The Missing Link in Leguminous Pterocarpan Biosynthesis is a Dirigent Domain-Containing Protein with Isoflavanol Dehydratase Activity

Kai Uchida1, Tomoyoshi Akashi1 and Toshio Aoki*
Department of Applied Biological Sciences, Nihon University, Fujisawa, Kanagawa, 252-0880 Japan
1These authors contributed equally to this work.
*Corresponding author: E-mail, aoki.toshio@nihon-u.ac.jp; Fax, +81-466-84-3353.
(Received August 24, 2016; Accepted November 25, 2016)

Pterocarpan forms the basic structure of leguminous phytoalexins, and most of the isoflavonoid pathway genes encoding the enzymes responsible for its biosynthesis have been identified. However, the last step of pterocarpan biosynthesis is a ring closure reaction, and the enzyme that catalyzes this step, 2′-hydroxyisoflavanol 4,2′-dehydratase or pterocarpan synthase (PTS), remains as an unidentified ‘missing link’. This last ring formation is assumed to be the key step in determining the stereochemistry of pterocarpons, which plays a role in their antimicrobial activity. In this study, a cDNA clone encoding PTS from Glycyrrhiza echinata (GpPTS1) was identified through functional expression fractionation screening of a cDNA library, which requires no sequence information, and orthologs from soybean (GmPTS1) and Lotus japonicus (LjPTS1) were also identified. These proteins were heterologously expressed in Escherichia coli and biochemically characterized. Surprisingly, the proteins were found to include amino acid motifs characteristic of dirigent proteins, some of which control stereospecific phenoxy radical coupling in lignan biosynthesis. The stereospecificity of substrates and products was examined using four substrate stereoisomers with hydroxy and methoxy derivatives at C-4′. The results showed that the 4R configuration was essential for the PTS reaction, and (−)- and (+)-pterocarpons were produced depending on the stereochemistry at C-3. In suspension-cultured soybean cells, levels of the GmPTS1 transcript increased temporarily prior to the peak in phytoalexin accumulation, strongly supporting the possible involvement of PTS in pterocarpan biosynthesis.

Keywords: Dirigent domain • 2′-Hydroxyisoflavanol 4,2′-dehydratase • Isoflavonoid • Functional expression fractionation screening • Phytoalexin • Pterocarpan synthase.

Abbreviations: CDS, coding sequence; DDBJ, DNA Data Bank of Japan; DIR, dirigent protein; DMI, 7,2′-dihydroxy-4′-methoxyisoflavonol; DMID, 7,2′-dihydroxy-4′-methoxyisoflavonol dehydratase; HID, 2-hydroxyisoflavonone dehydratase; IFR, isoflavone reductase; I2′H, isoflavone 2′-hydroxylase; IPTG, isopropyl β-D-thiogalactopyranoside; kcat, turnover number; LB, Luria broth; NMR, nuclear magnetic resonance; PPR, pterocarpan reductase; PTS, pterocarpan synthase; THI, 7,2′,4′-trihydroxyisoflavonol; TLC, thin-layer chromatography; Vmax, maximum velocity; YE, yeast extract.

Introduction

Plants produce antimicrobial compounds known as phytoalexins in response to biotic and abiotic stresses such as pathogen infection, ultraviolet irradiation and heavy metal ions. Different plant genera or tribes produce different phytoalexins, and consequently there are a wide variety of naturally occurring phytoalexin structures. Leguminous plants are known to produce pterocarpan-based isoflavonoid phytoalexins (Aoki et al. 2000).

Since the basic pterocarpan structure is chiral and can be diversely modified by hydroxylation, methylation, methylenedioxylation and prenylation, different leguminous species display specific pterocarpan phytoalexin profiles. Representative pterocarpan phytoalexins and their producer plants are shown in Fig. 1A. The majority of leguminous plants produce (−)-type pterocarpons, with an limited number of species producing the (+)-enantiomers (Ingham 1982). Soybean (Glycine max) produces a series of (−)-type pterocarpan phytoalexins that are collectively named glyceollins. Phaseolus species, which are closely related to Glycine since both genera belong to the Phaseoleae tribe, produce another type of pterocarpan phytoalexin, (−)-phaseololin. (−)-Maackiain is produced by distinct groups of legumes such as Maackia, Trifolium and Cicer, while (−)-medicarpin is found in legumes including Glycyrrhiza and Medicago. The isoflavon (−)-vestitol, which is biosynthesized by the reduction of (−)-medicarpin, is the major phytoalexin in Lotus (Fig. 1A). Examples of (−)-type pterocarpan phytoalexins include (−)-pisatin from pea (Pisum sativum), (−)-medicarpin from peanut (Arachis hypogaea) and (−)-maackiain from Sophora japonica (Ingham 1979, VanEtten et al. 1983, Strange et al. 1985).

(−)-Pterocarpons are biosynthesized from isoflavones via a number of reaction steps (Fig. 1B). A number of intensive studies published since the 1990s have, to date, identified enzymes involved in three reaction steps. Isoflavone...
2'-hydroxylase (I2'H), which catalyzes the first reaction step, belongs to the CYP81E subfamily of the Cyt P450 superfamily. The function of I2'H was identified through heterologous expression in yeast cells of cDNA clones isolated from *G. echinata* (CYP81E1), *L. japonicus* (CYP81E6) and barrel medic (*Medicago truncatula*; CYP81E7) (Akashi et al. 1998, Shimada et al. 2000, Liu et al. 2003). Soybean I2'Hs (CYP81E11, CYP81E12 and CYP81E18) have been expressed and characterized using an *Escherichia coli* expression system (Uchida et al. 2015). Isoflavone reductase (IFR), which catalyzes the second reaction step, was purified from soybean cultured cells (Fischer et al. 1990), and biochemical analyses have been performed on proteins expressed from cDNA clones from alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*) and pea (*P. sativum*) (Paiva et al. 1991, Tiemann et al. 1991, Paiva et al. 1994). IFR is of particular interest to pterocarpan biosynthesis researchers because it produces a chiral isoflavanone from an achiral 2'-hydroxyisoflavone. Paiva et al. (1991, 1994) reported the stereospecific production of (3R)-2'-hydroxyisoflavonones (vestitone and sophorain) from 2'-hydroxyisoflavonones (2'-hydroxyformononetin and 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone) using IFR of alfalfa and pea.

