

Molecular Cloning and Characterization of a cDNA for Pterocarpan 4-Dimethylallyltransferase Catalyzing the Key Prenylation Step in the Biosynthesis of Glyceollin, a Soybean Phytoalexin^{1[W]}

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Glyceollins are soybean (*Glycine max*) phytoalexins possessing pterocarpanoid skeletons with cyclic ether decoration originating from a C₅ prenyl moiety. Enzymes involved in glyceollin biosynthesis have been thoroughly characterized during the early era of modern plant biochemistry, and many genes encoding enzymes of isoflavonoid biosynthesis have been cloned, but some genes for later biosynthetic steps are still unidentified. In particular, the prenyltransferase responsible for the addition of the dimethylallyl chain to pterocarpan has drawn a large amount of attention from many researchers due to the crucial coupling process of the polyphenol core and isoprenoid moiety. This study narrowed down the candidate genes to three soybean expressed sequence tag sequences homologous to genes encoding homogentisate phytyltransferase of the tocopherol biosynthetic pathway and identified among them a cDNA encoding dimethylallyl diphosphate: (6aS, 11aS)-3,9,6a-trihydroxy-pterocarpan [(–)-glycinol] 4-dimethylallyltransferase (G4DT) yielding the direct precursor of glyceollin I. The full-length cDNA encoding a protein led by a plastid targeting signal sequence was isolated from young soybean seedlings, and the catalytic function of the gene product was verified using recombinant yeast microsomes. Expression of the *G4DT* gene was strongly up-regulated in 5 to 24 h after elicitation of phytoalexin biosynthesis in cultured soybean cells similarly to genes associated with isoflavonoid pathway. The prenyl part of glyceollin I was demonstrated to originate from the methylerythritol pathway by a tracer experiment using [1-¹³C]Glc and nuclear magnetic resonance measurement, which coincided with the presumed plastid localization of G4DT. The first identification of a pterocarpan-specific prenyltransferase provides new insights into plant secondary metabolism and in particular those reactions involved in the disease resistance mechanism of soybean as the penultimate gene of glyceollin biosynthesis.

Typical phytoalexins of the Leguminosae are isoflavonoid derivatives with characteristic species-specific modifications in both their skeletons and their decoration, e.g. prenylation (Dixon, 1999). Isoflavonoids are formed through an early branching pathway in flavonoid metabolism. The most abundantly found isoflavonoid skeleton of leguminous phytoalexins is pterocarpan, and more than one-half of these ptero-

carpanoids are decorated in a complex manner mainly by isoprenoid-derived substituents (Tahara and Ibrahim, 1995). Glyceollin is the collective name for soybean (*Glycine max*) phytoalexins with pterocarpanoid skeletons and cyclic ether decoration originating from C₅ prenyl substitutions (Fig. 1). The biosynthesis mechanism of soybean phytoalexins has been studied extensively during the 1970s to 1990s, most actively by Grisebach et al. (Ebel and Grisebach, 1988), and the pathway and biosynthetic enzymes involved have been characterized intensively at the biochemical level (Ebel, 1986; Dixon, 1999). More recent studies with leguminous plants such as alfalfa (*Medicago sativa*), licorice (*Glycyrrhiza echinata*), *Lotus japonicus*, and *Medicago truncatula* in addition to soybean have resulted in the identification of many genes encoding enzymes involved in isoflavonoid formation (Dixon, 1999; Shimada et al., 2007; Veitch, 2007). However, some genes encoding enzymes of the later stages of glyceollin biosynthesis, especially the crucial prenylation step, have remained uncharacterized until now.

During glyceollin biosynthesis, a dimethylallyl group is introduced at either C-4 or C-2 of the pterocarpan skeleton (C-8 or C-6 by isoflavone num-

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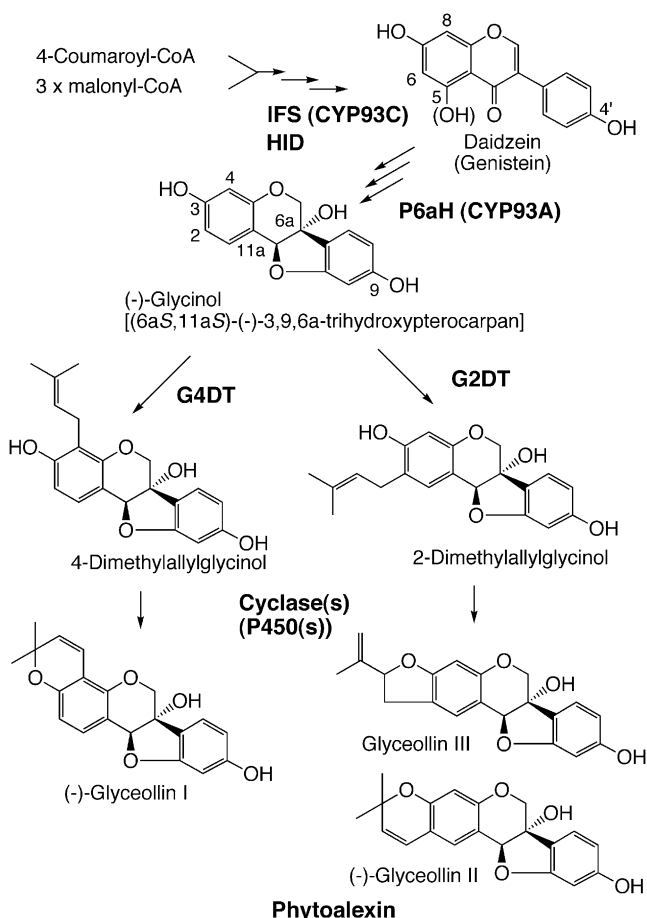


Figure 1. Biosynthesis of glyceollin isomers in soybean. Abbreviations not defined in the text: HID, 2-hydroxyisoflavanone dehydratase; IFS, 2-hydroxyisoflavanone synthase; P6aH, pterocarpan 6a-hydroxylase; G2DT, dimethylallyl diphosphate: (-)-glycinol 2-dimethylallyltransferase.

bering, respectively). A prenyltransferase activity catalyzing the dimethylallylation of (6aS, 11aS)-3,9,6a-trihydroxypterocarpan, (-)-glycinol, has been demonstrated in microsomal fractions of soybean cotyledons and cell cultures treated with a glucan elicitor derived from the cell walls of *Phytophthora sojae* (Zähringer et al., 1979). An increased toxicity of the prenylated pterocarpan against a phytopathogenic fungus was also demonstrated (Zähringer et al., 1981). An important finding was that the prenylation activity was localized to the chloroplast fraction of cotyledon cells in contrast to the endoplasmic reticulum (ER) where many of the cytochrome P450s (P450s) for glyceollin formation are localized (Welle and Grisebach, 1988; Biggs et al., 1990; Ayabe and Akashi, 2006). Efficient solubilization of the activity and partial purification of the enzyme have also been reported (Welle and Grisebach, 1991), but no complete purification was achieved to sequence the amino acids, and thus the gene responsible remains unidentified.

Recently, plant cDNAs of aromatic substrate prenyltransferases have been characterized, and their

nucleotide sequence information has become available (Yazaki et al., 2002; Sasaki et al., 2008). In view of the potential benefits of understanding the molecular mechanism underlying the phytopathogen resistance of soybean for the future disease-resistance breeding, studies toward the complete identification of the enzymes involved in glyceollin biosynthesis are important. Thus, this study undertook the molecular cloning and biochemical characterization of a soybean prenyltransferase involved in the glyceollin biosynthetic pathway.

RESULTS

Identification of cDNAs for (-)-Glycinol Prenyltransferase from a Soybean EST Library

Naringenin 8-dimethylallyltransferase of *Sophora flavescens* (SfN8DT), the first flavonoid-specific prenyltransferase, was revealed to share significant homology with homogentisate prenyltransferases responsible for the biosynthesis of vitamin E and plastoquinone (Sasaki et al., 2008). To find candidates of pterocarpan prenyltransferase, we employed homology searches using a soybean EST database with Arabidopsis (*Arabidopsis thaliana*) homogentisate prenyltransferase (VTE2-1) of the tocopherol biosynthesis pathway (AtVTE2-1, AY089963) as the query, which include a putative transit peptide. The DFCI soybean EST library yielded three sequences with more than 50% amino acid identity to AtVTE2-1, which were named PT1 (TC229226, 75% identity to AtVTE2-1), PT2 (TC227057, 50%), and PT3 (TC207773, 52%; see Supplemental Table S1). Among them, PT1 was predicted to be soybean VTE2-1 due to its high similarity with AtVTE2-1 and other orthologs. While PT3 did not have its full-length sequence information in the database, PT2 contained a full-length open reading frame (ORF) that encoded a polypeptide similar to VTE2-1 with a plastid targeting signal but was more divergent than PT1 (Fig. 2A) and was present in abundance in pathogen (*P. sojae*)-challenged soybean hypocotyls (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?tc=TC227057&species=soybean). In addition, highly homologous sequences to PT2 were not found in the databases for other leguminous plants like *L. japonicus* and *M. truncatula*, which do not produce prenylated isoflavonoids. Thus, we focused on the characterization of the catalytic function of PT2. The PT2 gene encoded for a polypeptide of 409 amino acids with nine putative transmembrane domains that were predicted by the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM/>; Fig. 2A). The polypeptide of PT2 possessed the conserved prenyltransferases motif, NQxxDxxxD, in the second loop (L2), in addition to another characteristic sequence conserved in the flavonoid and homogentisate prenyltransferases in loop 6 (L6), KD(I/L)xDx(E/D)GD.

