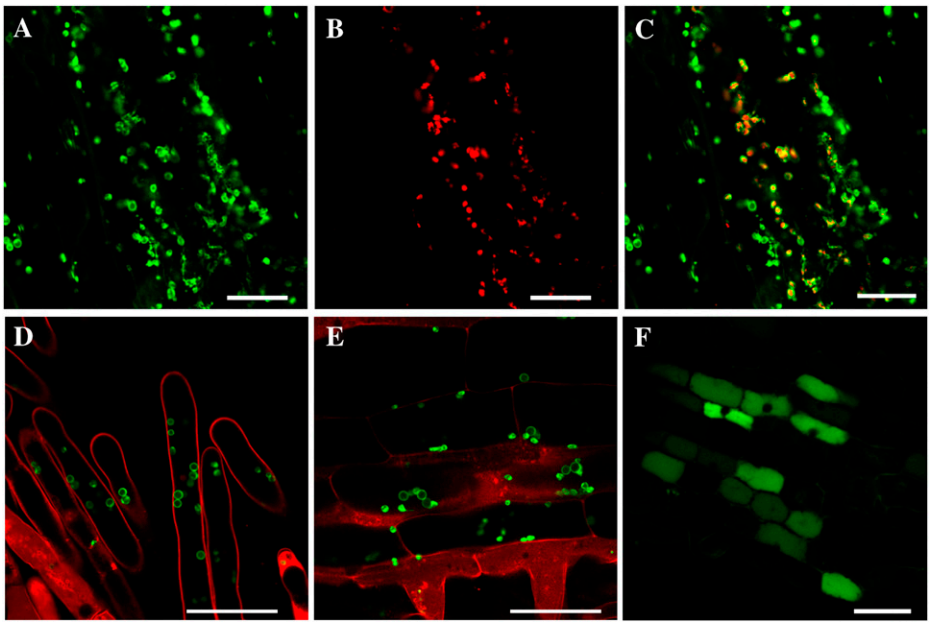

Figure 6. Confocal fluorescence microscopy images of LaPT1-GFP fusion protein in transgenic *M. truncatula* hairy roots. A, LaPT1-GFP fluorescence in the main body of the root. B, Chlorophyll autofluorescence in the main body of the root. C, Overlay of LaPT1-GFP fluorescence and chlorophyll autofluorescence. D, LaPT1-GFP signal in root hair cells. E, LaPT1-GFP signal in epidermal cells. F, Vacuolar localization of LaPT1Δ_{44}-GFP. The cell walls are shown by staining with 0.2 μg mL^{-1} propidium iodide in D and E. Bars = 5 μm in A to E and 50 μm in F.