It was previously believed that pterocarpan was formed in the next step that involves the direct conversion of 2'-hydroxyisoflavone into pterocarpan, catalyzed by the putative enzyme pterocarpan synthase (PTS). This hypothesis was based on the results of assays performed using crude enzyme preparations from chickpea and soybean (Bleß and Barz 1988, Fischer et al. 1990). However, it was later revealed that the conversion of 2'-hydroxyisoflavone (vestitone) to pterocarpan (medicarpin) is catalyzed by two distinct enzymes: one catalyzes the conversion of 2'-hydroxyisoflavone to 2'-hydroxyisoflvanol, and the other produces pterocarpan from 2'-hydroxyisoflvanol. The former enzyme, which converts vestitone to 7,2'-dihydroxy-4'-methoxyisoflavone (DMI), has been purified from alfalfa (Guo et al. 1994a), and cDNA clones encoding this enzyme, which has been designated vestitone reductase (VR) or isoflavanone reductase, have also been isolated from alfalfa and pea (Guo and Paiva 1995, DiCenzo and VanEtten 2006). The latter enzyme, which catalyzes the final step of pterocarpan biosynthesis, has been partially purified from alfalfa and designated DMI dehydratase (DMID; EC 4.2.1.139) (Guo et al. 1994a). DMID was assumed to generate the dihydrofuran structure by dehydrating between the two hydroxyl groups at C-4 and C-2' in isoflvanol. However, detailed information about the DMID protein or its coding gene is not yet available, 20 years after its initial discovery. In the present study, we characterized this enzyme and propose calling it 2'-hydroxyisoflavonol 4,2'-dehydratase or PTS, based on the reaction catalyzed and the substrate and product involved.

Our molecular characterization of PTS could impact research on plant specialized metabolism and natural products in two ways. First, it completes the long-standing exploration of the pterocarpan biosynthetic pathway, opening up possibilities for industrial production of pterocarpans and related bioactive...
compounds using microbial fermentation, and could also facilitate molecular breeding programs for pathogen-resistant crops. Secondly, our findings provide insight into the biosynthetic pathway by which (+)-pterocarpan compounds are produced, and these compounds reportedly possess potent antipathogenic activity (VanEtten et al. 1989). Because no sequence information for PTS was available, we employed functional expression fractionation screening, which requires no sequence information, only a cDNA library and appropriate enzyme assay conditions, in order to clone the PTS cDNA. This method allows isolation of the target cDNA clone through functional expression of pooled cDNA clones in E. coli or yeast, and by enriching cDNA clones expressing proteins possessing the enzymatic activity of interest through repeated fractionation of clone pools and enzyme assays. This approach has been successfully applied to the identification of 2-hydroxyisoflavanone 4′-O-methyltransferase and 2-hydroxyisoflavanone dehydratase (HID) in G. echinata (Akashi et al. 2003, Akashi et al. 2005).

In the present study, we identified the cDNA clone encoding PTS for the first time through functional expression fractionation screening using a G. echinata cDNA library. PTSs from soybean and L. japonicus were also identified by homology searching, cDNA cloning and biochemical assays. The stereospecificity of substrates and products was analyzed using a variety of substrates prepared by organic synthesis and in vivo bioconversion using E. coli cells heterologously expressing the biosynthetic enzymes, which provided insight into the discrete biosynthesis of (−)- and (+)-pterocarps. PTSs were unexpectedly found to possess amino acid motifs characteristic of dirigent protein (DIR), which determines the specificity of phenoxyl radical coupling in lignan biosynthesis but does not possess enzymatic activity, and was named ‘dirigent’ based on this mode of action (Latin: dirigere, to guide or align) (Davin et al. 1997, Pickel and Schaller 2013). Hundreds of proteins sharing this conserved amino acid motif (dirigent-domain-containing or DIR-like proteins) have since been identified and categorized into six phylogenetic subfamilies (a, b/d, c, e, f and g) (Ralph et al. 2007). Since PTS is the first dirigent-domain-containing protein shown to possess enzymatic activity, our results offer a new perspective on the reaction mechanism and the molecular evolution of dirigent-domain-containing proteins.

### Results

**Cloning of G. echinata PTS cDNA using functional expression fractionation screening**

For functional expression fractionation screening, a cDNA library was constructed using G. echinata AK-1 cultured cells treated with yeast extract (YE) as an elicitor for medicarpin induction (Nakamura et al. 1999). The substrate, a mixture of (3R,4R)-DMI and (3R,4S)-DMI, was prepared by chemical reduction of (3R)-vestitone made from formononetin using recombinant I2H and IFR. In the initial screening, the cDNA library comprising about 50,000 clones was fractionated into 10 pools, cultured in liquid medium, and the production of recombinant proteins was induced. Crude extracts prepared from each protein-expressing fractional pool were incubated with the substrate, and reaction products were extracted with ethyl acetate and determined using HPLC by comparison with the retention time and UV spectrum of an authentic sample of medicarpin (Akashi et al. 2006). Although medicarpin production was observed in all pools as a result of the non-enzymatic dehydration of DMI, only fractions in which medicarpin production was increased compared with the control were considered positive and were subsequently cultured on a Luria broth (LB) plate, and a reduced number (usually 1/10) of colonies was pooled into 10 aliquots. After four rounds of similar screening, a single positive clone was isolated from 40 single colonies by enzymatic assay.

**Amino acid motifs in GePTS1 and its soybean and L. japonicus orthologs**

The obtained cDNA clone GePTS1 [DNA Data Bank of Japan (DDBJ) accession No. LC121822] was 852 nucleotides in length, encoding a protein of 188 amino acids (Fig. 2A). A conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) predicted that GePTS1 includes the dirigent domain, and we found five amino acid motifs (I–V) characteristic of DIR and dirigent-domain-containing proteins (Ralph et al. 2007). Based on subcellular protein localization prediction using WolFPSORT (http://wolfpsort.org/), the presence of a putative signal peptide at the N-terminus indicated extracellular and/or vacuolar localization. The predicted molecular mass of the mature protein after truncation of the signal peptide was 18 kDa.

