Genome-wide Analyses of the Structural Gene Families Involved in the Legume-specific 5-Deoxyisoflavonoid Biosynthesis of *Lotus japonicus*

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Abstract

A model legume *Lotus japonicus* (Regel) K. Larsen is one of the subjects of genome sequencing and functional genomics programs. In the course of targeted approaches to the legume genomics, we analyzed the genes encoding enzymes involved in the biosynthesis of the legume-specific 5-deoxyisoflavonoid of *L. japonicus*, which produces isoflavan phytoalexins on elicitor treatment. The paralogous biosynthetic genes were assigned as comprehensively as possible by biochemical experiments, similarity searches, comparison of the gene structures, and phylogenetic analyses. Among the 10 biosynthetic genes investigated, six comprise multigene families, and in many cases they form gene clusters in the chromosomes. Semi-quantitative reverse transcriptase–PCR analyses showed coordinate up-regulation of most of the genes during phytoalexin induction and complex accumulation patterns of the transcripts in different organs. Some paralogous genes exhibited similar expression specificities, suggesting their genetic redundancy. The molecular evolution of the biosynthetic genes is discussed. The results presented here provide reliable annotations of the genes and genetic markers for comparative and functional genomics of leguminous plants.

Key words: 5-deoxyflavonoid; gene cluster; isoflavonoid; *Lotus japonicus*; phytoalexin

1. Introduction

The last decade has seen remarkable advances in structural analysis of model plant genomes. The complete whole-genome sequence data of *Arabidopsis thaliana* and *Oryza sativa* have been published together with the predicted gene structures and annotations,1,2 and the draft genome sequence of a model tree *Populus trichocarpa* (Torr. & Gray) was recently disclosed.3 Two leguminous plants, *Lotus japonicus* (Regel) K. Larsen and *Medicago truncatula* Gaertn., are also subjects of genome sequencing programs.4 The post-sequence functional genomic approaches to legumes will be important to uncover new functions of legume-specific genes and to establish the systems biology of legumes. At present, non-targeted approaches are mainly used for legume functional genomics. The EST macro-array analysis of *L. japonicus* became available recently.5 Data mining by multivariate analyses of the integrated data of transcriptomics and metabolomics is expected to facilitate our understanding of the physiology of legumes, as is being accomplished with *A. thaliana*.6 On the other hand, targeted approaches, that is, focusing on the genes of particular families or particular pathways, are practical to assess the functions and structures of individual genes, and thus they will complement the non-targeted ones.

The legume-specific 5-deoxyisoflavonoid pathway is one of the targets for functional legume genomics. 5-Deoxyisoflavonoids have two distinctive structural features: the B-ring attached to C-3 of the C-ring and a hydrogen attached to C-5 instead of a B-ring at C-2 and a hydroxyl group at C-5 in general flavonoids. To the best of our knowledge, no 5-deoxyisoflavonoid has been found in any plant other than the Leguminosae. About...
95% of isoflavonoids are found in legumes, and 60% of leguminous flavonoids are 5-deoxy series flavonoids. Some isoflavonoids, particularly those with pterocarpan and isoflavan skeletons, are typical leguminous phytoalexins active in the defense response against phytopathogenic organisms, whereas an isoflavone (daidzein) functions as a signal molecule toward symbiotic rhizobia to establish nitrogen-fixing root nodules in the soybean. Vestitol, an isoflavon phytoalexin of Lotus and other leguminous genera, is postulated to be biosynthesized from the phenylpropanoid precursor by a total of 11 enzymes (Fig. 1). The cDNAs encoding 10 of the 11 enzymes have been identified from L. japonicus or other leguminous plants, \(^\text{20-18}\) the exception is 7,2′-dihydroxy-4′-O-methoxyisoflavanol dehydratase, which has been purified from M. sativa, \(^\text{19}\) but for which no sequence data is available. We have reported the induction of vestitol accumulation by treatment with reduced glutathione (GSH) \(^\text{20}\) and the structures and functions of paralogous genes encoding catalytically distinct chalcone isomerase (CHI) isozymes of L. japonicus. \(^\text{21}\) The findings on the genes for CHI, together with those for dihydroflavonol-4-reductase \(^\text{22}\) of the general flavonoid pathway and oxidosqualene cyclase \(^\text{23}\) in the triterpenoid biosynthesis, suggest the functional diversification of the multigene families, due to gene duplication, followed by the accumulation of nucleotide substitutions. \(^\text{21}\)