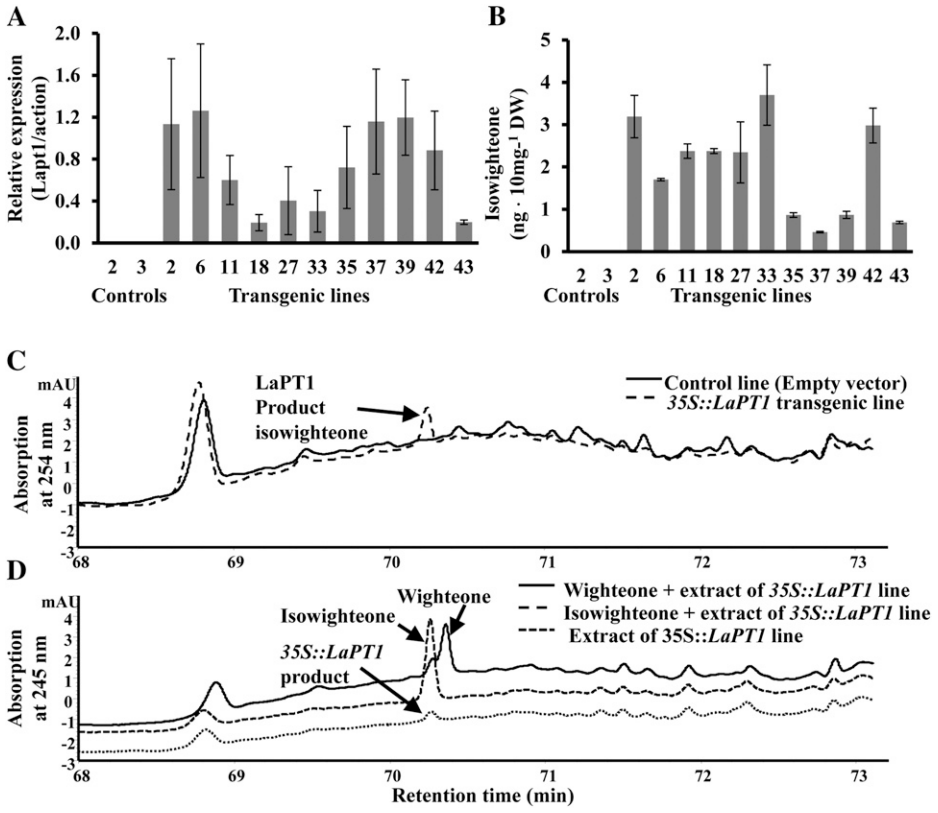

Figure 7. Reverse-phase HPLC of isowighteone produced in hairy roots of *M. truncatula* A17 overexpressing *LaPT1* under the control of the cauliflower mosaic virus 35S promoter. A, qRT-PCR analysis of *LaPT1* transcript levels in transgenic hairy roots. B, Reverse-phase HPLC analysis of isowighteone content in hairy root lines overexpressing *LaPT1*. DW, Dry weight. C, Overlap of HPLC traces of extracts from *LaPT1*-overexpressing lines and a control line transformed with empty vector. D, Overlap of HPLC traces of extracts from a *LaPT1*-overexpressing line supplemented with authentic isowighteone or wighteone standards. Results in A and B are means and SD of three biological replicates.

prenyl donor (usually DMAPP) is a crucial reaction in determining the complement of antimicrobial compounds in legumes. We here report an isoflavone prenyltransferase, LaPT1, expressed in the roots of white lupin.

White lupin radicles exhibit about 20-fold higher total isoflavone prenylation activity than hypocotyls (Laflamme et al., 1993), consistent with the tissue specificity of LaPT1 expression. Only minute amounts of prenylated isoflavonoids are detectable in ripening seeds of white lupin (Katagiri et al., 2000), also consistent with the transcript expression patterns reported here.

Previous studies have reported the molecular identification of four flavonoid prenyltransferases, three from *S. flavescens* (SfN8DT-1, SfG6DT, and SfiLDT) and one from soybean (G4DT; Sasaki et al., 2008, 2011; Akashi et al., 2009). G4DT transfers a prenyl group to the A-ring of a pterocarpan precursor of the glyceollin phytoalexins, SfN8DT-1 prenylates the flavanone naringenin on the A-ring, and SfG6DT prenylates the isoflavone genistein on the A-ring. LaPT1 is closely related to the above proteins but catalyzes the B-ring prenylation of genistein and 2′-hydroxygenistein to produce isowighteone, one of a series of prenylated isoflavones produced constitutively in the roots of white lupin.

LaPT1 has similar biochemical properties to other isoflavone prenyltransferases, such as an alkaline pH optimum and a requirement for divalent metal ions (Sasaki et al., 2008, 2011). However, although its affinity for the prenyl donor DMAPP is similar to that of other flavonoid prenyltransferases, its affinity for the prenyl acceptor is more than 1 order of magnitude greater than that of the previously characterized enzymes.

G4DT, SfN8DT-1, and SfG6DT, as well as isoflavone prenyltransferases previously identified only at the biochemical level, are localized in chloroplasts (Biggs et al., 1990; Sasaki et al., 2008, 2011; Akashi et al., 2009). Our studies here indicate that LaPT1 is likewise localized to plastids, both with and without chlorophyll, when expressed in *Medicago* hairy roots. The pattern of GFP fluorescence in plastids from roots expressing LaPT1:GFP suggests that the enzyme locates to the chloroplast membrane and is not found in the internal membranes of the organelle. Overall, these results are consistent with an early report suggesting that prenyltransferases for isoflavonoid phytoalexin formation are located in the envelope membrane of plastids in bean (*Phaseolus vulgaris*) and soybean (Biggs et al., 1990). The presence of the chloroplast transit peptide inactivates recombinant G4DT in vitro for unknown reasons (Akashi et al., 2009). Overall activity of LaPT1 is greater in yeast microsomes after removal of the transit peptide, but the K_m value is also significantly increased. The apparent increase in turnover may possibly be due to higher expression efficiency or better protein stability for LaPT1$\Delta_{1\text{-}44}$.