Database searches were conducted to investigate the distribution of GePTS1 orthologs in other pterocarpan-producing leguminous plants and their related genes in angiosperms. A TBLASTN search using GePTS1 as the query yielded a considerable number of proteins sharing >50% amino acid sequence identity with GePTS1, many of which were annotated as dirigent-domain-containing proteins. Phylogenetic analysis revealed that the GePTS1 orthologs belong to the DIR-b/d subfamily of dirigent-domain-containing proteins (Ralph et al. 2007), with some GePTS1 homologs of leguminous plants forming a monophyletic leguminous DIR-like clade. Fig. 2B shows a Neighbor-Joining phylogenetic tree composed of selected leguminous dirigent-domain-containing proteins together with a non-leguminous DIR-b/d member and a few members of the DIR-a subfamily (Ralph et al. 2007) as the outgroup. Some proteins particularly closely related to GePTS1 (sharing >75% sequence identity) constituted a further monophyletic clade. Two members of this clade, GmPTS1 from soybean and LjPTS1 from L. japonicus, which share 85% and 76% identity with GePTS1, respectively, were also experimentally confirmed to possess PTS activity as described below, and this clade was therefore referred to as the PTS clade. Other members of the leguminous DIR-like clade are referred to as PTS-like (Fig. 2B).

**Analysis of GmPTS1 expression by real-time PCR**

Expression of phytoalexin biosynthetic genes generally increases following stimuli by elicitors that induce phytoalexin production, of which soybean cell cultures treated with YE are
an example. Our previous studies demonstrated that glyceollins I and III become detectable at 10 h after YE treatment, and continue to increase up to 48 h after YE treatment. Additionally biosynthetic genes encoding IFS, HID, pterocarpan 6a-hydroxylase, glycinol 4-dimethylallyltransferase and glycinol 2-dimethylallyltransferase are transiently up-regulated (Akashi et al. 2009, Yoneyama et al. 2016). GmPTS1 transcript levels were therefore analyzed by real-time PCR in YE-treated soybean cultures, and were increased by approximately 30 and 100 at 5 and 10 h after YE treatment, respectively (Fig. 3).

Biochemical analysis of PTSs

DIRs in planta are reportedly translated with a signal peptide at the N-terminus and processed into mature active forms following truncation of the signal peptide. Consistent with this, the recombinant GhDIR4 was detected in a truncated form in cultured tomato cells (Effenberger et al. 2015). Thus, for biochemical analysis of GePTS1, GmPTS1 and LjPTS1, recombinant proteins were expressed as signal peptide-truncated and histidine-tagged forms in E. coli, and their enzymatic properties were examined after affinity purification.

The substrate of PTS, 2'-hydroxyisoflavanol, can exist as four stereoisomers owing to the presence of two chiral centers at C-3 and C-4. To examine the substrate stereoselectivity of GePTS1, a mixture of the four DMI stereoisomers was obtained (Fig. 2A). Amino acid sequence features of PTS and related dirigent domain-containing proteins. (A) Amino acid alignment of PTS orthologs in leguminous plants. Bars with roman numerals (I–V) below the sequences indicate motifs predicted from approximately 150 dirigent domain-containing proteins (Ralph et al. 2007). The motifs are as follows: I, LchYhHD; II, FGslsVhDDslT—G; III, ShhVGRAQGhY; IV, ThVFpsGcasGSTLslhG; V, ReHuVGGGTGcFphARG—Aph+T; c, charged; h, hydrophobic; s, small; l, aliphatic; —, negative charge; p, polar; a, aromatic; u, tiny; +, positive charge. (B) Phylogenetic tree of GePTS1 orthologs. The tree was created using the Neighbor–Joining method with 1,000 bootstrap replicates. The PTS activity of GePTS1, GmPTS1 and LjPTS1 was experimentally confirmed. Proteins in the leguminous DIR-like clade were classified into the DIR-b/d subfamily according to Ralph et al. (2007). Those in the DIR-a subfamily are included as the outgroup. AtDIR6 (Arabidopsis thaliana, NM_118500); CaPTS1 (Cicer arietinum, XM_004493567); GhDIR4 (Gossypium hirsutum, LN875776); FiDIR1 (Forsythia intermedia, AAF25357); GePTS1 (LC121822); GmPTS1 (AK245195); GmPTS2 (GR830455); GmPTS3 (XM_003554180); GmPTS4 (XM_003554181); GmPTS-L1 (XM_003521186); GmPTS-L2 (XM_003554178); GmPTS-L3 (XM_003554179); GmPTS-L4 (NM_001249325); LjPTS1 (Lotus japonicus, FS322418); LjPTS-L1 (BT147970; LjPTS-L2 (BT141593); MtPTS1 (Medicago truncatula, XM_003625269); PsDRR206 (Pisum sativum, U11716); VrPTS1 (Vigna radiata, XM_014640082).

Fig. 2 Analysis of GmPTS1 transcript levels in cultured soybean cells. Data shown are the mean ± SE (n = 3 biological replicates). Transcript levels were analyzed using the ΔΔCt method. Skip16 was used as the internal standard. Transcript levels were normalized against the 0 h value, which was set at 1.
by reduction of racemic (3RS)-vestitone with NaBH₄, which was prepared via two-step bioconversion from formononetin using recombinant I₂₀H and IFR, followed by alkaline racemization. The stereoisomeric mixture obtained by chemical reduction was then separated by thin-layer chromatography (TLC) into two mixtures composed of two enantiomers, (3R,4R) and (3S,4S), and (3S,4R) and (3R,4S), designated as cis-DMI and trans-DMI, respectively. Each racemic mixture was then incubated with recombinant GePTS1, and residual substrates and reaction products were analyzed by HPLC using a chiral separation column, and identified by comparing the retention times and UV spectra with those of the authentic compounds. To determine the stereochemistry of residual substrates, authentic samples of enantiospecific (3R,4R)-DMI and (3S,4R)-DMI were also prepared from (3R)-vestitone that was made by omitting the alkaline racemization step (Fig. 4A). When using cis-DMI as substrate, (−)-medicarpin was produced, accompanied by a decrease in (3S,4S)-DMI (Fig. 4A). These results suggest that GePTS1 recognized (3R,4R)-DMI and (3S,4R)-DMI and produced (−)-medicarpin and (+)-medicarpin, respectively (Fig. 4A, B).