The PT2 cDNA (GenBank accession no. AB434690) was isolated from young soybean seedlings. The PT2

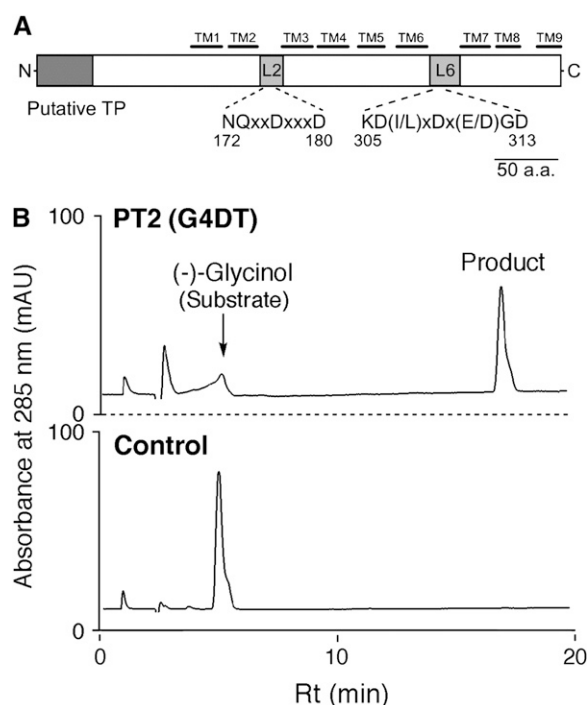


Figure 2. Properties of PT2 (G4DT). A, Structural features of PT2. Conserved amino acids for this prenyltransferase family are also shown. TP, Transit peptide; TM, transmembrane α -helix; L2, loop number 2; L6, loop number 6. B, HPLC chromatogram of the enzymatic reaction mixture of PT2. The assay mixture contained DMAPP, (–)-glycinol, and the microsomal fraction of yeast expressing PT2. For the negative control, the microsomal fraction of yeast cells transformed with pYES2 without a cDNA insert was used with the same substrates. The peak before the substrate glycinol is a solvent peak and not an enzymatic reaction product.

polypeptide possesses a putative transit peptide sequence (44 amino acids) at the N terminus, which was predicted by Wolf-PSORT (<http://psort.nibb.ac.jp/>). Both the full ORF and truncated PT2, in which its N-terminal putative transit peptide sequence was removed, were subcloned into a yeast shuttle vector pYES2.1 and were expressed in yeast strain BJ2168. In vitro assays using the microsomes of these recombinant yeasts were performed with pterocarpan skeleton substrates. The most probable substrate, (–)-glycinol, was prepared by purification from elicitor-treated soybean cell cultures in the presence of inhibitors of the isoprenoid pathway to increase the accumulation of this non-prenylated metabolite. The chemical structure of the isolated substance was confirmed by NMR (Supplemental Data S1) and $[\alpha]_D^{25}$ (-240° , $c = 0.0012$, ethanol). The incubation mixture of yeast microsomes expressing the truncated form of PT2, (–)-glycinol, and dimethylallyl diphosphate (DMAPP) with the addition of Mg^{2+} ion as a cofactor, gave a single product in an HPLC assay (Fig. 2B). The enzymatic reaction product was recovered from a large-scale assay, its chemical structure was analyzed by NMR, and the product was identified as 4-dimethylallylglycinol

from several lines of evidence (Supplemental Fig. S1), e.g. the addition of the signals of a dimethylallyl moiety, disappearance of the H-4 signal (δ 6.32) of the substrate, and a large down-field shift of C-4 (δ 103.8 to δ 116.6) in the product. The observation that both H-2 and C-2 signals did not change significantly between the substrate and product (substrate, δ 6.55 and 110.7; product, δ 6.59 and 110.2) clearly ruled out the possibility that the prenylation occurred at C-2. As such, the enzyme encoded by the PT2 cDNA was defined as DMAPP: (6aS, 11aS)-3,9,6a-trihydroxypterocarpan 4-dimethylallyltransferase (abbreviated as glycinol 4-dimethylallyltransferase or G4DT). Yeast microsomes expressing the full-length ORF of PT2 did not show detectable activity. The reason for this result is not clear, but the transit peptide may not be tolerated well by yeast as the heterologous host, which presumably resulted in a decrease in protein stability or incorrect folding, leading to reduced enzymatic activity.

(–)-Maackiain [(6aR, 11aR)-3-hydroxy-8,9-methylenedioxypterocarpan], another pterocarpanoid, was also employed in this study and yielded a single product in a radioactive assay, in which the radiolabeled prenyl donor ([1- ^{14}C]DMAPP) was used and the product was detected by thin-layer chromatography autoradiogram (Supplemental Fig. S2). However, the enzyme activity with maackiain seemed to be very weak, and product recovery from a nonlabeled large-scale experiment was unsuccessful. Genistein and daidzein, the isoflavones contained in soybean seeds in a large quantity, were not accepted as substrates. Divalent cations were definitely necessary for the enzyme activity, Mg^{2+} being most effective (100% ; 512 ± 15 pmol mg protein $^{-1}$ s $^{-1}$; $n = 3$) followed by Mn^{2+} (68%) and Co^{2+} (50%), which was a similar preference to *S. flavescens* flavanone dimethylallyltransferase (Sasaki et al., 2008), although an inconsistent requirement was previously observed for Mn^{2+} for the glycinol prenyltransferase activity in soybean microsomes (Welle and Grisebach, 1991). Kinetic analysis showed that the K_m values were 68 μM for (–)-glycinol and 150 μM for DMAPP using the recombinant yeast microsomes, which were comparable to the values [45 μM for (–)-glycinol and 180 μM for DMAPP] obtained with microsomes of cultured soybean cells treated with yeast extract elicitor for 24 h (Supplemental Fig. S3).

Expression Analysis and Elicitation Response in Cultured Soybean Cells

When cultured soybean cells were treated with yeast extract (0.3% w/v medium) as an elicitor, a rapid decrease in daidzin (daidzein 7-O-glucoside) from the culture and a transient increase of daidzein followed by a decrease in its level (10 h after elicitation) was observed (Fig. 3A). Accompanying the decrease in the isoflavone levels, the phytoalexins glyceollin I and III started to accumulate to reach a maximum at 48 h. Their precursor, glycinol, also accumulated, but showed its peak level at 24 h (Fig. 3B). The structures of these

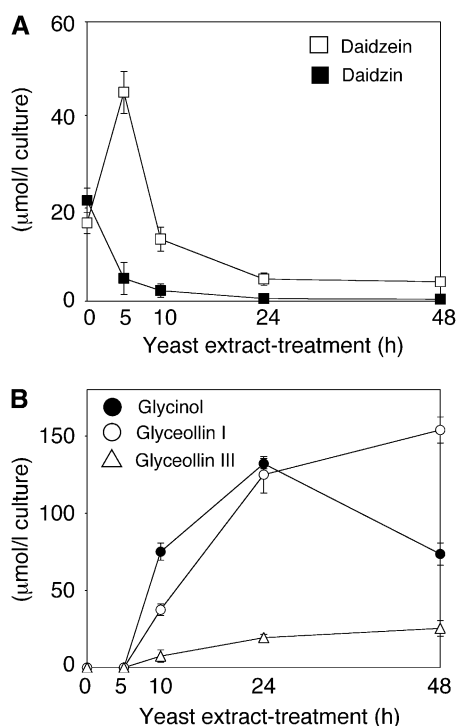


Figure 3. Time course of isoflavonoid formation in suspension-cultured soybean cells treated with yeast extract (0.3% w/v). A, Formation of daidzein and daidzin. B, Formation of glycinol and glyceollins. The values are the averages from four independent experiments. Vertical bars represent the SD.

metabolites were confirmed by NMR spectra (Supplemental Data S1). Glyceollin I, which is biosynthesized through the 4-dimethylallyl transfer reaction with glycinol, was the major product of the elicited cell culture ($154 \pm 9 \mu\text{mol L}^{-1}$ culture at 48 h after elicitation; Fig. 3B). The level of glyceollin III, which should be biosynthesized through dimethylallylation of glycinol at its 2 position, was about one-eighth of glyceollin I at the same period after elicitation, but glyceollin II, another final product of putative glycinol 2-dimethylallyltransferase (see Fig. 1), was not detectable in this study. Transient up-regulation of *G4DT* gene activity was observed at around 5 to 10 h postelicitation in a manner consistent with the accumulation pattern of the above metabolites in soybean cells (Fig. 4). This *G4DT* expression pattern occurred alongside other genes involved in isoflavonoid phytoalexin biosynthesis, i.e. two isoforms of 2-hydroxyisoflavanone synthase, 2-hydroxyisoflavanone dehydratase, and 3,9-dihydroxypterocarpan 6a-hydroxylase were clearly induced, which were monitored by reverse transcription (RT)-PCR using their respective specific primers.

Intracellular Localization of the G4DT-Reporter Construct in a Heterologous Plant Host

The multiple alignment of soybean candidate clones (*G4DT*, *PT1*, and *PT3*) and homogentisate prenyltrans-

ferase sequences such as phytyltransferases (*VTE2-1*) and geranylgeranyltransferases (*HGGT*) involved in vitamin E biosynthesis, and solanesyltransferases involved in plastoquinone biosynthesis was shown in Supplemental Figure S4. In the multiple alignment, the N-terminal region showed high divergence among them, and Wolf-PSORT predicted that the N-terminal 44 amino acids of *G4DT* is a plastid targeting signal. To confirm the subcellular localization of *G4DT*, a plasmid *G4DT*(TP)-GFP was constructed encoding a fusion protein between the *G4DT* N-terminal and green fluorescent protein (GFP) under the control of the cauliflower mosaic virus 35S (*CaMV35S*) promoter and introduced into onion (*Allium cepa*) epidermal peel by particle bombardment. As a positive control of plastid protein targeting, *Waxy* fused to DsRed (*WxTP*-DsRed) was used (http://podb.nibb.ac.jp/Organelle/bin/browseImage.php?ID=Image-t.mitsui_agr.niigata-u.ac.jp-20080314112511). In the transient expression experiment, the green fluorescence derived from *G4DT*(TP)-GFP was localized to dotted organelles in the onion epidermal peel cells, and the fluorescence pattern completely matched the red fluorescence derived from *WxTP*-DsRed (Fig. 5A). Control GFP showed a fluorescence pattern typical of cytosol localization (Fig. 5B). This result indicates that the N-terminal sequence of *G4DT* functions as a transit peptide for the plastid sorting of *G4DT*.