Chloroplast localization may be critical for the in vivo biological activity of flavonoid and isoflavonoid

prenyltransferases, because the chloroplast is a major intracellular site for the biosynthesis of prenyl moieties. If prenyl substrate required for LaPT1 originates from the methyl erythritol phosphate pathway in plastids, as proposed in the case of G4DT (Akashi et al., 2009), the localization of LaPT1 on the envelope membrane might facilitate access to both DMAPP and the prenyl acceptor genistein, which is formed from naringenin via isoflavone synthase that is attached to the cytoplasmic, outer surface of the endoplasmic reticulum (Liu and Dixon, 2001). However, it is currently unknown how genistein is transported from its site of synthesis to the plastid membrane.

Although LaPT1 was inactive with daidzein (5-deoxygenistein), daidzein can be prenylated by a microsomal extract from white lupin (Laflamme et al., 1993). LaPT1 was also unable to produce wighteone or lupiwighteone, although these are naturally occurring isoflavonoid compounds in white lupin (Schröder et al., 1979; Ingham et al., 1983; Gagnon et al., 1992; Shibuya et al., 1992; Laflamme et al., 1993). It has been reported that a crude microsomal membrane pellet from lupin radicle extracts can catalyze the prenylation of genistein at the 3′-, 6-, and 8-positions, but the microsomal fraction from suspension cultures only catalyzes prenylation at the 3′- and 6-positions (Laflamme et al., 1993), and an enzyme extract from hypocotyls of white lupin produces only 6-prenylated genistein (Schröder et al., 1979). These results suggested that white lupin and other legume species likely possess multiple flavonoid and isoflavonoid prenyltransferase enzymes with different substrate specificities. The identification of LaPT1 provides a basis for the characterization of the other prenyltransferases responsible for 8- and 6-prenylation of genistein.

LaPT1 as a Tool for Metabolic Engineering

Tomato (*Solanum lycopersicum*) plants expressing either a microbial prenyltransferase from *Streptomyces coelicolor* with broad substrate specificity or *Sophora* naringenin dimethylallyl transferase accumulated low levels of 3′-prenylnaringenin or 8-prenylnaringenin, respectively, in fruit tissue (Koeduka et al., 2011). Interestingly, the *Streptomyces* prenyltransferase produced 6-prenylnaringenin in vitro, suggesting that the substrate preference of prenyltransferases might be affected by in vivo conditions. This was supported by another study in which prenylated flavonoids were observed after the feeding of various flavonoid substrates to transgenic *Lotus japonicus* and soybean. LaPT1 appears to make the same prenylated isoflavone derivative in vivo as in vitro.

Isowighteone has been proven to be both antibacterial (Hatano et al., 2000) and antifungal (Ingham, 1990; Máximo et al., 2002; Queiroz et al., 2002; Hostettmann et al., 2005). However, it is expensive to extract directly from plant materials because of its low level. The identification of LaPT1 provides a new tool for the biochemical synthesis of isowighteone, either enzymatically or in a microbe such as yeast. LaPT1 and other known flavonoid and isoflavonoid prenyltransferases share high homology but produce largely varied products, implying that it should also be possible to engineer flavonoid and isoflavonoid prenyltransferases with altered substrate specificity for producing natural or completely artificial compounds with stronger pharmaceutical or agrochemical activity for the control of animal or plant diseases. Based on previous studies (Sugiyama et al., 2011), it may be possible to increase the levels of isowighteone in transgenic *Medicago* hairy roots by feeding with genistein precursor.

Alfalfa (*Medicago sativa*) is the fourth highest value crop in the United States and is prone to many fungal and bacterial diseases (Porto et al., 1988; Pennypacker et al., 1990; Esnault et al., 1993). The naturally occurring isoflavonoid phytoalexins of alfalfa are not prenylated, and alfalfa pathogens, therefore, may be less able to detoxify prenylated isoflavonoids. Although the results presented here indicate only low levels of isowighteone production in *M. truncatula* hairy roots, the formation of this compound in *Medicago* species can likely be optimized through the use of better tissue-specific promoters as well as by up-regulation of the formation of prenyl donor and acceptor.