Leguminous isoflavonoids are categorized by the functional group at the C-4₀ (corresponding to C-9 of pterocarpan) position, hydroxy or methoxy forms, and the type that principally accumulates varies between leguminous species. For instance, soybean produces glyceollins of the 9-hydroxy pterocarpan type from the 4₀-hydroxyisoflavone (daidzein), whereas G. ecchinoidea and L. japonicus produce the 9-methoxy pterocarpan medicarpin from formononetin (Fig. 1A, B). The substrate specificity of GePTS1, GmPTS1, and LjPTS1 was examined using (3R,4R)-DMI, (3R,4S)-DMI, and (3R,4R)-7,2₀,4₀-trihydroxyisoflavone (THI). THI is a precursor of glyceollins, and its preparation requires an IFR with high activity towards 7,2₀,4₀-trihydroxyisoflavone, but no such IFR was known at the time of our experiments. Thus, we identified a soybean cDNA clone encoding IFR (GmIFR; Supplementary Fig. S1). (3R,4R)-DMI and (3R,4R)-THI were prepared from the products of IFR, (3R)-vestitone and
Table 1 Substrate specificity of PTS from three leguminous species

| Substrates     | Products                           | Specific activity\(^b\) (µmol min\(^{-1}\) mg\(^{-1}\)) |
|----------------|------------------------------------|-----------------------------------------------------|
| (3R,4R)-DMI    | (−)-Medicarpin                     | GePTS1 384.7 ± 11.0, GmPTS1 246.8 ± 8.7, LjPTS1 32.6 ± 4.8 |
| trans-DMI\(^c\) | (+)-Medicarpin                     | GePTS1 169.0 ± 21.8, GmPTS1 117.8 ± 14.1, LjPTS1 31.3 ± 3.7 |
| (3R,4R)-THI    | (−)-3,4-Dihydroxypterocarpan       | GePTS1 79.5 ± 9.8, GmPTS1 717.7 ± 7.2, LjPTS1 16.4 ± 1.7 |

Each value is the mean ± SD (n = 3).
\(^a\) Concentrations are as follows: 50 µM (3R,4R)-DMI, 68 µM trans-DMI and 50 µM (3R,4R)-THI.
\(^b\) Note that these values are tentative because of the triphasic kinetic properties of PTS shown in Fig. 4C.
\(^c\) Only (3S,4R)-DMI in this sample was conserved as substrate.

Table 2 Primers for cDNA cloning and real-time PCR analysis

| Primer name            | Primer sequence                                      |
|------------------------|------------------------------------------------------|
| MsrFR-LIC-Fw            | GACGACGCAAGATGGCACTGAAAAACAAAAATCTTCATC             |
| MsrFR-LIC-Rv            | GAGGAAGCCGCGTTAGACAAATTTGCAATATTCTC                |
| GmIFR (AK245728)-Fw     | ATGGCTGCGAAATGAAATTC                                |
| GmIFR (AK245728)-Rv     | TCAGACAAACTGATTCATATC                                |
| Δ2-23-GePTS1-LIC-Fw     | GAGCGAGCAAGATGACTACTCTAAACGATGC                     |
| GePTS1-LIC-Rv          | GAGGAAGCCGCGTTAGACAAATTTGCAATATTCTC                |
| Δ2-21-GmPTS1-LIC-Fw     | GAGCGAGCAAGATGACTACTCTAAACGATGC                     |
| GmPTS1-LIC-Rv          | GAGGAAGCCGCGTTAGACAAATTTGCAATATTCTC                |
| Δ2-27-LjPTS1-LIC-Fw     | GAGCGAGCAAGATGACTACTCTAAACGATGC                     |
| LjPTS1-LIC-Rv          | GAGGAAGCCGCGTTAGACAAATTTGCAATATTCTC                |
| GmSKIP16-realtime-Fw   | GACATATACACATATCAATTACATC                           |
| GmSKIP16-realtime-Rv   | GCAAGTATCTCCATGAGGATGACTACTCTAAACGATGC             |
| GmSKIP16-realtime-Fw   | AAAAAGGTTGGCTGGGATAGAATAAAAA                       |
| GmSKIP16-realtime-Rv   | GGCCTCGCGTTACGAAAA                           |

Δ: Region of deleted amino acids.
Sequences for the ligation-independent cloning (LIC) method are underlined; bold type and double underlining indicate initiation and stop codons, respectively.

(3R)-7,2′,4′-trihydroxyisoflavanone, respectively, by chemical reduction and TLC separation. Recombinant PTS from each of the three plant species was then incubated with the substrates, and residual substrates and products were similarly determined by HPLC (Table 1). The results showed that all PTSs displayed the highest activity with (3R,4R)-DMI as substrate. In assays with trans-DMI, GePTS1 and GmPTS1 exhibited approximately half of the activity displayed with (3R,4R)-DMI, but the activity with LjPTS1 was almost the same with (3R,4R)-DMI or trans-DMI. All three PTSs displayed the lowest activity with (3R,4R)-THI, which was only 20–50% of that obtained with (3R,4R)-DMI (Table 1).

To measure the reaction kinetics of GePTS1, assays were carried out using various concentrations of (3R,4R)-DMI. The Hanes–Woelf (S/v versus S) plot exhibited a triphasic pattern in which the slope varied in the substrate concentration ranges of 1–20, 20–500 and 500–1,300 µM (Fig. 4C). Data in only the 20–500 µM range fitted well to Michaelis–Menten kinetics, hence parameters were tentatively obtained from data within this range, and were as follows: \( K_m = 0.20 \text{ mM}, V_{max} = 2.0 \text{ mmol min}^{-1} \text{ mg}^{-1} \) and \( k_{cat} = 5.9 \times 10^4 \text{ s}^{-1} \). DMI is readily converted to medicarpin by non-enzymatic dehydration due to its instability in solution, and the rate constant of the spontaneous dehydration of (3R,4R)-DMI in buffer at pH 6.5 and 30°C was examined and shown to fit a first-order reaction with a rate constant of \( 2.2 \times 10^{-6} \text{ s}^{-1} \), which was much smaller than the \( k_{cat} \) value of \( 5.9 \times 10^4 \text{ s}^{-1} \) displayed by GePTS1 towards the same substrate. The half-life of (3R,4R)-DMI under these conditions was 96.3 h. The pH dependence of GePTS1 was examined using 23 microM (3R,4R)-DMI. The optimum pH range was pH 6.5–7.5, and activity decreased rapidly outside this range (Fig. 4D).