Biosynthetic Origin of the Dimethylallyl Moiety of Glyceollin Molecule

The isoprenoid unit is biosynthesized via either the mevalonate pathway or the methylerythritol phosphate (MEP) pathway depending on the organism and intracellular compartment where it is produced. In plant cells, the mevalonate pathway operates in the cytosol/ER, while enzymes in the MEP pathway are localized to plastids (Lichtenthaler et al., 1997). It is interesting to determine the origin of the dimethylallyl part of the glyceollin molecule in view of the plastid localization of *G4DT*. Employing the standard tracer method, [$1\text{-}^{13}\text{C}$]Glc was administered to soybean cell cultures, and ^{13}C -enrichment in the recovered glyceollin I was measured by ^{13}C -NMR (Supplemental Fig. S5). As shown in Table I, carbons of 1' (5.4% abundance), 4' (3.1%), and 5' (4.6%) of the dimethylallyl portion of the molecule showed 3- to 5-fold enrichment of ^{13}C compared to the natural abundance (1.1%). In case the MEP pathway provides the prenyl moiety, these carbons are derived from C-1 and C-5 of DMAPP (C-4' and C-5' of glyceollin I should be equivalent during the biosynthesis), respectively, which in turn come from C-1 of pyruvate and C-3 of glyceraldehyde 3-phosphate, both of which are derived from C-1 of Glc (Fig. 6). Therefore, the observed enrichment is consistent with the involvement of the MEP pathway in the formation of the isoprenoid unit for the dimethylallyl group. No enrichment of ^{13}C was observed at the C-2' position, which excludes the possibility of the

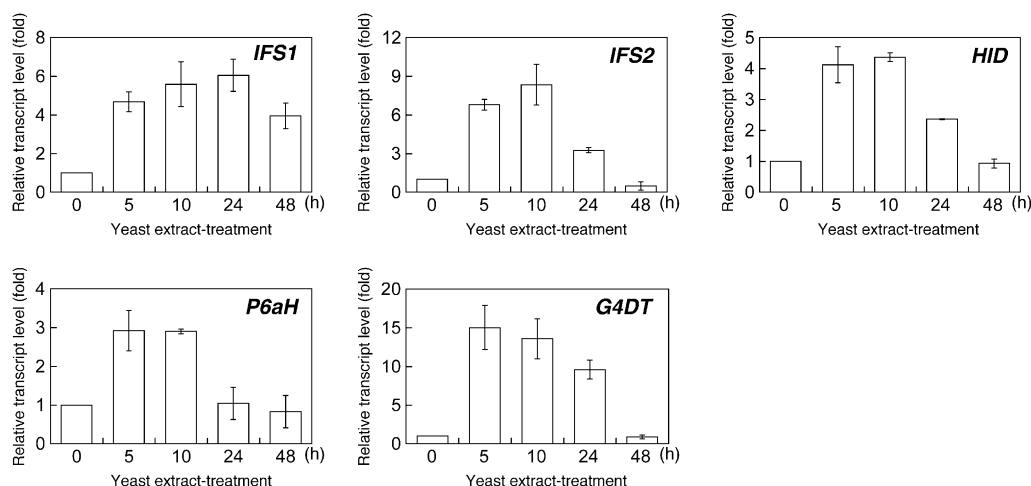


Figure 4. Semiquantitative RT-PCR analysis of gene expression of isoflavonoid biosynthetic enzyme in soybean cells upon yeast extract treatment. Transcripts levels were normalized against those of actin in the respective cells. The basal level of the control cells (time 0) was set at 1.0. The values are the averages of three independent experiments. Vertical bars represent the SD. Abbreviations not defined in the text: HID, 2-hydroxyisoflavanone dehydratase; IFS, 2-hydroxyisoflavanone synthase; P6aH, pterocarpin 6a-hydroxylase.

involvement of the mevalonate pathway. Other carbons enriched in glyceollin I can be reasonably explained by the incorporation of $1\text{-}^{13}\text{C}$ enriched pyruvate.

Phylogenetic Analysis of Flavonoid Dimethylallyltransferases and Related Enzymes

A phylogenetic tree composed of flavonoid prenyltransferases and those involved in vitamin E and plastoquinone biosynthesis in plants is shown in Figure 7. This indicates that both G4DT and PT3 of soybean belong to the same clade of flavonoid prenyltransferases from *S. flavescentis*, which is clearly divergent from that of homogentisate prenyltransferases for vitamin E and plastoquinone biosynthesis. Although the deposited PT3 sequence in the EST database does not contain the full ORF (lacking about five amino acids at the N terminus) and its full sequence could not be obtained in this study, PT3 shares higher similarity with G4DT than prenyltransferases of homogentisate. In the soybean database, two putative homogentisate prenyltransferases have been deposited, and PT1 described in this study is highly similar to GmVTE2-1 (DQ231059, 97% identity at the amino acid level), which is clustered with phytyltransferases of tocopherol biosynthesis (Sasaki et al., 2008). The other soybean prenyltransferase GmVTE2-2 is in the clade of plastoquinone biosynthetic enzymes (Fig. 7).

DISCUSSION

Prenylation as a Typical Modification of Flavonoids

Most of the phytoalexins of leguminous plants have isoflavonoid skeletons with species-specific modifications. For example, methoxy or methylendioxy groups

at C-4' (isoflavone numbering) are the characteristic features of phytoalexins of pea (*Pisum sativum*), alfalfa, and licorice (Tahara and Ibrahim, 1995; Dixon, 1999). Another typical modification of the isoflavonoid skeleton is prenylation in the phytoalexins of soybean, bean (*Phaseolus vulgaris*), and lupin (*Lupinus albus*; Schröder et al., 1979; Biggs et al., 1987; Laflamme et al., 1993). However, the molecular basis of the prenyl transfer reaction onto the isoflavonoid skeleton has not been determined previously. This study has identified a

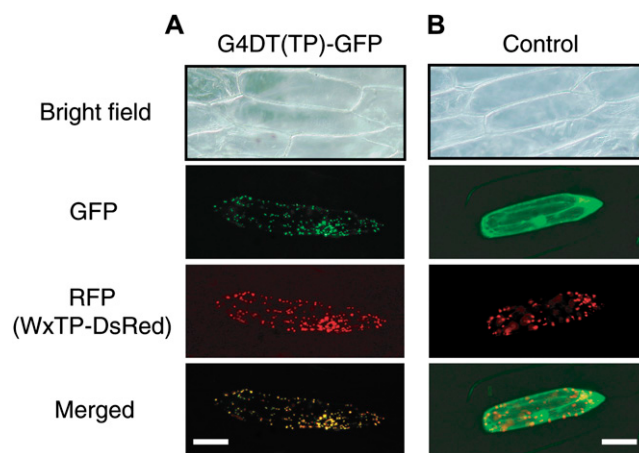


Figure 5. Transient expression of G4DT(TP)-GFP fusion protein in onion epidermal peels. A, G4DT(TP)-GFP and WxTP-DsRed plasmids were cotransformed into onion epidermal peels by particle bombardment. B, For the control, pGWB5 (CaMV35S promoter + GFP) and WxTP-DsRed plasmids were double transformed into onion epidermal peels. The images were obtained at 24 h after bombardment. WxTP-DsRed (red fluorescence) was used as a control for plastid targeting. All scale bars = 100 μm .

Table 1. ^{13}C abundance in glyceollin I after feeding of $[1-^{13}\text{C}]\text{Glc}$

The relative ^{13}C abundance of individual carbon atoms was calculated from the integrals of the labeled sample by comparison with the natural abundance sample (see Supplemental Fig. S5). The values were referenced to 1.1% for the carbon with the lowest ^{13}C enrichment.

Carbon	δ	Abundance
C-1	132.2	1.3
C-2	111.1	5.0
C-3	154.8	1.4
C-4	110.6	5.3
C-4a	151.6	1.1
C-6	70.8	5.2
C-6a	76.7	1.5
C-6b	121.3	1.1
C-7	125.2	4.5
C-8	108.9	1.6
C-9	160.8	2.1
C-10	98.7	1.4
C-10a	161.9	4.9
C-11a	86.0	1.6
C-11b	114.4	4.6
C-1'	117.0	5.4
C-2'	130.1	1.1
C-3'	76.8	1.7
C-4'	27.9	3.1
C-5'	28.1	4.6

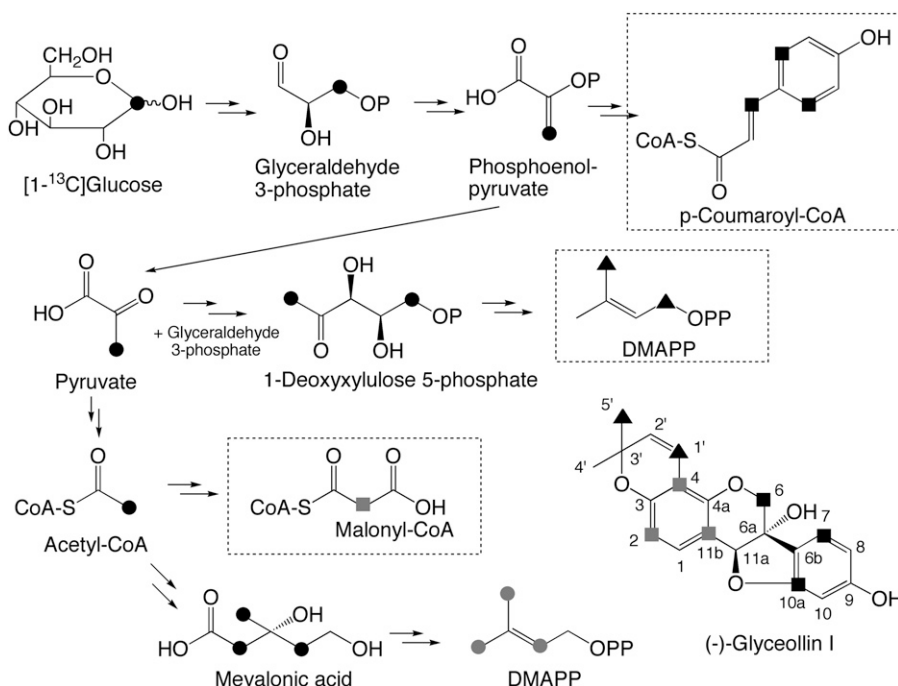
cDNA encoding G4DT of soybean through a homology search for VTE2-1 using soybean EST libraries and carried out functional analysis of the cloned cDNA for the catalytic activity of the recombinant protein with a yeast expression system. The yeast recombinant protein showed an approximately 400-fold stronger specific activity ($512 \pm 15 \text{ pmol mg protein}^{-1} \text{ s}^{-1}$) compared to