CONCLUSION

We have characterized, at the molecular level, a plastid-localized flavonoid prenyltransferase from white lupin with strict specificity for B-ring 3′ prenylation of the isoflavones genistein and 2′-hydroxygenistein. Expression of the gene encoding this enzyme in *M. truncatula* leads to the accumulation of isowighteone, an antimicrobial compound that is not naturally occurring in this species.

MATERIALS AND METHODS

Chemicals

Authentic standards of isowighteone, lupiwighteone, and wighteone were obtained from Plantech. Other flavonoids and isoflavonoids were purchased from Indofine Chemical Company. Stock solutions of each standard were prepared in dimethyl sulfoxide. DMAPP was synthesized according to a previously described method (Keller and Thompson, 1993). Synthetic dextrose (SD) medium was purchased from Sigma-Aldrich.

Plant Materials

White lupin (*Lupinus albus* 'Ultra') was obtained from the U.S. Department of Agriculture Soybean Germplasm Collection. The seeds were scarified with sandpaper, sterilized with 10% bleach, and rinsed thoroughly with clean water. Seeds were germinated for 4 d on moist filter paper in the dark at 25°C and then transplanted to pots.

For phosphorus deficiency treatment, germinated seeds were planted in pots filled with sea sand that had been thoroughly washed with water and grown in a growth chamber at 20°C/15°C with 16-h/8-h day/night cycles. To maintain proper moisture levels, plants were watered every other day with 200 mL of the appropriate nutrient solution based on the volume of the pots.

Detailed recipes of control (+P) nutrient solution and phosphorus deficient (−P) nutrient solution were as described previously (Johnson et al., 1996; Gilbert et al., 2000). Five-week-old plants were harvested, and the sand was washed quickly from the roots. Plants were dissected into roots and aerial parts, which were flash frozen in liquid nitrogen and stored at −80°C until further processing.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated using modified cetyl-trimethyl-ammonium bromide extraction as described previously (Pang et al., 2007). Total RNA was purified and concentrated using the RNeasy MiniElute Cleanup Kit (Qiagen) and then treated with DNase I (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 4 μg of total RNA in a total volume of 20 μL using SuperScript III reverse transcriptase (Invitrogen).

Primers for quantitative real-time PCR were designed using Primer3 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The forward and reverse primers for the amplification of LaPT1 were WLrtFPTF3 and WLrtFPTR3; primers for the amplification of the *Actin* control gene were WLactinRTF2 and WLactinRTR2. Each primer pair was confirmed to give a single PCR product. All primers for PCR amplification are listed in Supplemental Table S1. The parameters and analysis of the qRT-PCR were as described previously (Pang et al., 2007).

3′ RACE Amplification of *LaPT1*

EST clone FF836984 was kindly provided by Dr. Carroll P. Vance (U.S. Department of Agriculture-Agricultural Research Service) and sequenced. The sequence was aligned with G4DT, SfN8DT-1, and several other plant homogentisate phytyltransferase genes, and two degenerate forward primers, Lpt3F1 and Lpt3F2, were designed for first- and second-round nested PCR. RACE reactions were set up as follows: 3 μL of cDNA, 0.2 μM forward primer, 0.2 μM reverse poly(T)$_{30}$ primer, 5 μL of 10× reaction buffer, 0.2 mM deoxyribonucleoside triphosphate, and 1 μL of ExTaq (TaKaRa) in a final volume of 50 μL. PCR was conducted on an Applied Biosystems Thermal 2720 cycler, and reaction cycles were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 2 min; and a final extension of 7 min at 72°C. After first-round PCR, 0.1 μL of PCR products was used as a template for second-round PCR under the same PCR conditions. PCR product was cloned into pGEMT-easy vector (Promega) and sequenced.