**Discussion**

In this study, the cDNA for 2′-hydroxyisoflavanol 4,2′-dehydrationase, responsible for the last step of pterocarpan biosynthesis and therefore referred to as PTS, was cloned and characterized for the first time using functional expression fractionation screening of a *G. echinata* cDNA library. Homology searching using the deduced amino acid sequence of the enzyme (GePTS1) led to the identification of orthologous enzymes in soybean (GmPTS1) and *L. japonicus* (LjPTS1). The amino acid sequences were found to include the characteristic motif (Ralph et al. 2007) originally reported in DIR, which governs stereospecific phenoxyl radical coupling in collaboration with an oxidase to form enantiopure (+)-pinoresinol during lignan biosynthesis (Davin et al. 1997).
The molecular characteristics of other DIRs have since been investigated (Pickel and Schaller 2013), and some are involved in the formation of the lignin-based Casprian strip in roots, while others play a role in cotton phytoalexin biosynthesis (Hosmani et al. 2013, Effenberger et al. 2015). Before the present study, it was believed that DIR did not possess enzymatic activity, and simply regulated product configuration.

PTS is the first dirigent domain-containing protein for which enzymatic activity has been demonstrated, and the triphasic pattern displayed in the kinetic analysis makes this enzyme particularly unusual. As shown in the Hanes–Woolf (S/v versus S) plot, only data within the particular concentration range of 20–500 μM conform to Michaelis–Menten kinetics (Fig. 4C). Tentative kinetic parameters were therefore calculated using data within this concentration range. However, in the enzymatic assays, determining the excess substrate concentration required to reach the maximum reaction velocity was impossible due to the triphasic nature, and the specific activities shown in Table 1 and Fig. 4D are therefore tentative. The triphasic kinetic profile indicates multiple (two or three) mechanistic reaction steps or allosteric regulation by substrates, multiple different conformational states of PTS, or both. Further investigation is required to elucidate the exact reaction mechanism of PTS.

Phylogenetic analysis of proteins with high sequence similarity to GePTS1 revealed a monophyletic group comprised of dirigent domain-containing proteins from leguminous plants that was designated the leguminous DIR-like clade. The PTS cluster within this clade is comprised of GePTS1, GmPTS1, LjPTS1 and several dirigent domain-containing proteins that share >75% amino acid sequence identity (Fig. 2B). The PTS clade also includes three closely related soybean proteins, GmPTS2, GmPTS3 and GmPTS4, which are >95% identical to GmPTS1 and were possibly generated by local gene and/or whole-genome duplications, as has occurred for other soybean genes involved in plant specialized metabolism (Yoneyama et al. 2016). The high sequence identity implied very similar enzymatic properties, but functional characterization was not performed for enzymes other than GmPTS1 because of difficulties in discerning the closely related paralogous cDNAs in PCR amplification. Other members of the PTS clade are predicted to possess PTS activity based on the high sequence identity and the monophyletic relationship. However, little is known about the function of the other PTS-like proteins listed in Fig. 2B, but an intriguing possibility is that they are involved in legume-specific metabolism.

The expression of phytoalexin biosynthetic genes can be induced by elicitor treatment, and an increase in transcription of such genes is generally observed prior to the maximum accumulation of phytoalexins. The potential role of the PTS gene identified in this study in phytoalexin biosynthesis was therefore tested by comparing its transcript levels with those of other biosynthetic genes using a suitable phytoalexin induction system. In this study, the expression of GmPTS1 was tested using YE-treated soybean suspension-cultured cells in which glyceollins I and III were induced (Akashi et al. 2009). Real-time PCR analysis revealed an increase in GmPTS1 transcription at 5 and 10 h after YE treatment (Fig. 3), which preceded an increase in phytoalexin accumulation, and the GmPTS1 expression profile was similar to those of other genes involved in glyceollin biosynthesis (Akashi et al. 2009, Yoneyama et al. 2016). These results strongly support the possibility that GmPTS1 is involved in pterocarpan biosynthesis in soybean.

Detailed biochemical characterization of PTS was performed using recombinant GePTS1 expressed in E. coli. Assays using the four DMI stereoisomers revealed a preference for (3R,4R)-DMI and (3S,4R)-DMI as substrate and their conversion to (−)- and (+)-medicarpin, respectively. This confirmed that the 4R substrate configuration was essential for the PTS reaction, and the configuration at C-3 determines the enantiospecificity of pterocarpan production (Fig. 4A, B). Subsequent examination of the pH dependence revealed an optimum pH range of 6.5–7.5 (Fig. 4D).

Taking into consideration previously proposed mechanisms for pterocarpan formation (Guo et al. 1994b) and DIR-mediated (+)-piroresinol formation in lignan biosynthesis (Halls et al. 2004), we propose a PTS reaction model for the formation of (−)- and (+)-pterocarps via quinone methide intermediates (Fig. 5). Of the two chiral centres of 2′-hydroxyisoflavanol at C-3 and C-4, the C-4 chiral centre is lost in the quinone methide intermediate because this carbon atom becomes olefinic. As previously discussed (Guo et al. 1994a, Guo et al. 1994b), the stereochemistry at the C-3 carbon (corresponding to pterocarpan C6α) is likely to determine that at C-11a in pterocarpan (corresponding to isoflavonoid C-4), i.e. the subsequent cyclization proceeds so as to minimize ring strain, forming the 6a,11α-cis configuration. However, the C-3 configuration of the substrate will probably have no influence on product stereochemistry and instead be essential for substrate recognition.

The stereochemistry of pterocarpan is reportedly important because it determines the antimicrobial activity against various pathogens (VanEtten et al. 1989). At the simplest level, our results suggest that (+)-pterocarpan is probably biosynthesized via (3S)-isoflavone and subsequently via (3S,4R)-isoflavonol. However, an IFR that produces (3S)-isoflavonol has not yet been reported. In the isoflavonoid pathway of pea, (+)-pisatin is reportedly biosynthesized from (3R)-isoflavonol (sophorol) and subsequently from (3S,4R)-isoflavonol (7′,2′-dihydroxy-4′,5′-methylendioxyisoflavonol) via an achiral intermediate of isoflav-3-ene (7′,2′-dihydroxy-4′,5′-methylendioxyisoflav-3-ene) (Paiva et al. 1994, DiCenzo and VanEtten 2006, Kaimoyo and VanEtten 2008, Cely and VanEtten 2014). According to this hypothesis, the last steps of (+)-pterocarpan formation should involve unidentified enzymes (Cely and VanEtten 2014). However, (+)-pterocarpan-producing plants such as Arachis, Pisum and Sophora (Fig. 1A) are distinctly located in the molecular phylogenetic tree of leguminous plants (The Legume Phylogenetic Working Group 2013). We suspect, therefore, that the ability to produce (+)-pterocarpan has been independently acquired in each leguminous lineage by convergent evolution, making it possible for some legumes to produce (+)-pterocarpan differently from pea (e.g. by using the same type of PTS as reported here).