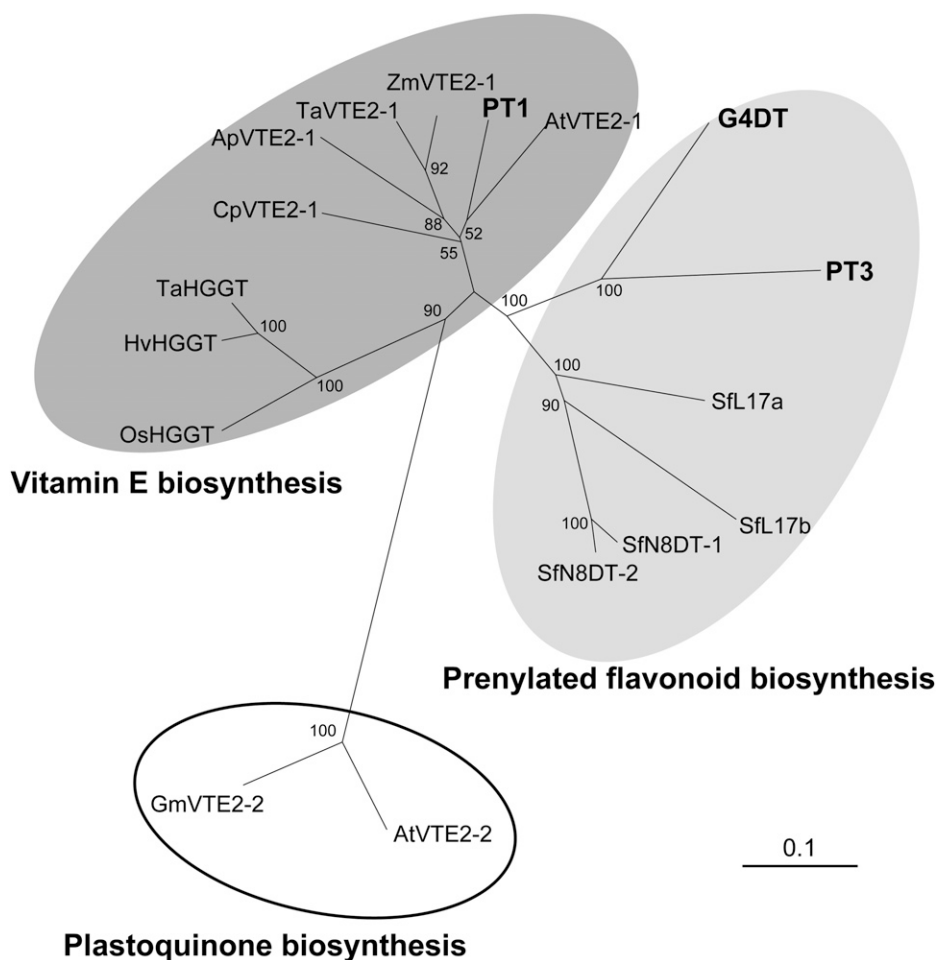
the native G4DT measured in the microsome of soybean cells treated with yeast extract for 24 h ($1.25 \pm 0.16 \text{ pmol mg microsome protein}^{-1} \text{ s}^{-1}$; $n = 3$). The regio-specificity of prenylation to C-4 of the pterocarpin skeleton indicates that the reaction represents the penultimate step of glyceollin I biosynthesis (see Fig. 1), which is completed by the final formation of an additional ring from the dimethylallyl side chain and the hydroxyl group at C-3 (Welle and Grisebach, 1988).

Involvement of G4DT in Phytoalexin Biosynthesis

The involvement of the G4DT gene in phytoalexin biosynthesis is supported by coordinated transcriptional up-regulation with other enzymes of the phytoalexin pathway upon elicitation (Fig. 4) in cultured soybean cells concomitant with glyceollin I accumulation (Fig. 3B). In addition to such a transcriptional regulation of phytoalexin biosynthesis, the release of daidzein from its stored form (glycosyl conjugates) was also observed at 5 h after yeast extract treatment as an early response of the cells to elicitation (Fig. 3A), as shown previously in pathogen-challenged soybean tissues (Graham et al., 1990; Hsieh and Graham, 2001; Suzuki et al., 2006). A similar phenomenon was observed in the medicarpin metabolism in *M. truncatula* cell cultures, i.e. this isoflavonoid phytoalexin is accumulated in response to yeast extract and methyl jasmonate, which is accompanied by the decrease of isoflavone glycosides (Naoumkina et al., 2007; Farag et al., 2008). These results suggest that intermediates of phytoalexins are stored as glycoside forms, which facilitates the rapid synthesis of phytoalexins upon pathogen attack in legume plants.

Figure 6. Incorporation of $[1-^{13}\text{C}]\text{Glc}$ into glyceollin I. The labeled carbon (^{13}C) shown in black dot in Glc is incorporated into each metabolite. Expected labeling patterns of DMAPP via the MEP (black triangles) and mevalonate pathways (gray dots) and of *p*-coumaroyl-CoA (black squares) and malonyl-CoA (gray squares) are also shown.





agrees with the observations for the biosynthesis of glabrol (a flavanone with two dimethylallyl substituents) in licorice hairy root cultures (Asada et al., 2000) and sophoraflavanone G (a lavandulyl flavanone) and lupalbigenin (a diprenyl isoflavone) in *S. flavescens* cells (Yamamoto et al., 2002). It is likely that the prenylating enzymes for flavonoids are generally localized to plastids and utilize a prenyl donor from MEP pathway, at least in the Leguminosae.

In glyceollin I biosynthesis, the direct flavonoid substrate of G4DT is produced at the ER by the P450 3,9-dihydroxypterocarpan 6a-hydroxylase (Schopfer et al., 1998), and the product of G4DT supplies the substrate of another P450 enzyme, a cyclase, in the ER again (Welle and Grisebach, 1988). Although the physiological meaning of the intracellular movement of biosynthetic intermediates is unknown, the present study may enable the characterization the P450 cyclase at the molecular level by providing the appropriate substrate. Furthermore, the interaction of ER and plastid during the production of plant secondary metabolites can be investigated in detail with G4DT and soybean cultures as a model system in the future.

Prenyltransferase Family Accepting Aromatic Substrates

Prenyltransferases mediating aromatic proton substitution are divided into two large groups, either membrane bound or soluble. The former can be further classified into two subgroups: those for ubiquinone biosynthetic enzymes, whose common substrate is *p*-hydroxybenzoate (Ashby et al., 1992; Ohara et al., 2006), or those accepting homogentisate and flavonoid prenyltransferases characterized thus far. While *p*-hydroxybenzoate prenyltransferases occurring from bacteria to human have a similar membrane topology to flavonoid prenyltransferases, i.e. containing 7 to 9 transmembrane α -helices (Yazaki et al., 2002; Bräuer et al., 2008), their amino acid identity levels with homogentisate prenyltransferases are fairly low (<20%). It is generally accepted that the genes of secondary metabolism have evolved from those of primary metabolism through gene duplication and recruitment (Ober, 2005). In the phylogenetic tree of homogentisate and flavonoid prenyltransferases (Fig. 7), G4DT and PT3 are clustered on a single branch, and this branch has a common origin with the *S. flavescens* proteins, including SfN8DT. PT1 is within the branch of phytyltransferases involved in tocopherol biosynthesis, while the amino acid identity between PT1 and G4DT is 50%. It is very likely that flavonoid and pterocarpan (isoflavonoid) prenyltransferase have evolved through recruitment from the vitamin E and plastoquinone biosynthetic pathways in plants.

By crystallographic analysis of FPP synthases (Tarshis et al., 1994), the Asp-rich motif (DDxxDxxxD) was shown to be responsible for DMAPP binding via Mg^{2+} ion, and the corresponding region in the G4DT polypeptide is NQxxDxxxD in loop 2 (Fig. 2A), and a similar sequence is also conserved in loop 2 of

p-hydroxybenzoate prenyltransferases, NDxxDxxxD (Yazaki et al., 2002). The role of the other conserved region, KD(I/L)xDx(E/D)GD, in loop 6 of flavonoid prenyltransferase (Fig. 2A) is unpredictable, because this conserved sequence is only observed in the flavonoid/homogentisate prenyltransferases whose three-dimensional structures are currently uncharacterized. These seem to be related to the amino acid sequence differences between flavonoid-specific and homogentisate prenyltransferases (Supplemental Fig. S4): Y123 for the former (G4DT), H127 for the latter (PT1); K/R197 for the former, V263 and R264 for the latter; and P/S284 for the former, but their functions in substrate recognition are as yet unclarified.

Although G4DT did not employ isoflavones as a substrate, many prenylated isoflavones in bean, lupin, licorice, and other legumes are known (Dewick, 1993), and a prenyltransferase activity acting on the isoflavones genistein and 2'-hydroxygenistein has been reported in lupin (Schröder et al., 1979). In addition, a pterocarpanoid dimethylallyltransferase with different regiospecificity (3,9-dihydroxypterocarpan 10-dimethylallyltransferase) has been characterized in bean (Biggs et al., 1987). A large family of isoflavonoid prenyltransferases can thus be envisaged.

Application of Prenyltransferase Genes for Metabolic Engineering

Soybean is undoubtedly one of the most important leguminous crops, and extensive basic information regarding its disease resistance is available, including the intensive work on the significance of phytoalexin production in differential fungal resistance among the soybean cultivars (Yoshikawa et al., 1978) and also glucan elicitor recognition (Umemoto et al., 1997). The ongoing project of sequencing the whole genome of soybean in Japan and the US will bring about rapid advances in the understanding of these processes from elicitor perception and signal transduction to the biosynthesis of phytoalexins and the evolution of the stress resistance machinery. While a preliminary examination of the soybean genome database for the cDNAs PT1 to PT3 showed that the corresponding genes are mapped on different scaffolds and not arranged in tandem on a single chromosome (Soybean Genome Project, DoE Joint Genome Institute <http://www.phytozome.net/soybean.php>; Supplemental Table S1), more detailed information is expected to reveal the organization of related genes and their evolution in the near future. Further studies on pterocarpan prenyltransferases should also be useful in the functional evaluation of phytoalexins with unique structures in terms of soybean physiology.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

cDNAs are extracted from young soybean (*Glycine max*) seedling as reported previously (Akashi et al., 2005). The callus culture of soybean was

donated by Dr. T. Yoshikawa of Kitasato University and grown on Murashige and Skoog medium containing 9 g L⁻¹ agar, 30 g L⁻¹ Suc, 1 mg L⁻¹ kinetin, and 0.1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid in the dark at 25°C with a 4-week culture cycle. Suspension culture was started by inoculating 3-week-old callus (about 10 g) into 200 mL of the same medium without agar. After 2 weeks, a portion of the culture was diluted with 4 volumes of freshly prepared medium, grown for 2 further weeks, and used for the induction experiments. Elicitation was performed with 0.3% (w/v) yeast extract (Difco Laboratories).

EST Database Search, cDNA Cloning, and Heterologous Expression

The EST database of soybean (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gmain.pl?gudb=soybean>) was searched for cDNAs homologous to VTE2-1 of *Arabidopsis* (*Arabidopsis thaliana*; AtVTE2-1, AY089963). The full ORF of PT2 was amplified by RT-PCR with KOD polymerase (Toyobo), primers (PT2-F and PT2-R), and a cDNA template from soybean seedlings (Akashi et al., 2005). The truncated form of PT2, lacking the region encoding the 44 amino acids of the putative transit peptide sequence at the N terminus, was obtained according to the procedure mentioned above except for the use of primers (PT2/d44-F and PT2-R). Both PCR products were cloned into pYES2.1/TOPO vector (Invitrogen). Introduction of the plasmid into *Saccharomyces cerevisiae* BJ2168, selection of the transformant, and induction of the recombinant protein were performed as described previously (Akashi et al., 1998). Yeast cells were suspended in 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. The cells were disrupted by vigorous shaking with glass beads, and microsomes were prepared by ultracentrifugation (Akashi et al., 1998). The microsomal precipitates were homogeneously suspended in the same buffer as above (approximately 0.5 mg mL⁻¹ microsome protein). The protein content was calculated by the method of Bradford (1976). Yeast cells transformed with pYES2 were used as controls in the enzyme assay.