In the present study, we clarified the structures of the genes encoding the enzymes of the legume-specific 5-deoxyisoflavonoid pathway as comprehensively as possible, and semi-quantified the transcripts during phytoalexin induction and in several organs. The gene identification was based on a biochemical assay using heterologously expressed enzymes, comparison of the gene structures, and the phylogenetic relationship. The results offer reasonable gene annotations and genetic markers for comparative and functional genomics of legumes, as well as insights into the molecular evolution of the legume-specific biosynthetic genes.

2. Materials and methods

2.1. Genome structure and genetic mapping

Generation of genomic libraries of L. japonicus (Regel) K. Larsen accession Miyakojima MG-20, sequence strategy, and gene assignment were carried out as described elsewhere. \(^\text{24}\) The libraries were screened for each gene by the PCR method using primer sets based on the sequences of the cDNAs isolated (Table 1). Putative genes for chalcone synthase (CHS) were in part discovered in the course of the whole-genome sequencing program of L. japonicus. \(^\text{25}\) Genetic mapping was carried out with the simple sequence repeat markers found in each TAC clone. \(^\text{26}\)

2.2. Functional characterization of paralogous genes

mRNA was isolated from 4-week-old whole plants of L. japonicus accession Gifu B-129 using the Straight A’s mRNA isolation system (Novagen, Madison, WI, USA). Single-strand cDNAs were synthesized using Superscript II RNase H\(^{-}\) Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA). To amplify the full ORF of 2-hydroxyisoflavonone synthase 2 (IFS2, GenBank accession no. AB279984), a primer set was designed based on the genomic sequence: 5′-AGATCAAAACAAAGCTTATG TTTGTTGGAAC-3′ and 5′-GAAAATCGAAAGGT TTATTTGTGTGTACTT-3′, which included HindIII and XbaI sites (underlined), respectively. The PCR product was cloned into a pT7Blue T-vector (Novagen). EST clones corresponding to polyketide reductase 5 (PKR5, GenBank accession no. AV424286; synonyms with chalcone reductase, CHR) and 2-hydroxyisoflavonone dehydratase (HID, GenBank accession no. AV425709) are in the collection of Kazusa DNA Research Institute. The construction of expression vectors, heterologous expression, and in vitro assays of IFS and HID have been described previously. \(^\text{13,22}\) The PKR activity was tested by assaying 6′-deoxychalcone synthase activity as described previously, \(^\text{28}\) except that the CHS source was the enzyme solution prepared from recombinant Escherichia coli cells expressing a cDNA for CHS obtained from licorice, instead of plant cell-free extracts.

2.3. Semi-quantitative RT-PCR

To analyze the expression patterns of each paralogous gene in GSH-treated seedlings and various organs, specific primers were designed (Table 1). mRNA isolation from L. japonicus Gifu B-129 and first-strand cDNA synthesis were performed as described previously. \(^\text{20,22}\) The absence
| Target | Primer sequence |
|--------|----------------|
| PKR (LjT38O12) | 5'-TGGAAAGACTATAGCTCAGGTCAG-3' |
| PKR (LjT10F22) | 5'-TTTAAACCACAAAGGAGACACG-3' |
| PKR | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| PKR | 5'-AGGTAGCTCAGGGAGGATGAG-3' |
| PKR1 | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| PKR2 | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| PKR4 | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| PKR5 | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| PKR6 | 5'-TTCTCACTTGGAGAAGGTTGAG-3' |
| IFS (LjT24P23) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFS (LjT46B17) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFS | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFS1 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFS2 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| HID (LjB01D01) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| HID | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| I2H (LjT07D18) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| I2H | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFR (LjT32H22) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFR | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFR1 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| IFR2 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| VR (LjT43J18) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| VR | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| VR1 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| VR2 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| PTR (LjT44D07) | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| PTR | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| PTR1 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| PTR2 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |
| PTR3 | 5'-AGGAATTAATTCAAGGCAACACAT-3' |