GePTS1, GmPTS1 and LjPTS1 displayed similar substrate specificities, with the strongest preference for (3S,4R)-DMI, while (3R,4R)-THI was the least preferred substrate (Table 1).
Pterocarpans are classified by the type of modification at the D-ring (corresponding to the B-ring of isoflavonoids) into 9-methoxy, 9-hydroxy or 8,9-methylenedioxy types such as medicarpin, glyceollins and pisinat, respectively (Fig. 1A). DMI and THI are the intermediates for 9-methoxy and 9-hydroxy pterocarpan biosynthesis, respectively. The major phytoalexins in G. echi-
nata, soybean and L. japonicus are (−)-medicarpin, (−)-glyceollins and (−)-vestitol, respectively (Aoki et al. 2000), the last of which is produced by reductive ring opening of (−)-medicarpin catalyzed by pterocarp transductase (PTR) (Akashi et al. 2006). Based on the three substrates tested, the most likely natural substrates for GePTS1, GmPTS1, and LjPTS1 were deduced to be (3R,4R)-DMI, (3S,4R)-THI and (3R,4R)-DMI, respectively. Notably, soybean GmPTS1, which is most likely to produce glyceollins from (3R,4R)-DMI, showed a stronger preference for (3R,4R)-DMI than for (3S,4R)-THI. This contrasts with the high specificity observed for the deduced natural substrates of I1/H and HID (Akashi et al. 1998, Akashi et al. 2005). Another noteworthy finding is that these PTSs have the potential to produce (+)-pterocarpan biosynthesis, which are not actually produced by the three plant species. In contrast, PTR1 and PTR2 of L. japonicus showed high activity only towards (−)-medicarpin to form (−)-vestitol, the major phytoalexin produced by this plant (Akashi et al. 2006). The relatively low specificity of PTS for the deduced natural substrates suggests that the enzyme has not been highly optimized during molecular evolution compared with other biosynthetic genes in the isoflavonoid pathway.

In previous studies, some of the biochemical properties of the two-step VR/PTS reaction catalyzed by chickpea, soybean and alfalfa PTS (referred to as DMID) were investigated using crude and partially purified enzyme preparations (Bleß and Barz 1988, Fischer et al. 1990, Guo et al. 1994a). The molecular characterization of GePTS1 and its orthologs reported herein raises the question of whether they are the same enzymes as those previously investigated, but the evidence available at present is not conclusive. The molecular mass of alfalfa DMID estimated by gel filtration chromatography was 38 ± 2 kDa (Guo et al. 1994a), and the deduced molecular mass of signal peptide-truncated GePTS1 was 18 kDa. Both of the estimated molecular masses would be consistent assuming that GePTS1 forms a homodimer, but this evidence is not sufficient to conclude that GePTS1 and DMID are the same enzyme. Indeed, optimum pH and K_m data indicate different enzymatic properties, since the optimum pH of DMID was reported to be 6.0 (Bleß and Barz 1988, Fischer et al. 1990, Guo et al. 1994a), compared with 6.5–7.5 for GePTS1 (Fig. 4D), and the K_m value of 0.20 mM for GePTS1 derived using Michaelis–Menten kinetics (Fig. 4C) was larger than the K_m of 5 μM reported previously for DMID in a crude alfalfa extract. These inconsistencies in pH dependency and K_m values are, however, inconclusive, because they may be the result of different assay conditions, or the presence of other interfering factors in the crude or partially purified preparations.

In conclusion, the discovery of PTS as the missing link in (−)-pterocarp biosynthesis offers insight into (−)- and (+)-pterocarp biosynthesis. The presence of a dirigent domain and enzymatic activity in PTS raise intriguing questions about its kinetic properties and molecular evolution. The knowledge could be applied to develop pterocarp-producing microorganisms and plants with improved disease resistance.

Materials and Methods

Chemicals

Daidzein and formomononetin were purchased from LC Laboratories and Toronto Research Chemicals, respectively. 2’-Hydroxymononetin and 2’-hydroxydaidzein were prepared from formomononetin and daidzein by bioconversion using E. coli expressing L. japonicus I2/H (CYP81E6) and soybean I2/H (CYP81E18) (Uchida et al. 2015), respectively. (4S)-Medicarpin and (4R)-medicarpin were obtained from our laboratory stocks (Akashi et al. 2006).
(3R)-Vestitone (7,2′,4′-trihydroxyisoflavonone) and (3R)-7,2′,4′-trihydroxyisoflavonone were made from 2′-hydroxymonoflorone and 2′-hydroxyladzein by conversion using E. coli expressing alfalfa IFR (MifIR) and soybean IFR, respectively. The coding sequence (CDS) of MsIFR (GenBank accession No. CAAA41106) (Paiva et al. 1991) was amplified by PCR with KOD polymerase (Toyobo) using the primers shown in Table 2 and CdNAs prepared from young alfalfa seedlings (up to 5 d old) and introduced into the pET46 Ek/LIC vector (Novagen). For soybean IFR, a search of the soybean CdNA clone database of the National Bioresource Project (http://resourcedb.nbrp.jp/top.jsp) yielded CMFLO1-39-M10 (GenBank accession No. AK245728) as the CdNA clone sharing the highest sequence identity (~81%) with MsIFR. Biochemical analysis confirmed that it encoded IFR, and it was therefore designated GmIFR (Supplementary Fig. S1). Escherichia coli strain Rosetta2 (DE3) (Novagen) was transformed with the expression vectors containing the CDSs of MsIFR and GmIFR, and cultured at 37°C in 200 ml of LB medium (50 mg l−1 carbenicillin) to an OD600 value of 0.4–0.6. Subsequently, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 2 ml of 2′-hydroxyisoflavonones (<20 mg) dissolved in ethanol were added, and culturing continued for a further 2–3 h. (3R)-Vestitone and (3R)-7,2′,4′-trihydroxyisoflavonone were extracted with an equal volume of ethyl acetate, purified by TLC and confirmed by 1H-nuclear magnetic resonance (NMR; Supplementary text). For racemization, purified (3R)-vestitone was dissolved in 1 M NaOH and incubated overnight at room temperature. The resultant racemic (3RS)-vestitone was extracted with ethyl acetate after neutralization with HCl.