Construction of Yeast Expression Vector and Yeast Transformation

Yeast expression vectors were constructed using the native and truncated forms of LaPT1 that had been amplified by high-fidelity PCR using *Pfu* DNA polymerase (Stratagene). The PCR conditions with primers WlFPT2F and WlFPTSacI3R (for the full-length clone) were as follows: initial denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s; and a final extension step of 72°C for 7 min. The resulting PCR product was introduced into the entry vector pENTR/D-TOPO (Invitrogen), and the construct was confirmed by sequencing. The entry vector was recombined into the destination vector pDR196GW by site-specific recombination with LR Clonase according to the manufacturer's instructions (Invitrogen). pDR196GW, a yeast expression vector carrying the constitutive yeast PAM1 promoter, was kindly provided by Dr. Kazafumi Yazaki (Kyoto University).

Two truncated forms of LaPT1 were obtained as above by PCR amplification with the forward primers WlFPT17F and WlFPT44F starting at 51 or 132 bp downstream from the ATG start codon and the reverse primer WlFPTSacI3R (Supplemental Table S1), which resulted in the removal of 17 or 44 amino acids from the N-terminal transit peptide, respectively. The destination vectors were transformed into yeast strain W303A1 using the modified LiCl method (Pompon et al., 1996; Liu et al., 2003), and the transformants were selected on SD (−uracil) plates. Colonies confirmed by colony PCR were used for enzyme assay.

Preparation of Microsomes and Enzyme Assay

A yeast microsomal fraction expressing prenyltransferase was prepared as described previously (Yazaki et al., 2002). In brief, a single yeast clone was picked and cultured in 5 mL of SD (−uracil) broth overnight at 28°C. The overnight culture was inoculated into 50 mL of SD (−uracil) broth and grown at 28°C overnight. Finally, this culture was inoculated into a 2-L baffled flask containing 200 mL of fresh SD (−uracil) broth. The yeast cells were harvested by centrifugation at 3,795 g at 4°C for 15 min. The pellet was washed once with extraction buffer, resuspended in 20 mL of extraction buffer, and lysed with glass beads (Sigma) five times for 30 s each (each time with a 1-min cooling interval on ice) on a vortex mixer. The extraction buffer was 20 mM Tris-HCl (pH 7.5), 0.6 M sorbitol, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The microsomal fraction containing the recombinant LaPT1 protein was obtained by ultracentrifugation and resuspended in 1 mL of Tris-HCl buffer (pH 7.5). The total protein concentration was determined by the method of Bradford (1976).

The basic prenyltransferase assay reaction contained 1 mM dithiothreitol, 25 mM MOPS, pH 7.0, 10 mM Mg^{2+}, 160 μM genistein, and 400 μM DMAPP. The reaction mixtures were incubated in a total volume of 250 μL at 30°C. The reaction was terminated by adding 250 μL of methanol and centrifuged for 30 min, and then 100 μL of supernatant was injected for reverse-phase HPLC analysis. To determine whether the N-terminal transit peptide would affect the activity of LaPT1, microsomal preparations containing 40 μg of full-length or truncated LaPT1-containing microsomal proteins (LaPT1Δ$_{1\text{-}17}$ and LaPT1Δ$_{1\text{-}44}$) were added into a 250-μL enzyme reaction and incubated at 30°C for 1 h. To test the requirement of LaPT1 activity for divalent cations, 40 μg of full-length protein was assayed in a reaction mixture supplemented with 10 mM $MgCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $ZnCl_2$, $CuCl_2$, or $NiCl_2$ and incubated at 30°C for 1 h.

The apparent K_m value for genistein was determined by incubating 10 μg of recombinant yeast microsomes with various concentrations of genistein (0.5, 1, 2.5, 5, 10, 20, 40, 80, 160, and 400 μM) and a fixed concentration of DMAPP (1 mM), whereas the apparent K_m for DMAPP was determined with varying concentrations of DMAPP (5, 10, 20, 40, 80, 160, and 400 μM) and a fixed concentration of genistein (1 mM). The reaction product was quantified using a standard curve generated from known concentrations of wighteone (rather than isowighteone, which is very expensive), which has a very similar UV spectrum and extinction coefficient as isowighteone. Apparent K_m values were calculated from Lineweaver-Burk plots using Hyper32 software (http://homepage.ntlworld.com/john.easterby/hyper32.html).