Continued
of contamination with genomic DNA in the cDNA samples was confirmed by PCR with a primer set designed to overlap one of the exon–intron junctions of the isoflavone reductase 1 (IFR1) gene. The quantity of each template was adjusted to give roughly equal amplification of β-tubulin cDNA. RT (reverse transcriptase)–PCR was carried out with 0.5 pmol of each specific primer using ExTaq DNA polymerase (Takara-Bio Inc., Shiga, Japan) in a final volume of 20 μl according to the manufacturer’s protocol. The products (5 μl) were separated on 1.2% (w/v) agarose gel and stained with ethidium bromide.

2.4. Construction of phylogenetic tree

Neighbor-joining trees were generated from the results of 1000 bootstrap replicates using the CLUSTAL W program29 of the DNA Data Bank of Japan (Shizuoka, Japan). The phylogenetic trees were displayed by Njplot software.30

A CHS phylogenetic tree was constructed based on the coding sequences of CHSs and plant polyketide synthases, i.e. acridone synthase, bibenzyl synthase, and stilbene synthase. GenBank accession numbers for the nucleotide sequences of polyketide synthases are Antirrhinum majus CHS (X03710), Arabidopsis thaliana CHS (M20308), Camellia sinensis CHS1 (D26593), Cicer arietinum CHS (AJ012690), Daucus carota CHS2 (AJ006780), Gerbera hybrida CHS1 (Z38096), Glycine max CHS2 (X65636), G. max CHS3 (X53958), G. max CHS4 (X52097), G. max CHS5 (L07647), G. max CHS6 (L03352), G. max CHS7 (M98871), G. max CHS8 (AY237728), Malus x domestica CHS1 (DQ026297), Ipomoea purpurea CHS1 (U81158), I. purpurea CHSB (U15947), Lycopepsicon esculentum CHS1 (X55194), L. esculentum CHS2 (X55195), Medicago sativa CHS1 (L02901), M. sativa CHS2 (L02902), M. sativa CHS4 (L02903), M. sativa CHS8 (L02904), M. sativa CHS9 (L02905), M. sativa CHS12-1 (U01021), Perilla frutescens CHS (AB002815), Petunia x hybrida CHS (AF233638), Phalaenopsis sp. bibenzyl synthase (X79903), Pinus strobus CHS (AJ004800), P. sylvestris bibenzyl synthase (X60753), P. strobos stilbene synthase (Z46914), Pisum sativum CHS2 (X63334), Pueraria lobata CHS (D10223), Ruta graveolens acridone synthase (Z34088), Trifolium subterraneum CHS1 (M91193), T. subterraneum CHS2 (M91194), T. subterraneum CHS3 (L24515), T. subterraneum CHS5 (L24517), T. subterraneum CHS6 (M91195), Vitis stilbene synthase (S63221), and Vitis vinifera CHS (X75969).