2′-Hydroxyisoflavonones (DMI and THI) were prepared from 2′-hydroxyisoflavonones (3S)-vestitone, (3S)-vestitone and (3R)-7,2′,4′-trihydroxyisoflavonone by chemical reduction with NaBH4 (Kaimoyo and E. coli database of the National Bioresource Project (http://resourcedb.nbrp.jp/top.jsp) and its orthologs in soybean (GMFL01-23-H19, GenBank accession No. AK245195) and L. japonicus (LjFL3-013-AG1, GenBank accession No. FS322418) were PCR-amplified using the primers shown in Table 2 so that putative signal peptides (GePT5, 23 amino acids; GmPT5, 21 amino acids; LjPT5, 27 amino acids) were truncated. The resultant PCR products were introduced into the pET46 Ek/LIC vector according to the manufacturer’s protocol. His-tagged PTS proteins were expressed in E. coli strain C41 (DE3) and purified using a HisTrap Kit (GE Healthcare) as described previously (Akashi et al. 2003).

Phylogenetic analysis

Deduced amino acid sequences of the selected GePT5s orthologs were analyzed using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/), and a phylogenetic tree was produced using the Neighbor–Joining method in MEGA version 6.0 (Tamura et al. 2013) from the results of 1,000 bootstrap replicates.

Enzyme assays

The standard PTS assay was carried out using purified recombinant enzymes as described in the above section on functional expression fractionation screening of G. echinata PTS, but with an incubation time of 5 min instead of 15 min. Except when otherwise indicated, 1 ng of purified recombinant GePT5 protein was used. The stereoselectivity of GePT5 was determined using cis- and trans-DMI with 1 μg of the purified recombinant protein. The reaction product and residual substrates were analyzed by HPLC with a Chiral RU-2 column (4.6 × 150 mm; Shiseido) at a flow rate of 0.5 ml min−1 at 40°C via isocratic elution (40% acetonitrile in water) for 50 min. To determine the relative activity of PTSs, 50 μM (3R,4R)-DMI, 50 μM (3R,4R)-THI or 68 μM trans-DMI was incubated with 1–10 ng of recombinant enzyme for (3R,4R)-DMI and (3R,4R)-THI, and 5–50 ng for trans-DMI. To determine the kinetic parameters of GePT5, 2–1,300 μM (3R,4R)-DMI was used. Kd and kcat values were measured using linear regression of a Hanes–Woolf (S/V versus S) plot in the 20–500 μM substrate concentration range. To measure the rate of spontaneous dehydration, 78 μM (3R,4R)-DMI was incubated in buffer for 0.5, 1, 2, 4, and 24 h at 30°C. The optimum pH of GePT5 was determined in 23 μM (3R,4R)-DMI in potassium phosphate buffers at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

Analysis of GmPT51 expression by real-time PCR

Real-time PCR was performed using the Power SYBR Green PCR Master Mix and a 7500 Real-Time PCR system (Applied Biosystems) with 200 ng of primers (Table 2) and CdNA samples generated from total RNA periodically prepared from cultured soybean cells induced with yeast extract as described previously (Akashi et al. 2009). PCR conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the ΔΔCt method with the housekeeping gene SKIPT6 (GenBank accession No. NM_001255441) as an internal standard.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan [a Grant-in-Aid for
Effenberger, I., Zhang, B., Li, L., Wang, Q., Liu, Y., Klaiber, I., et al. (2015)

DiCenzo, G.L. and VanEtten, H.D. (2006) Studies on the late steps of

Fischer, D., Ebenau-Jehle, C. and Grisebach, H. (1990) Purification and

glycosylation of cDNA clones (GMFL01-39-M10, GMFL01-23-H19 and

Glycine (https://www.legumebase.brc.miyazaki-u.ac.jp/) for pro-

viding cDNA samples for real-time PCR.

We are grateful to the National BioResource Project–Lotus/

Machinery, No. 25108723]).

References

Akashi, T., Aoki, T. and Ayabe, S. (1998) CYP81E1, a cytochrome P450
cDNA of licorice (Glycyrrhiza echinata L.), encodes isoflavone 2'-hydro-

xylase. Biochem. Biophys. Res. Commun. 251: 67–70.

Akashi, T., Aoki, T. and Ayabe, S. (2005) Molecular and biochemical char-

acterization of 2'-hydroxysflavonol dehydratase. Involvement of car-

boxyesterase-like proteins in leguminous isoflavone biosynthesis. Plant

Physiol. 137: 882–891.

Akashi, T., Koshimizu, S., Aoki, T. and Ayabe, S. (2006) Identification of
cDNAs encoding pterocarpan reductase involved in isoflavon phyto-

alexin biosynthesis in Lotus japonicus by EST mining. FEBS Lett. 580:

5666–5670.

Akashi, T., Sasaki, K., Aoki, T., Ayabe, S. and Yazaki, K. (2009) Molecular
cloning and characterization of a cDNA for pterocarpan 4-dimethyl-

allyltransferase catalyzing the key prenylation step in the biosynthesis of
glyceollin, a soybean phytoalexin. Plant Physiol. 149: 683–693.

Akashi, T., Sawada, Y., Shimada, N., Sakurai, N., Aoki, T. and Ayabe, S.
(2003) cDNA cloning and biochemical characterization of S-adenosyl-

l-methionine: 2',7',4'-trihydroxysflavonol 4'-O-methyltransferase, a

critical enzyme of the legume isoflavonoid phytoalexin pathway.

Plant Cell Physiol. 44: 103–112.

Aoki, T., Akashi, T. and Ayabe, S. (2000) Flavonoids of leguminous plants:
structure, biological activity, and biosynthesis. J Plant Res. 113: 475–488.

Bleß, W. and Barz, W. (1988) Isolation of pterocarpan synthase, the ter-

tinal enzyme of pterocarpan phytoalexin biosynthesis in cell suspen-

sion cultures of Cicer arietinum. FEBS Lett. 235: 47–50.

Celoy, R.M. and VanEtten, H.D. (2014) +=Pisatin biosynthesis: from (−)

enantiomeric intermediates via an achiral 7,2'-dihydroxy-4',5'-methylene-

nedioxysflav-3-ene. Phytochemistry 98: 120–127.

Davin, L.B., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen,
S., et al. (1997) Stereoselective bimolecular phenoxy radical coupling by

an auxiliary (dirigent) protein without an active center. Science 275:

362–366.

Dewick, P.M. (1976) Biosynthesis of pterocarpan phytoalexins in Trifolium
pretense. Phytochemistry 16: 93–97.