The prenyl acceptor specificity was studied in reaction mixtures containing microsomal enzyme preparation, various flavanones, flavones, chalcones, flavonols, or isoflavones (1 mM), with DMAPP as prenyl donor. The prenyl donor specificity was tested with isopentenyl diphosphate, geranyl diphosphate, farnesyl diphosphate, and DMAPP with genistein as prenyl acceptor (1 mM). The enzymatic reactions were extracted twice with an equal volume of ethyl acetate and dried under nitrogen gas. The residues were then dissolved in 100 μL of 80% methanol containing 0.018 mg mL^{-1} umbelliferone (internal standard), and the samples were analyzed by UPLC-ESI-qTOF-MS and/or reverse-phase HPLC.

Subcellular Localization of LaPT1 Protein

The full-length open reading frame of LaPT1 without the stop codon was cloned into the entry vector pENTR/D-TOPO (Invitrogen) and then recombined into the Gateway destination vector pK7FWG2 (Karimi et al., 2002) in frame with GFP at the C-terminal end. The resulting destination vector was confirmed by sequencing, introduced into *Agrobacterium rhizogenes* strain ARqual1 by electroporation, and used to inoculate radicles of *Medicago truncatula* using a method described previously (Limpens et al., 2004).

The roots from transformed plants were observed with an Olympus SZX 12 stereo fluorescence microscope, and the positive transgenic hairy roots with GFP fluorescence were subcultured on B5 medium (Gamborg et al., 1968). Confocal fluorescence images were obtained with a Bio-Rad MRC-1024 ES confocal laser scanning microscope.

Liquid Chromatography and Mass Spectrometry

Twenty milligrams of powdered plant material was extracted in 1 mL of 80% methanol containing 0.018 mg mL^{-1} umbelliferone (internal standard) at room temperature for 2 h on an orbital shaker. After centrifugation, 5 μL of supernatant was analyzed by UPLC coupled to both photodiode array and mass spectrometry detection. Mass spectra were acquired in the negative electrospray ionization mode on a hybrid quadrupole time-of-flight mass spectrometer. Experimental details of negative-ion UPLC-ESI-qTOF-MS and HPLC-ESI-MS spectra have been given in a previous publication (Farag et al., 2007).

Traditional reverse-phase HPLC of enzyme products was performed on an Agilent 1100 HPLC apparatus as described previously (Pang et al., 2008).

Phylogenetic Analysis

Protein sequences of LaPT1 and related prenyltransferase proteins of plants were aligned using ClustalX (Thompson et al., 1997). From this alignment, a consensus phylogenetic tree was generated by the neighbor-joining method using MEGA4 (Tamura et al., 2007). All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Bootstrap values in percentage (only those greater than 70% are presented) are indicated on the nodes. The bootstrap values were obtained from 1,000 bootstrap replicates. The scale bar corresponds to 0.05 estimated amino acid changes per site.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number JN228254.

Supplemental Data

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

Supplemental Figure S1. UPLC-ESI-qTOF-MS analysis of the prenylated product generated in vitro by full-length and truncated variants of LaPT1.

Supplemental Figure S2. UPLC-ESI-qTOF-MS analysis of LaPT1 enzyme product.

Supplemental Figure S3. Comparison of UV spectra of the LaPT1 enzyme product and authentic standards.

Supplemental Figure S4. TOF MS/MS chromatogram (*m/z* 337.11) of the enzyme product of LaPT1.

Supplemental Figure S5. Effects of temperature and pH on enzyme activities of full-length LaPT1 and two truncated variants of LaPT1.

Supplemental Figure S6. Effects of various divalent metal ions on LaPT1 activity.

Supplemental Figure S7. Kinetic analysis of intact and truncated LaPT1.

Supplemental Figure S8. Identification of the enzyme products of LaPT1 protein with 2′-hydroxygenistein as substrate by reverse-phase HPLC.

Supplemental Figure S9. Presence of chlorophyll in *M. truncatula* hairy roots.

Supplemental Table S1. PCR primers used in this work.