A phylogenetic tree of PKR cDNAs was constructed based on the 384 bp region from the initiation codon of aldo/keto reductase (AKR) and PKR/CHR, because full-length ORF sequences of EST clones were unavailable. GenBank accession numbers for the nucleotide sequences used for the construction of a PKR phylogenetic tree are A. thaliana AKR (NM104687), Fragaria x ananassa AKR (AY703448), G. max PKR (X55730), G. max PKR-like (BG882535), Glycyrrhiza echinata PKR (D83718), Glycyrrhiza glabra PKR2 (D86559), Hydrangea macrophylla AKR (AY382665), M. sativa CHR (X82368), M. truncatula PKR-like (TC100399), Papaver somniferum codeinone reductase (AF108435), P. lobata CHR (AF462632), Orzya sativa AKR (XM462652), and Sesbania rostrata CHR (AJ223291).

A phylogenetic tree of the genes for short-chain dehydrogenase/reductase (SDR) was constructed based on full-length ORFs of IFR, leucoanthocyanidin reductase, phenylcoumaran benzylic ether reductase (PCBER), pinoresinol–larciresinol reductase (PLR), and pterocarpan reductase (PTR). GenBank accession numbers for the nucleotide sequences of these are C. artemis CHS (X60755), Desmodium uncinatum leucoanthocyanidin reductase (AJ550154), Forsythia x intermedia PLR (U81158), G. max IFR (AJ003245), M. sativa IFR (X58078), M. truncatula IFR (AF277052), Pinus taeda PCBER (AF242490), P. sativum IFR (S72472), Populus trichocarpa PCBER (AJ132262), Tsuga heterophylla PLR1 (AF242501), T. heterophylla PLR2 (AF242502), Thuja plicata PLR1 (AF242503), T. plicata PLR2 (AF242504), T. plicata PLR3 (AF242505), and T. plicata PLR4 (AF242506).

| Target | Primer sequence |
|--------|-----------------|
| PTR4   | 5'-GAGGAGAGCTGCTAAGGA-3' |
| β-tublin | 5'-CTCTCTGACACTGCTAAAGCTC-3' |
|        | 5'-CCTTCACGAACACCGCGTGTTT-3' |
|        | 5'-AATTCTGGGAAGTCATCTGCAGAG-3' |
|        | 5'-CTGGTGCACTGAAAGGGTAGCATTAT-3' |

*Common primer for the amplification of PKR1 and PKR4 cDNAs.

*Common primer for the amplification of PKR5 and PKR6 cDNAs.

*Common primer for the amplification of IFR1 and IFR2 cDNAs.