Enzyme Assay

The standard assay conditions in this study were as follows: 400 μ M (–)-glycinol, 400 μ M DMAPP, and 10 mM MgCl₂ were incubated with recombinant yeast microsomes (40 μ g microsome protein) in a total volume of 250 μ L at 30°C for 10 min. An ethyl acetate extract of the reaction mixture was analyzed by HPLC with LC-2000 system (JASCO). HPLC was performed using a CAPCELL PAK C18 MG column (4.6 $\times$ 150 mm; Shiseido) at 40°C with a flow rate of 1 mL min⁻¹ and a linear gradient elution for 20 min from 40% to 80% (v/v) methanol in water. The eluate was monitored using a multi-wavelength detector (MD-2010, JASCO). For divalent cation requirement experiments, 10 mM MnCl₂ or 10 mM CoCl₂ instead of MgCl₂ was added to the reaction mixture as above, and activity was assayed. For kinetic studies, varying concentrations (5, 10, 20, 40, 80, 160, and 400 μ M) of (–)-glycinol versus a fixed concentration of DMAPP (400 μ M) and varying concentrations of DMAPP (10, 20, 40, 80, 160, and 400 μ M) versus a fixed concentration of (–)-glycinol (400 μ M) were incubated with recombinant yeast microsome expressing the truncated form of PT2 (10 μ g microsome protein) in a total volume of 250 μ L at 30°C for 10 min to calculate K_m values using Lineweaver-Burk plots. For NMR identification of the reaction product, 1 mg (–)-glycinol, 1 mg DMAPP, and 3 mg recombinant yeast microsomes expressing the truncated form of PT2 were used for the assay. After 3 h incubation, an ethyl acetate extract of the products was subjected to silica gel (Wako gel C-200, Wako Pure Chemical; 10 g) column chromatography with hexane:ethyl acetate (3:7, v/v) as the eluting solvent, and a fraction containing the reaction product was obtained.

Purification and Identification of Pterocarpanoids in Soybean Cells

Yeast extract-treated suspension cultures (48 h, 1,000 mL) were used for the partial purification of glyceollins. The cells (approximately 50 g) were lyophilized and extracted with ethyl acetate, and the medium was partitioned with ethyl acetate. The combined ethyl acetate extracts (1 g) were subjected to silica gel (100 g) column chromatography with hexane:ethyl acetate (3:7, v/v) as the eluting solvent. A fraction (40 mg) containing glyceollin I and III (approximately 70% purity) was then applied onto a second silica gel (50 g) column with CHCl₃:methanol (8:2, v/v) to obtain a fraction (20 mg) containing

glyceollin I and III (>95% purity). Further separation of glyceollin I from glyceollin III was only achieved by HPLC. For the purification of (–)-glycinol, a suspension culture of soybean (500 mL) was treated with 0.3% yeast extract (w/v), 100 μ M lovastatin (mevalonate-pathway inhibitor), and 100 μ M fosmidomycin (MEP-pathway inhibitor) for 24 h. The medium was collected and partitioned with ethyl acetate. The ethyl acetate extract (0.4 g) was subjected to silica gel column chromatography with hexane:ethyl acetate (3:7, v/v). A fraction (20 mg) containing (–)-glycinol (approximately 70% purity) was then applied to silica gel thin-layer chromatography [Kieselgel F254 (Merck); solvent, CHCl₃:methanol (8:2, v/v)], and a product [(–)-glycinol, 10 mg, >95% purity, R_f 0.40] was collected. NMR spectra were recorded on a JMN ECA-500 system (JEOL) in acetone-*d*₆.

HPLC Analysis of Isoflavonoids

A suspension culture (200 mL) was treated with 0.3% (w/v) yeast extract. Portions of the culture (10 mL) were periodically collected and extracted with ethyl acetate (10 mL) using a Polytron homogenizer. After centrifugation (3,000g for 2 min), the ethyl acetate extracts were collected and analyzed by HPLC for pterocarpan and daidzin, as mentioned above in detail. For the analysis of daidzin, portions of the culture (1 mL) were homogenized and extracted with 4 mL methanol. An aliquot of the methanol extract was heated at 70°C for 10 min and cooled to room temperature to remove the unstable malonyl group attached to the sugar moiety and analyzed by HPLC as above. The content of isoflavonoids was determined from the peak areas of the compounds calibrated with those of known concentrations of the samples. The eluate was monitored at 250 nm for daidzin and daidzein or at 285 nm for glycinol and glyceollins. Retention times of isoflavonoids: daidzin (3.5 min), glycinol (5.0 min), daidzein (11.4 min), glyceollin III (17.7 min), and glyceollin I (18.2 min).

Administration of [1-¹³C]Glc into Suspension-Cultured Soybean Cells

Soybean cells were cultured in liquid medium (200 mL) as described above with [1-¹³C]Glc (250 mg) for 1 week. [1-¹³C]Glc (250 mg) and 0.3% (w/v) yeast extract were administered to the culture, and the culture was maintained for 48 h. Glyceollins were purified as above.

RT-PCR Analysis

Total RNA was isolated from the soybean cells using an RNeasy Plant Mini kit (Qiagen), and cDNAs were synthesized using a Ready-To-Go T-Primed First Strand kit (BD Biosciences). DNase treatment was done before column purification of RNA according to the manufacturer's instruction. RT-PCR was carried out using *ExTaq* DNA polymerase and specific primers. The reaction was initiated with denaturation at 94°C for 1 min, followed by 25 or 30 cycles of 3-step incubation (94°C, 1 min; 55°C, 1 min; 72°C, 1 min). The quantity of each template was adjusted to give equal amplification of actin cDNA. Amplification efficiency was considered to be linear during the PCR (25 cycles for *IFS1*, *HID*, *P6aH*, and *G4DT*, and 30 cycles for *IFS2* and *actin*). The products after electrophoresis on 1.2% (w/v) agarose gel were stained with ethidium bromide. The intensity of bands was quantified by using Quantity One software (Bio-Rad).

Phylogenetic Analysis

The amino acid sequences were analyzed using the ClustalW program (Thompson et al., 1994) of the DNA Data Bank of Japan (Shizuoka, Japan). A neighbor-joining tree was produced from the results of 1,000 bootstrap replicates. The tree was displayed using TreeView (Page, 1996).

Construction of GFP Fusion Proteins

The coding sequence for the putative transit peptide sequence at the N terminus of G4DT (44 amino acids) was amplified by PCR with KOD polymerase, primers (G4DT-F1 and G4DT-R2), and G4DT cDNA as the template. The PCR products were recombined into pDONR221 (Invitrogen) and then into modified pGWB5 vector (Sasaki et al., 2008), and the fusion protein G4DT(TP)-GFP was expressed from the CaMV35S promoter. Recombination reactions were performed according to the manufacturer's protocol

(Invitrogen). WxTP-DsRed as a control plastid targeted protein was a kind gift from Dr. Toshiaki Mitsui, Niigata University. Ten micrograms of both GFP and WxTP-DsRed plasmids were precipitated onto 1.0- μ m spherical gold beads (Bio-Rad). Onion (*Allium cepa*) peels were bombarded using a particle gun PDS-1000 (Bio-Rad) according to the manufacturer's instructions. After 24 h, GFP and RFP fluorescence in the onion cells was observed using an Axioskop 2 microscope (Zeiss).

DDBJ accession number for the gene isolated in this article is AB434690 (G4DT cDNA).

Supplemental Data

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

Supplemental Figure S1. Long-range correlations observed in the HMBC spectrum of 4-dimethylallylglycinol.

Supplemental Figure S2. Thin-layer chromatography autoradiograms of products from the incubation of flavonoids and [14 C]DMAPP with microsomal fractions of yeast cells expressing PT2.

Supplemental Figure S3. Lineweaver-Burk plots.

Supplemental Figure S4. Multiple alignment of the amino acid sequences among flavonoid and homogentisate prenyltransferases.

Supplemental Figure S5. 13 C-NMR spectra of glyceollin I at natural 13 C abundance and from the experiment with [13 C]Glc.

Supplemental Table S1. Database search results.

Supplemental Data S1. PCR primers and NMR data.