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LITERATURE CITED

Akashi T, Sasaki K, Aoki T, Ayabe S, Yazaki K (2009) Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin. Plant Physiol **149:** 683–693

Bednarek P, Frański R, Kerhoas L, Einhorn J, Wojtaszek P, Stobiecki M (2001) Profiling changes in metabolism of isoflavonoids and their conjugates in Lupinus albus treated with biotic elicitor. Phytochemistry **56:** 77–85

Biggs DR, Welle R, Grisebach H (1990) Intracellular localization of prenyltransferases of isoflavonoid phytoalexin biosynthesis in bean and soybean. Planta **181:** 244–248

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Esnault R, Buffard D, Breda C, Sallaud C, el Turk J, Kondorosi A (1993) Pathological and molecular characterizations of alfalfa interactions with compatible and incompatible bacteria, *Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*. Mol Plant Microbe Interact **6:** 655–664

Farag MA, Huhman DV, Lei Z, Sumner LW (2007) Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of Medicago truncatula using HPLC-UV-ESI-MS and GC-MS. Phytochemistry **68:** 342–354

Ferro M, Salvi D, Riviere-Rolland H, Vermat T, Seigneurin-Berny D, Grunwald D, Garin J, Joyard J, Rolland N (2002) Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. Proc Natl Acad Sci USA **99:** 11487–11492

Gagnon H, Ibrahim RK (1997) Effects of various elicitors on the accumulation and secretion of isoflavonoids in white lupin. Phytochemistry **44:** 1463–1467

Gagnon H, Seguin J, Bleichert E, Tahara S, Ibrahim RK (1992) Biosynthesis of white lupin isoflavonoids from [U-^{14}C]L-phenylalanine and their release into the culture medium. Plant Physiol **100:** 76–79

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158

Gilbert GA, Knight JD, Vance CP, Allan DL (2000) Proteoid root development of phosphorus deficient lupin is mimicked by auxin and phosphonate. Ann Bot (Lond) **85:** 921–928

Harborne JB, Ingham JL, King L, Payne M (1976) The isopentenyl isoflavone luteone as a pre-infectional antifungal agent in the genus Lupinus. Phytochemistry **15:** 1485–1487

Hatano T, Shintani Y, Aga Y, Shiota S, Tsuchiya T, Yoshida T (2000) Phenolic constituents of licorice. VIII. Structures of glicophenone and glicoisoflavanone, and effects of licorice phenolics on methicillin-resistant *Staphylococcus aureus*. Chem Pharm Bull (Tokyo) **48:** 1286–1292

Hostettmann K, Marston A, Wolfender J-L (2005) Strategy in the search for new lead compounds and drugs from plants. CHIMIA Int J Chem **59:** 291–294

Ingham J, Tahara S, Harborne J (1983) Fungitoxic isoflavones from *Lupinus albus* and other *Lupinus* species. Z Naturforsch C **38C:** 194–200

Ingham JL (1990) Systematic aspects of phytoalexin formation within tribe Phaseoleae of the Leguminosae (subfamily Papilionoideae). Biochem Syst Ecol **18:** 329–343

Johnson JF, Vance CP, Allan DL (1996) Phosphorus deficiency in *Lupinus albus*: altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. Plant Physiol **112:** 31–41

Karimi M, Inzé D, Depicker A (2002) Gateway vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7:** 193–195

Katagiri Y, Ibrahim RK, Tahara S (2000) HPLC analysis of white lupin isoflavonoids. Biosci Biotechnol Biochem **64:** 1118–1125

Keller RK, Thompson R (1993) Rapid synthesis of isoprenoid diphosphates and their isolation in one step using either thin layer or flash chromatography. J Chromatogr A **645:** 161–167

Koeduka T, Shitan N, Kumano T, Sasaki K, Sugiyama A, Linley P, Kawasaki T, Ezura H, Kuzuyama T, Yazaki K (2011) Production of prenylated flavonoids in tomato fruits expressing a prenyltransferase gene from *Streptomyces coelicolor* A3(2). Plant Biol (Stuttg) **13:** 411–415

Laflamme P, Khouri H, Gulick P, Ibrahim R (1993) Enzymatic prenylation of isoflavones in white lupin. Phytochemistry **34:** 147–151

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Geurts R (2004) RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J Exp Bot **55:** 983–992

Liu CJ, Dixon RA (2001) Elicitor-induced association of isoflavone *O*-methyltransferase with endomembranes prevents the formation and 7-*O*-methylation of daidzein during isoflavonoid phytoalexin biosynthesis. Plant Cell **13:** 2643–2658