*Common primer for the amplification of VR1 and VR2 cDNAs.
| Gene | Genome clone (accession number) | Location (initiation → termination codon) | cDNA accession | Map position | Biochemical identification |
|------|---------------------------------|------------------------------------------|----------------|-------------|--------------------------|
| CHS1 | LjT09H02 (AP006710) | 6693 → 8287 | BP033951<sup>a</sup> | Chr. I, 51.8cM |  |
| CHS2 | LjT30A24 (AP009237) | 47359 → 48662 | BP034657<sup>a</sup> | Chr. I, 26.1cM |  |
| CHS3 | LjT30A24 (AP009237) | 51392 → 52763 | AV767686<sup>a</sup> | Chr. I, 26.1cM |  |
| CHS4 | LjT01O03 (AP006702) | 14266 → 15581 |  | Chr. II, 61.7cM |  |
| CHS5 | LjT01O03 (AP006702) | 9231 → 10524 |  | Chr. II, 61.7cM |  |
| CHS6 | LjT01O03 (AP006702) | 5437 → 6749 |  | Chr. II, 61.7cM |  |
| CHS7 | LjT08P04 (AP006701) | 8189 → 9571 | BP076978<sup>a</sup> | Chr. II, 62.1cM |  |
|      | LjT41D19 (AP006709) | 47678 → 49060 |  |  |  |
| CHS8 | LjT08P04 (AP006701) | 3147 → 4437 |  | Chr. II, 62.1cM |  |
|      | LjT41D19 (AP006709) | 42636 → 43926 |  |  |  |
| CHS9 | LjT41D19 (AP006709) | 32090 → 34006 |  | Chr. II, 62.1cM |  |
|      | (pseudogene) |  |  |  |  |
| CHS10 | LjT41D19 (AP006709) | 26974 → 28272 |  | Chr. II, 62.1cM |  |
| CHS11 | LjT10F20 (AP007302) | 17404 → 18703 | BP083372<sup>a</sup> | Chr. II, 62.1cM |  |
| CHS12 | LjT09I23 (AP006706) | 8878 → 10177 | BP051174<sup>a</sup> | Chr. II, 62.1cM |  |
| CHS13 | LjT03B03 (AP004528) | 8283 → 9582 |  | Chr. VI, 27.6cM |  |
| PKR1 | LjT38O12 (AP009072) | 80096 → 81290 | AB263016 | Chr. I (B-129) | Shimada et al.<sup>31</sup> |
|      | (pseudogene) |  |  |  |  |
| PKR2 | LjT38O12 (AP009072) | 68792 → 69986 |  | Chr. I (B-129) |  |
|      | (pseudogene) |  |  |  |  |
| PKR3 | LjT38O12 (AP009072) | 55902 → 62367 |  | Chr. I (B-129) |  |
|      | (pseudogene) |  |  |  |  |
| PKR4 | LjT38O12 (AP009072) | 23657 → 24849 | AW428662<sup>a</sup> | Chr. I (B-129) |  |
|      | (pseudogene) |  |  |  |  |
| PKR5 | LjT10F22a (AP009238) | 54908 → 56168 | AV424286<sup>a</sup> | Chr. II (MG-20, 72.5cM) |  |
| PKR6 | LjT10F22a (AP009238) | 62291 → 63545 | AV428662<sup>a</sup> | n.d. |  |
| CHI1 | LjT47K21 (AP004250) | 17264 → 18700 | AB054801 | Chr. V, 1.2cM | Shimada et al.<sup>21</sup> |
| CHI2 | LjT47K21 (AP004250) | 26521 → 28478 | AB054802 | Chr. V, 1.2cM | Shimada et al.<sup>21</sup> |
| CHI3 | LjT47K21 (AP004250) | 14079 → 15609 | AB073787 | Chr. V, 1.2cM | Shimada et al.<sup>21</sup> |
| CHI4 | LjT47K21 (AP004250) | 24721 → 25965 |  | Chr. V, 1.2cM |  |
| IFS1 | LjT34P23B (AP009063) | 31597 → 33467 | AB024931 | n.d. | Shimada et al.<sup>20</sup> |
| IFS2 | LjT46B17B (AP009070) | 53978 → 55659 | AB279984 | Chr. IV, 11.6cM | This work |
| IFS3 | LjT46B17C (AP009071) | 2325 → 3979 |  | Chr. IV, 11.6cM |  |
|      | (pseudogene) |  |  |  |  |
| H14/OMT | LjT24P23C (AP009064) | 13643 → 15476 | AB091686 | n.d. | Akashi et al.<sup>11</sup> |
| HID | LjB01D01 (AP009065) | 80214 → 81203 | AV425769<sup>a</sup> | n.d. | This work |
| I2 | LjT07D18 (AP009066) | 77648 → 80381 | AB025016 | Chr. IV, 5.6cM | Shimada et al.<sup>20</sup> |
| IFR1 | LjT32H22B (AP009068) | 4305 → 5851 | BP081283<sup>a</sup> | Chr. II, 61.7cM |  |
| IFR2 | LjT32H22B (AP009068) | 9850 → 11396 | AB265595 | Chr. II, 61.7cM |  |
| VR1 | LjB20B09 (AP009074) | 25385 → 29733 | BP073948<sup>a</sup> | Chr. I, 23.3cM |  |
| VR2 | LjB20B09 (AP009074) | 21816 → 23749 | BP051977<sup>a</sup> | Chr. I, 23.3cM |  |