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LITERATURE CITED

- Akashi T, Aoki T, Ayabe S (1998) Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.; Fabaceae) which represents licodione synthase and flavone synthase II. *FEBS Lett* **431**: 287–290
- Akashi T, Aoki T, Ayabe S (2005) Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. *Plant Physiol* **137**: 882–891
- Asada Y, Li W, Yoshikawa T (2000) Biosynthesis of the dimethylallyl moiety of glabrol in *Glycyrrhiza glabra* hairy root cultures via a non-mevalonate pathway. *Phytochemistry* **55**: 323–326
- Ashby MN, Kutsunai SY, Ackerman S, Tzagoloff A, Edwards PA (1992) COQ2 is a candidate for the structural gene encoding para-hydroxybenzoate:polyprenyltransferase. *J Biol Chem* **267**: 4128–4136
- Ayabe S, Akashi T (2006) Cytochrome P450s in flavonoid metabolism. *Phytochem Rev* **5**: 271–282
- Biggs D, Welle R, Grisebach H (1990) Intracellular localization of prenyltransferases of isoflavonoid phytoalexin biosynthesis in bean and soybean. *Planta* **181**: 244–248
- Biggs DR, Welle R, Visser FR, Grisebach H (1987) Dimethylallylpyrophosphate:3,9-dihydroxypterocarpan 10-dimethylallyl transferase from *Phaseolus vulgaris*. Identification of the reaction product and properties of the enzyme. *FEBS Lett* **220**: 223–226
- 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
- Bräuer L, Brandt W, Schulze D, Zakharaova S, Wessjohann L (2008) A structural model of the membrane-bound aromatic prenyltransferase UbiA from *E. coli*. *ChemBioChem* **9**: 982–992
- Dewick PM (1993) Isoflavonoids. In JB Harborne, ed, *The Flavonoids*. Advances in Research since 1986. Chapman and Hall, London, pp 117–238
- Dixon RA (1999) Isoflavonoids: biochemistry, molecular biology, and biological functions. In U Sankawa, ed, *Comprehensive Natural Products Chemistry*, Vol 1. Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives. Elsevier, Oxford, pp 773–823
- Ebel J (1986) Phytoalexin synthesis: the biochemical analysis of the induction process. *Annu Rev Phytopathol* **24**: 235–264
- Ebel J, Grisebach H (1988) Defense strategies of soybean against the fungus *Phytophthora megasperma* f.sp. *glycinea*: a molecular analysis. *Trends Biochem Sci* **13**: 23–27
- Farag MA, Huhman DV, Dixon RA, Sumner LW (2008) Metabolomics reveals novel pathways and differential mechanistic and elicitor-specific responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures. *Plant Physiol* **146**: 387–402
- Graham TL, Kim JE, Graham MY (1990) Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Mol Plant Microbe Interact* **3**: 157–166
- Hsieh MC, Graham TL (2001) Partial purification and characterization of a soybean β -glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **58**: 995–1005
- Laflamme P, Khouri H, Gulick P, Ibrahim R (1993) Enzymatic prenylation of isoflavones in white lupin. *Phytochemistry* **34**: 147–151
- Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol Plant* **101**: 643–652
- Nauumkina M, Farag MA, Sumner LW, Tang Y, Liu CJ, Dixon RA (2007) Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*. *Proc Natl Acad Sci USA* **104**: 17909–17915
- Ober D (2005) Seeing double: gene duplication and diversification in plant secondary metabolism. *Trends Plant Sci* **10**: 444–449
- Ohara K, Yamamoto K, Hamamoto M, Sasaki K, Yazaki K (2006) Functional characterization of *OsPPT1*, which encodes *p*-hydroxybenzoate polyprenyltransferase involved in ubiquinone biosynthesis in *Oryza sativa*. *Plant Cell Physiol* **47**: 581–590
- Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**: 357–358
- 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
- Schopfer CR, Kochs G, Lottspeich F, Ebel J (1998) Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (*Glycine max* L.). *FEBS Lett* **432**: 182–186
- 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
- Shimada N, Sato S, Akashi T, Nakamura Y, Tabata S, Ayabe S, Aoki T (2007) Genome-wide analyses of the structural gene families involved in the legume-specific 5-deoxyisoflavonoid biosynthesis of *Lotus japonicus*. *DNA Res* **14**: 25–36
- Suzuki H, Takahashi S, Watanabe R, Fukushima Y, Fujita N, Noguchi A, Yokoyama R, Nishitani K, Nishino T, Nakayama T (2006) An isoflavone conjugate-hydrolyzing β -glucosidase from the roots of soybean (*Glycine max*) seedlings: purification, gene cloning, phylogenetics, and cellular localization. *J Biol Chem* **281**: 30251–30259
- Tahara S, Ibrahim RK (1995) Prenylated isoflavonoids—an update. *Phytochemistry* **38**: 1073–1094
- Tarshis LC, Yan M, Poulter CD, Sacchettini JC (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution. *Biochemistry* **33**: 10871–10877
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through se-

- quence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680
- Umemoto N, Kakitani M, Iwamatsu A, Yoshikawa M, Yamaoka N, Ishida I** (1997) The structure and function of a soybean β -glucan-elicitor-binding protein. *Proc Natl Acad Sci USA* **94**: 1029–1034
- Veitch NC** (2007) Isoflavonoids of the Leguminosae. *Nat Prod Rep* **24**: 417–464
- Welle R, Grisebach H** (1988) Induction of phytoalexin synthesis in soybean: enzymatic cyclization of prenylated pterocarpan to glyceollin isomers. *Arch Biochem Biophys* **263**: 191–198
- Welle R, Grisebach H** (1991) Properties and solubilization of the prenyltransferase of isoflavonoid phytoalexin biosynthesis in soybean. *Phytochemistry* **30**: 479–484
- Yamamoto H, Zhao P, Inoue K** (2002) Origin of two isoprenoid units in a lavandulyl moiety of sophoraflavanone G from *Sophora flavescens* cultured cells. *Phytochemistry* **60**: 263–267
- Yazaki K, Kuniyama 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
- Yoshikawa M, Yamauchi K, Masago H** (1978) Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. *Physiol Plant Pathol* **12**: 73–82
- Zähringer U, Ebel J, Mulheim LJ, Lyne RL, Grisebach H** (1979) Induction of phytoalexin synthesis in soybean. Dimethylallylpyrophosphate: trihydroxypterocarpan dimethylallyl transferase from elicitor-induced cotyledons. *FEBS Lett* **101**: 90–92
- Zähringer U, Schaller E, Grisebach H** (1981) Induction of phytoalexin synthesis in soybean. Structure and reactions of naturally occurring and enzymatically prepared prenylated pterocarpan from elicitor-treated cotyledons and cell cultures of soybean. *Z Naturforsch [C]* **36**: 234–241

Supplementary data

1. PCR primers

Three specific primers were designed from PT2 of soybean: PT2-F, 5'-AAAATGGATTGGGGGCTTGCTATATCTTC-3'; PT2/d44-F, 5'-AAAATGCACAAAAGGGAACTCAAATAGAACATAATG-3'; PT2-R, 5'-TCCTCATCTAATTAATGCCATGAG-3'.

The following PCR primers were used for RT-PCR; IFS1/F (5'-GTTGTTAGAGAATCATCAAAAAGTGCG-3') and IFS1/R (5'-TGGTTATTGAATAACATTCGGAGTC-3') for IFS1, IFS2/F (5'-CAACAAGGAAATCGGTGGAACGCG-3') and IFS2/R (5'-AAGGTGTTTAATGTCATTTGGAGCG-3') for IFS2, HIDH/F (5'-CGTAGGAGGTGAAACCAGCGGTGC-3') and HIDH/R (5'-TTGGAATCTCTCATGACAAGCCATC-3') for HID, P6aH/F (5'-ATTGGACATGCACGAAGATGAGAATG-3') and P6aH/R (5'-CACTGGTTATTGAATAACATTCGGAGTC-3') for P6aH, G4DT/F (5'-CCACATCTTCCAATGGCATCTGGAC-3') and G4DT/R (5'-GCCCCGTTTCTGACCTAGACGTACTG-3') for G4DT and Act/F (5'-ATGGCAGATGGTGAGGATATTCAACC-3') and Act/R (5'-CGTAATCAAGGGCAATATAAGCCAGC-3') for actin.

The following PCR primers were used for the construction of GFP fusion proteins; G4DT-F1

(5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCAT
GGATTGGGGGCTTGCTATATCTTC-3'), G4DT-R2

(5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCGTGAAGCTTTTGATATCC
AAGAAC-3') and G4DT-R1

(5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTAATTAATGCCATGAGA
AAGAACCCTGC-3')

2. NMR data

4-Dimethylallylglycinol (G4DT reaction product)

^1H -NMR [(acetone-*d*6); δ : 1.60 (3H, *s*, H-5'), 1.72 (3H, *s*, H-4'), 3.29 (2H, *d*, $J = 6.9$ Hz, H-1'), 4.06 (1H, *d*, $J = 11.5$ Hz, H-6), 4.14 (1H, *d*, $J = 11.5$ Hz, H-6), 5.19 (1H, *m*, H-2'), 5.28 (1H, *s*, H-11a), 6.22 (1H, *d*, $J = 2.3$ Hz, H-10), 6.42 (1H, *dd*, $J = 2.3, 8.0$ Hz, H-8), 6.59 (1H, *d*, $J = 8.0$ Hz, H-2), 7.14 (1H, *d*, $J = 8.0$ Hz, H-1), 7.21 (1H, *d*, $J = 8.0$ Hz, H-7). ^{13}C -NMR δ : 17.9 (C-4'), 22.9 (C-1'), 25.9 (C-5'), 71.2 (C-6), 76.9 (C-6a), 86.7 (C-11a), 98.5 (C-10), 108.8 (C-8), 110.2 (C-2), 113.7 (C-11b), 116.6 (C-4), 121.7 (C-6b), 123.8 (C-2'), 125.2 (C-7), 129.9 (C-1), 131.1 (C-3'), 154.9 (C-4a), 156.8 (C-3), 160.7 (C-9), 162.1 (C-10a).

(-)-Glycinol

^1H -NMR [(acetone-*d*6); δ : 4.02 (1H, *d*, $J = 11.5$ Hz, H-6), 4.12 (1H, *d*, $J = 11.5$ Hz, H-6), 5.26 (1H, *s*, H-11a), 6.24 (1H, *d*, $J = 2.3$ Hz, H-10), 6.32 (1H, *d*, $J = 2.3$ Hz, H-4), 6.42 (1H, *dd*, $J = 2.3, 8.0$ Hz, H-8), 6.55 (1H, *dd*, $J = 2.3, 8.6$ Hz, H-2), 7.20 (1H, *d*, $J =$

8.0 Hz, H-7), 7.30 (1H, *d*, *J* = 8.6 Hz, H-1). ¹³C-NMR δ: 70.6 (C-6), 76.5 (C-6a), 86.0 (C-11a), 98.7 (C-10), 103.8 (C-4), 108.8 (C-8), 110.7 (C-2), 112.5 (C-11b), 121.5 (C-6b), 125.2 (C-7), 133.2 (C-1), 157.1 (C-5), 159.6 (C-3), 160.7 (C-9), 162 (C-10a).

Glyceollin I

¹H-NMR [(acetone-*d*₆); δ: 1.35 (3H, *s*, H-4'), 1.39 (3H, *s*, H-5'), 4.11 (1H, *d*, *J* = 11.5 Hz, H-6), 4.19 (1H, *d*, *J* = 11.5 Hz, H-6), 5.27 (1H, *s*, H-11a), 5.66 (1H, *d*, *J* = 10.3 Hz, H-2'), 6.25 (1H, *d*, *J* = 2.3 Hz, H-10), 6.43 (1H, *dd*, *J* = 2.3, 8.0 Hz, H-8), 6.47 (1H, *d*, *J* = 8.0 Hz, H-2), 6.57 (1H, *d*, *J* = 10.3 Hz, H-1'), 7.22 (1H, *d*, *J* = 8.0 Hz, H-7), 7.24 (1H, *d*, *J* = 8.0 Hz, H-1).

Glyceollin III

¹H-NMR [(acetone-*d*₆); δ: 1.74 (3H, *s*, H-5'), 2.99 (1H, *ddd*, *J* = 1.1, 8.0, 15.4 Hz, H-1'), 3.37 (1H, *dd*, *J* = 9.7, 15.4 Hz, H-1'), 4.05 (1H, *d*, *J* = 11.5 Hz, H-6), 4.11 (1H, *d*, *J* = 11.5 Hz, H-6), 4.88 (1H, *m*, H-4'), 5.05 (1H, *m*, H-4'), 5.23 (1H, *dd*, *J* = 8.0, 9.7 Hz, H-2'), 5.27 (1H, *s*, H-11a), 6.24 (1H, *d*, *J* = 2.3 Hz, H-10), 6.24 (1H, *s*, H-4), 6.42 (1H, *dd*, *J* = 2.3, 8.0 Hz, H-8), 7.20 (1H, *s*, H-1), 7.22 (1H, *d*, *J* = 8.0 Hz, H-7).