Liu CJ, Huhman D, Sumner LW, Dixon RA (2003) Regiospecific hydroxylation of isoflavones by cytochrome p450 81E enzymes from *Medicago truncatula*. Plant J **36:** 471–484

Máximo P, Lourenço A, Feio SS, Roseiro JC (2002) Flavonoids from *Ulex airensis* and *Ulex europaeus* ssp. *europaeus*. J Nat Prod **65:** 175–178

Pang Y, Peel GJ, Sharma SB, Tang Y, Dixon RA (2008) A transcript profiling approach reveals an epicatechin-specific glucosyltransferase expressed in the seed coat of *Medicago truncatula*. Proc Natl Acad Sci USA **105:** 14210–14215

Pang Y, Peel GJ, Wright E, Wang Z, Dixon RA (2007) Early steps in proanthocyanidin biosynthesis in the model legume *Medicago truncatula*. Plant Physiol **145:** 601–615

Pennypacker BW, Knievel DP, Leath KT, Pell EJ, Hill RRJ (1990) Analysis of photosynthesis in resistant and susceptible alfalfa clones infected with *Verticillium albo-atrum*. Phytopathology **80:** 1300–1306

Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol **272:** 51–64

Porto MDM, Grau CR, de Zoeten GA, Gaard G (1988) Histopathology of *Colletotrichum trifolii* on alfalfa. Phytopathology **78:** 345–349

Queiroz EF, Wolfender JL, Atindehou KK, Traore D, Hostettmann K (2002) On-line identification of the antifungal constituents of *Erythrina vogelii* by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation. J Chromatogr A **974:** 123–134

Sasaki K, Mito K, Ohara K, Yamamoto H, Yazaki K (2008) Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of *Sophora flavescens*. Plant Physiol **146:** 1075–1084

Sasaki K, Tsurumaru Y, Yamamoto H, Yazaki K (2011) Molecular characterization of a membrane-bound prenyltransferase specific for isoflavone from *Sophora flavescens*. J Biol Chem **286:** 24125–24134

Schröder G, Zähringer U, Heller W, Ebel J, Grisebach H (1979) Biosynthesis of antifungal isoflavonoids in *Lupinus albus*: enzymatic prenylation of genistein and 2′-hydroxygenistein. Arch Biochem Biophys **194:** 635–636

Shibuya Y, Sugimura Y, Tahara S, Mizutani J (1992) Accumulation of isoflavones in lupin seedlings treated with copper chloride. Biosci Biotechnol Biochem **56:** 690–691

Sugiyama A, Linley PJ, Sasaki K, Kumano T, Yamamoto H, Shitan N, Ohara K, Takanashi K, Harada E, Hasegawa H, et al (2011) Metabolic engineering for the production of prenylated polyphenols in transgenic legume plants using bacterial and plant prenyltransferases. Metab Eng **13:** 629–637

Tahara S, Ibrahim RK (1995) Prenylated isoflavonoids: an update. Phytochemistry **38:** 1073–1094

Tahara S, Ingham JL, Nakahara S, Mizutani J, Harborne JB (1984) Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus*. Phytochemistry **23:** 1889–1900

Tahara S, Orihara S, Ingham JL, Mizutani J (1989) Seventeen isoflavonoids from *Lupinus albus* roots. Phytochemistry **28:** 901–911

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:** 1596–1599

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:** 4876–4882

Tian L, Peel GJ, Lei Z, Aziz N, Dai X, He J, Watson B, Zhao PX, Sumner LW, Dixon RA (2009) Transcript and proteomic analysis of developing white lupin (*Lupinus albus* L.) roots. BMC Plant Biol **9:** 1

Venkatesh TV, Karunanandaa B, Free DL, Rottnek JM, Baszis SR, Valentin HE (2006) Identification and characterization of an Arabidopsis homogentisate phytyltransferase paralog. Planta **223:** 1134–1144

Yazaki K, Kunihisa M, Fujisaki T, Sato F (2002) Geranyl diphosphate: 4-hydroxybenzoate geranyltransferase from Lithospermum erythrorhizon: cloning and characterization of a key enzyme in shikonin biosynthesis. J Biol Chem **277:** 6240–6246