Continued
Table 2. Continued

| Gene | Genome clone (accession number) | Location (initiation → termination codon) | cDNA accession | Map position | Biochemical identification |
|------|---------------------------------|------------------------------------------|----------------|--------------|---------------------------|
| VR3  | LjT43D08 (AP009073)             | 124819 ← Not found                     |                | Chr. I, 23.3 cM | Akashi et al.              |
| PTR1b| LjT44D07 (AP009075)             |                                          | AB265589       | Chr. III, 75.6 cM | Akashi et al.              |
| PTR2b| LjT44D07 (AP009075)             |                                          | AB265590       | Chr. III, 75.6 cM | Akashi et al.              |
| PTR3b| LjT44D07 (AP009075)             |                                          | AB265591       | Chr. III, 75.6 cM | Akashi et al.              |
| PTR4 | LjT37D14 (AP009195)             | 58138 ← 59854                            | AB265592       | Chr. I, 17.7 cM  | Akashi et al.              |

n.d., not determined.

*aEST clones.

*bPositions of PTR1-3 genes have not been determined due to the high number of repeated sequences in LjT44D07.

3. Results

3.1. Assignment of legume-specific 5-deoxyisoflavonoid genes

The structural genes involved in vestitol biosynthesis except for CHS genes were found in the genomic clones isolated by PCR-based screening of L. japonicus genome libraries.24 Some of the CHS genes had already been reported,25 and, in the present study, the other paralogous genes were discovered in the genomic sequences obtained in the course of the whole-genome sequencing program of this plant.24 The chromosomal localization and accession numbers of all genes identified are summarized in Table 2.

Those that have been functionally characterized before are PKR1, CHI1, CHI3, IFS1, 2, 7, 4-3-trihydroxyisoflavone 4‘-O-methyltransferase (HL4’OMT), isoflavone 2‘-hydroxylase (I2‘H), and PTR1–PTR4.11–13,20,31 The genes for PKR5, HID, and another IFS (IFS2) were functionally assigned in this study by in vitro assays using the heterologously expressed proteins (Supplementary Fig. S1). The other paralogous genes and putative genes for CHS, IFR, and vestitone reductase (VR) were assigned based on the sequence similarity to those identified from other plants, the resemblance of exon/intron structures, and the phylogenetic relationship of the predicted coding sequences as described in what follows. IFS1, I2‘H, and IFR1 correspond to the previously reported cDNAs, LjCYP-1, LjCYP-2, and LjIFR, respectively.20

3.2. Structures of isoflavonoid biosynthetic genes

3.2.1. CHS Structural analysis of putative CHS genes of L. japonicus revealed 13 paralogs, named CHS1–CHS13 (Fig. 2A). Two clusters, composed of three (CHS4, CHS5, and CHS6) and five (CHS7, CHS8, CHS9, CHS10, and CHS11) genes, respectively, are found on chromosome II. CHS9 should be a pseudogene because the initiation codon (ATG) is changed to ATA by a transition. The other five CHS genes, CHS1 to CHS3, CHS12 and CHS13, are separately located on chromosome I, III, and VI, respectively. The structure of the CHS genes is highly conserved: all the CHS genes are composed of two exons, i.e. the first exon (178 bp) and second exon (992 bp), except for CHS1, which has a second exon of 998 bp.

3.2.2. PKR Four PKR genes, PKR1–PKR4, compose a cluster in a 50 kb region of a genomic clone LjT38O12 mapped in the south end of chromosome II in accession Miyakojima MG-20 and chromosome I in accession Gifu B-129 (Fig. 2B), which is known as the reciprocal translocated region.26 They are composed of three exons, and the lengths of the first (323 bp) and second (244 bp) exons are conserved. The first exon of PKR3 contains ca. 5.3 kb sequence in which a terminal-repeat retrotransposon in miniature-like sequence is found, and thus PKR3 should be a pseudogene. The coding sequences of PKR1, PKR2, and PKR4 are more than 90% identical to each other. Two additional PKR genes, PKR5, and PKR6, are found in LjT10F22a, whose chromosome location is unknown. PKR5 and PKR6 are composed of four exons, and the corresponding exons of the two genes are identical in length.