Supplementary Table

Supplementary Table 1. Database search results

	EST database ¹ accession no.	Identity with AtVTE2-1 (%) ²	Genome database Scaffold no. (length) ³	Identity with each PT (%) ⁴
PT1	TC229226	75	scaffold_84 (3.6Mb) scaffold_25 (9.4Mb)	>95 >90
PT2	TC227057	50	scaffold_52 (5.9Mb)	>95
PT3	TC207773	52	scaffold_101 (2.9Mb)	>95
¹	DFCI		EST	database

<<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=soybean>>.

² At the amino acid level.

³ Phytozome soybean genome database (Soybean Genome Project, DoE Joint Genome Institute <<http://www.phytozome.net/soybean.php>>) was aligned using the amino acid sequence of each PT, and the scaffold containing over 90% identical sequences is shown. Length (bp) of each scaffold is shown in the parenthesis.

⁴ At the amino acid level.

Supplementary Figure legends

Supplementary Figure 1. Long range correlations observed in the HMBC spectrum of 4-dimethylallylglycinol.

Supplementary Figure 2. TLC autoradiograms of products from the incubation of flavonoids and [1-¹⁴C]-DMAPP with microsomal fractions of yeast cells expressing PT2.

(Method)

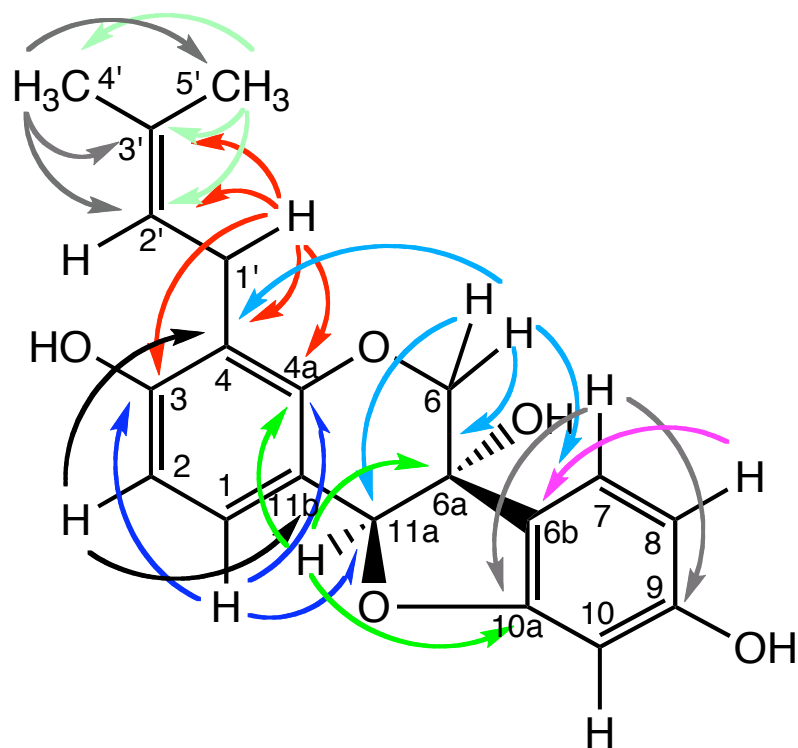
The full ORF and the truncated form of PT2 (G4DT) were expressed as yeast transformants (W303-1A-Δcoq2) by culturing in SD (-ura) liquid media (180 mL) to reach mid-log phase, and from which the microsomal fraction was prepared as described previously (Yazaki et al., 2002). The membrane fraction from each transformant was resuspended in 500 μL 0.1 mM Tris-HCl buffer (pH 7.5), which was then used as an enzyme solution for the prenyltransferase assay using flavonoids and 4.5 μM [1-¹⁴C]-DMAPP (specific activity 2.0 GBq mmol⁻¹, American Radiolabeled Chemicals, Inc. MO, USA) as substrates, as described previously [Yamamoto et al., (2000) Flavanone 8-dimethylallyltransferase in *Sophora flavescens* cell suspension cultures. *Phytochemistry* **54**: 649-655]. After incubation, the ethyl acetate-soluble portion was evaporated to dryness, dissolved in 20 μL of methanol, and spotted onto a silica gel TLC plate (20 x 20 cm, Silica gel 60 F₂₅₄, MERCK). TLC was developed with toluene:ethyl acetate:acetic acid (70:30:0.3), and subjected to autoradiography to

detect the reaction products. The radioactivity of the products was quantified using a BAS1800 Bio-image Analyzer (Fuji Film, Japan).

Supplementary Figure 3. Lineweaver-Burk plots. Apparent K_m values were calculated from the Lineweaver-Burk plots with varying concentrations of (-)-glycinol (5-400 μ M) (A, C) and of DMAPP (10 20, 40, 80, 160, and 400 μ M) (B, D). Recombinant yeast microsome expressing the truncated form of PT2 (A, B) and microsome of cultured soybean cells treated with yeast extract elicitor for 24 h (C, D) were used. The values are the averages of two independent experiments (maximum deviation was about 10%).

Supplementary Figure 4. Multiple alignment of the amino acid sequences among flavonoid and homogentisate prenyltransferases. Sequences were aligned using the ClustalW program and MacBoxshade. Identical, similar, and conserved amino acids are indicated by black, dark grey, and light grey shading, respectively, using a 60% consensus threshold. A dot represents a gap. Asterisks show differences in the amino acid sequence between flavonoid-specific and homogentisate prenyltransferases. The plant species names are given in Figure 7 legend.

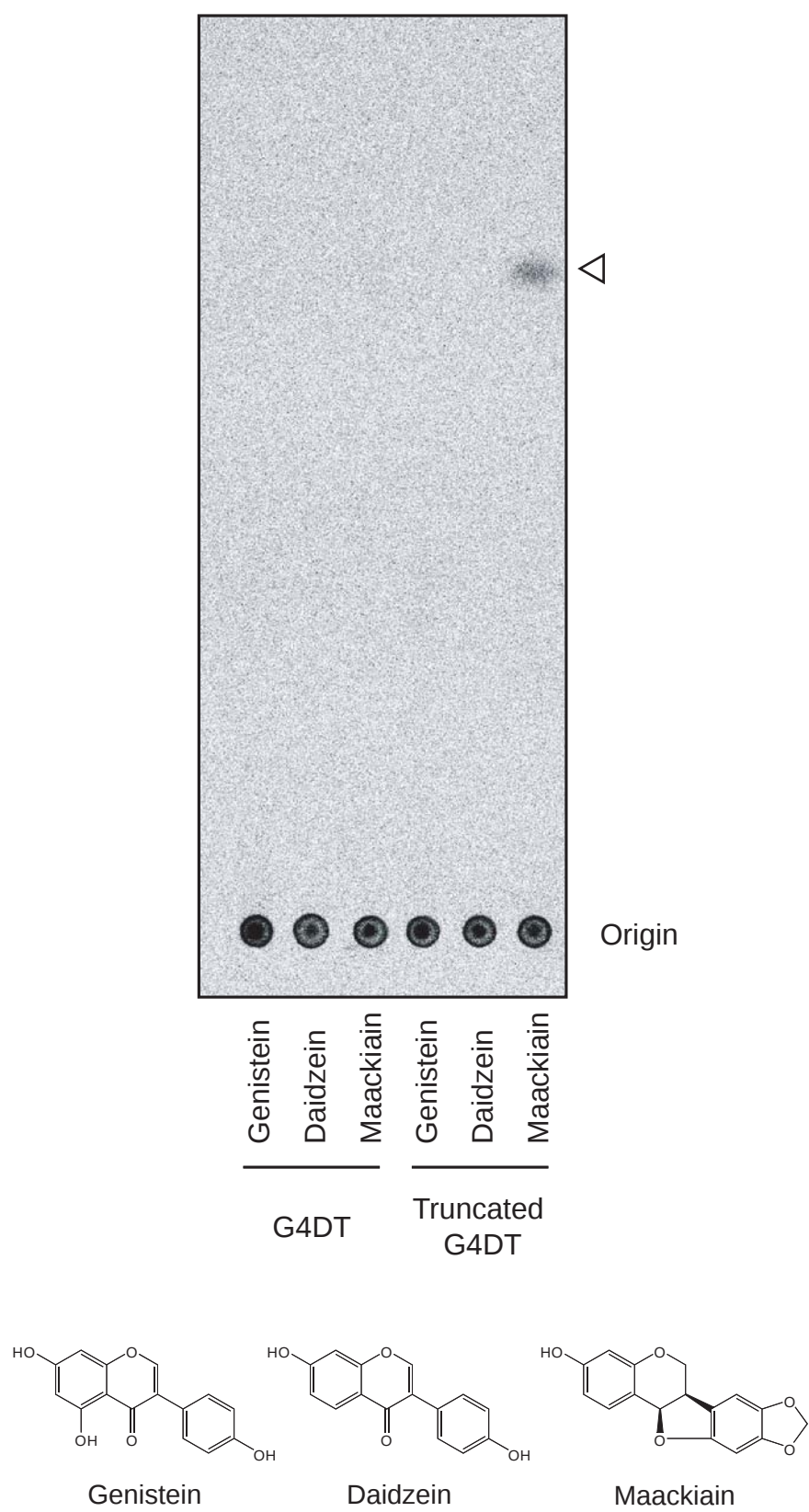
Supplementary Figure 5. ^{13}C -NMR spectra of glyceollin I at natural ^{13}C -abundance (A) and from the experiment with $[1-^{13}\text{C}]$ glucose (B). Asterisks indicate significantly enriched ^{13}C atoms.