DiCenzo, G.L. and VanEtten, H.D. (2006) Studies on the late steps of (+)

pisatin biosynthesis: evidence for (−) enantiomeric intermediates.

Phytochemistry 67: 675–683.

Effenberger, I., Zhang, B., Li, L., Wang, Q., Liu, Y., Klaiber, I., et al. (2015)

Dirigent proteins from cotton (Gossypium sp.) for the atropselective

generation of (+)-pinoresinol forming dirigent enzymes. FEBS Lett.

586: 2215–2219.

Guo, L. and Paiva, N.L. (2015) Molecular cloning and expression of alfalfa

(Medicago sativa L.) vestitone reductase, the penultimate enzyme in

medicarpin biosynthesis. Arch. Biochem. Biophys. 320: 353–360.

Halls, S.C., Davin, L.B., Kramer, D.M. and Lewis, N.G. (2004) Kinetic study

of coniferyl alcohol radical binding to the (+)-pinoresinol forming dirigent

protein. Biochemistry 43: 2587–2595.

Hosmani, P.S., Kamiya, T., Danku, J., Naseer, S., Geldner, N., Guerinot, M.L.,
et al. (2013) Dirigent domain-containing protein is part of the machin-

ery required for formation of the lignin-based Casparian strip in the

root. Proc. Natl. Acad. Sci. USA 110: 14498–14503.

Ingham, J.L. (1979) Phytoalexin production by flowers of garden pea

(Pisum sativum). Z. Naturforsch. 34c: 296–298.

Ingham, J.L. (1982) Phytoalexins. In Phytoalexins from the Leguminosae.
Edited by Bailey, J.A. and Mansfield, J.W. pp. 21–80. Blackie and Son,

Glasgow, UK.

Kaimoyo, E. and VanEtten, H.D. (2008) Inactivation of pea genes by RNAi

supports the involvement of two similar O-methyltransferases in the

biosynthesis of (−)-pisatin and of chiral intermediates with a configura-

tion opposite that found in (+)-pisatin. Phytochemistry 69: 76–87.

Liu, C.J., Huhman, D., Sumner, L.W. and Dixon, R.A. (2003) Regiospecific

hydroxylation of isoflavones by cytochrome p450 81E enzymes from

Medicago truncatula. Plant J. 36: 471–484.

Nakamura, K., Akashi, T., Aoki, T., Kawaguchi, K. and Ayabe, S. (1999)

Induction of isoflavonoid and retrochalcone branches of the flavonoid

pathway in cultured Glycrrhiza echinata cells treated with yeast ex-

tract. Biosci. Biotechnol. Biochem. 63: 1618–1620.

Paiva, N.L., Edwards, R., Sun, Y.J., Hrazdina, G. and Dixon, R.A. (1991) Stress

responses in alfalfa (Medicago sativa L.). 11. Molecular cloning and

expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid

phytoalexin biosynthesis. Plant Mol. Biol. 17: 653–667.

Paiva, N.L., Sun, Y., Dixon, R.A., VanEtten, H.D. and Hrazdina, G. (1994)

Molecular cloning of isoflavone reductase from pea (Pisum sativum L):
evidence for a 3R-isoflavonolamine intermediate in (−)-pisatin biosynthesis.

Arch. Biochem. Biophys. 312: 501–510.

Pickel, B. and Schaller, A. (2013) Dirigent proteins: molecular characteris-
tics and potential biotechnological applications. Appl. Microbiol.

Biotechnol. 97: 8427–8438.

Ralph, S.G., Jancsik, S. and Bohlmann, J. (2007) Dirigent proteins in conifer

defense II: Extended gene discovery, phylogeny, and constitutive and

stress-induced gene expression in spruce (Picea spp.). Phytochemistry

68: 1975–1991.

Shimada, N., Akashi, T., Aoki, T. and Ayabe, S. (2000) Induction of isofla-

vonoid pathway in the model legume Lotus japonicus: molecular char-

acterization of enzymes involved in phytoalexin biosynthesis. Plant

Sci. 160: 37–47.

Strange, R.N., Ingham, J.L., Colec, D.L., Cavill, M.E., Edwards, C., Cooksey, C.J.,
et al. (1985) Isolation of the phytoalexin medicarpin from leaflets of

Arachis hypogaea and related species of the tribe Aeschynomeneae. Z.

Naturforsch. 40c: 313–316.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013)

MEGA6: molecular evolutionary genetics analysis version 6.0. Mol.

Biol. Evol. 30: 2725–2729.

The Legume Phylogeny Working Group (2013) Legume phylogeny and
classification in the 21st century: progress, prospects and lessons for

other species-rich clades. Taxon 62: 217–248.

The authors have no conflicts of interest to declare.

Acknowledgments

We are grateful to the National BioResource Project–Lotus/

Glycine (https://www.legumebase.brc.miyazaki-u.ac.jp/) for pro-

viding cDNA clones (GMFL01-39-M10, GMFL01-23-H19 and

LjFL3-013-A11) and to Keisuke Yoneyama (Nihon University)
for providing soybean cDNA samples for real-time PCR.

Disclosures

Scientific Research on Innovation Areas (Biosynthetic

Machinery, No. 25108723)].

407
Tiemann, K., Inzé, D., Van Montagu, M. and Barz, W. (1991) Pterocarpan phytoalexin biosynthesis in elicitor-challenged chickpea (Cicer arietinum L.) cell cultures. Purification, characterization and cDNA cloning of NADPH:isoflavone oxidoreductase. Eur. J. Biochem. 200: 751–757.

Uchida, K., Akashi, T. and Aoki, T. (2015) Functional expression of cytochrome P450 in Escherichia coli: an approach to functional analysis of uncharacterized enzymes for flavonoid biosynthesis. Plant Biotechnol. 32: 205–221.

VanEtten, H.D., Matthews, P.S. and Mercer, E.H. (1983) (+)-Maackiain and (+)-medicarpin as phytoalexin in Sophora japonica and identification of the (−) isomers by biotransformation. Phytochemistry 22: 2291–2295.

VanEtten, H.D., Matthews, D.E. and Matthews, P.S. (1989) Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu. Rev. Phytopathol. 27: 143–164.

Yoneyama, K., Akashi, T. and Aoki, T. (2016) Molecular characterization of soybean pterocarpan 2-dimethylallyl transferase in glyceollin biosynthesis: local gene and whole-genome duplications of prenyltransferase genes led to the structural diversity of soybean prenylated isoflavonoids. Plant Cell Physiol. 57: 2497–2509.