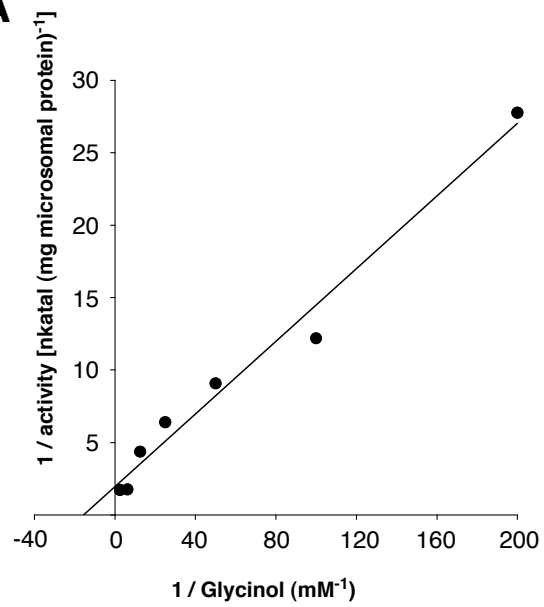
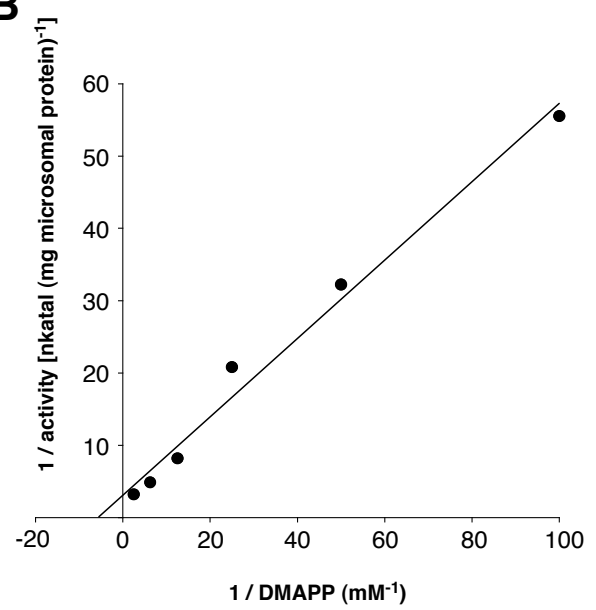
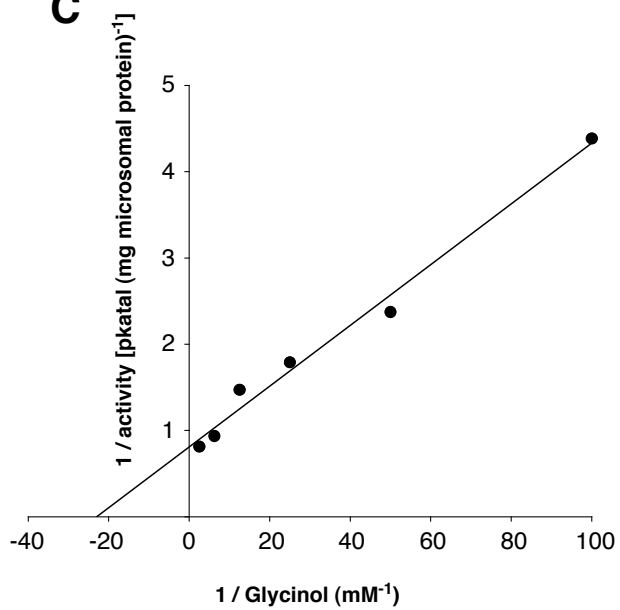
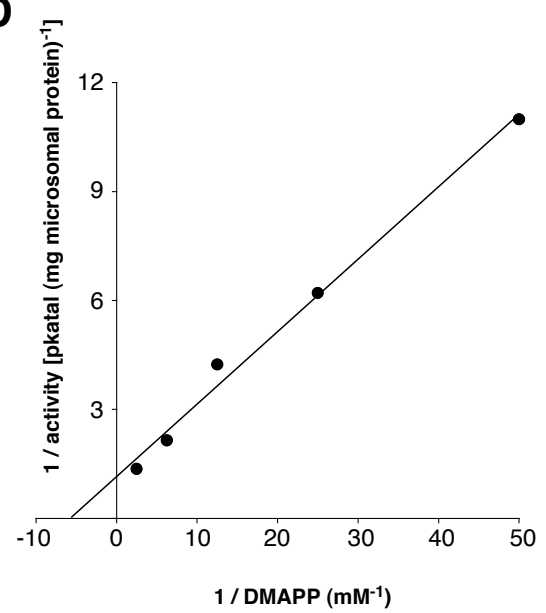
4-Dimethylallylglucitol

Position	HMBC correlations
H-1	C-3, C-4a, C-11a
H-2	C-4, C-11b
H-6	C-4a, C-6a, C-6b, C-11a
H-7	C-9, C-10a
H-8	C-6b
H-10	
H-11a	C-4a, C-6a, C-11b
H-1'	C-3, C-4, C-4a, C-2', C-3'
H-2'	
H-4'	C-2', C-3', C-5'
H-5'	C-2', C-3', C-4'

Akashi et al., Supplementary Fig. 1



Akashi et al., Supplementary Fig. 2

A**B****C****D**

Akashi et al., Supplementary Fig. 3

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      . . . . .10 . . . . .20 . . . . .30 . . . . .40 . . . . .50 . . . . .60
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SfN8DT-2 . . . . .MGFVLPASFPGASSITTTGGSCLRSKQYAKN.YYASSYVTTTLWHKKGKIQKEYCAVI: 55
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SfL17a . . . . .MDSVLRASFPRVSSISTGGSCWRSKQNAKH.YFTSSNVTTTSWHKTGKIQKEYCVVM: 55
G4DT . . . . .MDWGLAISSHPKPYSVTGGNLWRSKHTTKNIYFASSWISKASRHKRETQIEHNVLR: 57
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ZmVTE2-1 . . . . .MDALRLRPSLLSVRP..GAARPRDHFLPPCCSIQRNGEGRICFSSQ: 44
ApVTE2-1 . . . . .MLSMDSLLTKPVIPLPSPVCSLPILRGSSAPGGQYSCRNYNPIRIQ: 46
CpVTE2-1 . . . . .MRMESLILLNSFSPSPAGGKICRADTYKKAYFATAR.CNTLNSLNKN: 45
PT1 . . . . .MKFKSQQTQTQPPQHYCIWIWIRCFDFLFLILTTLHLSPPLVLICQMLHGTEIKIQKEYK: 58
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TaHGGT . . . . .MQATTAAAAAQLLTDTRRGPRCSRARLGATRLSWPGRFAVEAFAGRCQSSATT: 53
OsHGGT . . . . .MQASSAAAAAACSAIKPAAHQHTVQVQEDKRGSEFRARFGTRKLS.....WGGK: 49
GmVTE2-2 . . . . .MELSLSPTSSTRVPS.....TIPTLNSAKLSSTKATKSQQP: 35
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GmVTE2-2 LFLGFSGKHFNISIGLHHHSYRCCSNAVPERPQRPSSIRACTGVGASGSDRPLAERLLDLKD: 95
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CpVTE2-1 LSDIDIDKVNKPYLPLASGEFSVGTGVTIVTSFLIMSFWLGWVVGSPPLFWALFISFVLG:217
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CpVTE2-1 MSFFSIVIALFKDIPDIEGDKIYFGIRSFVRLGQERVFWICISLLEMAYAVAILVGSTSP:337
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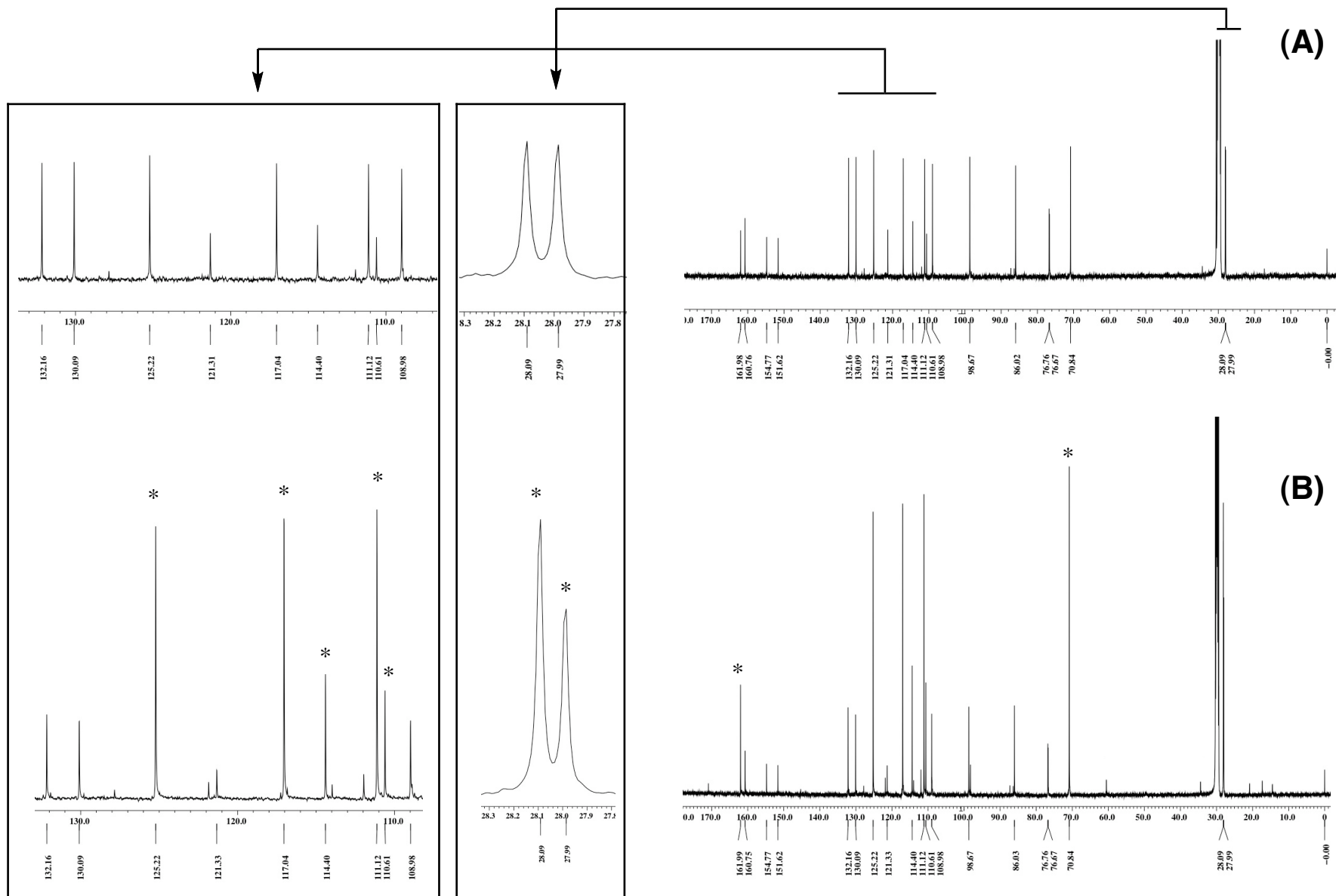
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KD(I/L)xDx(E/D)GD

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Akashi et al., Supplementary Fig. 5