3.2.3. IFS The IFS1 gene encoding CYP93C17 is found in LjT24P23, and IFS2 and IFS3 are located in LjT46B17 (Fig. 2C). The map position of LjT24P23 has not been clarified because no linkage marker was found in LjT24P23.

The multiple alignments of the IFS sequences revealed insertion/deletion (I/D) polymorphisms among the IFS genes. Fig. 3 shows the alignments of a partial nucleotide (A) and amino acid (B) sequences of IFS from L. japonicus (IFS1–IFS3) together with two genes for functionally identified IFS, CYP93C2 of Glycyrhiza echinata18 and CYP93C1v2 of Glycine max.17 The cladistic relationships of the IFS genes were elucidated using the I/D polymorphisms (Fig. 3C), demonstrating that the IFS3 of L. japonicus is the most derived. In IFS3, a frame shift is caused by a 4-bp insertion at 1217, and a non-sense codon (TGA) is formed next to His427 (asterisk in Fig. 3B). The heme-binding amino acid residue, which is critical for the activity of cytochrome P450, was
predicted to be Cys445 in IFS1 and Cys449 in IFS2, but is lacking in IFS3. IFS3 is thus considered to be a pseudo-gene. On the other hand, the 3- or 12-base deletions found in IFS1, IFS2, and CYP93C2 at the other sites cause no frameshift, and the amino acid residues encoded by deletion sites are probably unessential.

3.2.4. HI4'OMT, HID, and I2'H HI4'OMT catalyzes the reaction step next to IFS. The HI4'OMT gene is found in the same clone as IFS1 (LjT24P23), although the detailed organization of the genes has not been clarified (Fig. 2C). It is noteworthy that a cluster contains non-homologous genes encoding different enzymes of sequential reactions in the same pathway. Similar gene organizations and the implications for the evolution of metabolic diversity have also been reported.32,33

The genes for HID and I2'H (CYP81E6) are located in LjB01D01 (chromosome location unknown) and

Figure 2. Structures of genes encoding enzymes involved in the biosynthesis of 5-deoxyisoflavonoids in *L. japonicus* genome. The figures show only the structural features and relative lengths of exon (black boxes) and intron (white boxes) sequences of each gene, not distances between the genes.
mapped on chromosome IV (Fig. 2F). The structures of the two genes are highly similar: all the exons and introns are exactly the same in length, and the nucleotide sequences between the initiation and termination codons, including both exons and introns, are 99.7% identical. On the other hand, the identities of the 5′- and 3′-untranslated regions are less than 50%. Another IFR-like sequence was found ca. 18 kb distant from IFR2 but is not thought to be IFR because of the low sequence identity (ca. 60%).

Two putative VR genes (VR1 and VR2) are within ca. 17 kb in a contig comprised of LjT43J18 and Ljb20B09 mapped on chromosome I (Fig. 2G). Another VR-like gene (VR3) is at ca. 10 kb from VR2, but it is judged to be a pseudogene because it lacks an initiation codon.

3.2.6. PTR Four genes are shown to encode PTR.12 Three of these, PTR1–PTR3, are located within a 15 kb region in LjT44D07 on chromosome III, and a reductase-like gene is also found in the same clone (Fig. 2H). Because of the high number of repeated sequences in LjT44D07, the nucleotide sequence of the clone has been deposited in the database as a draft sequence. The positions of PTR1, PTR2, and PTR3 genes described in Table 2 are based on the draft sequence. PTR4 gene is located in LjT37D14 on chromosome I. The PTR1–PTR3 genes are composed of five exons, but PTR4 has four. Their exon lengths are not conserved (Fig. 2H). The identities of the deduced amino acid sequences of the four PTRs are 60–67%.

3.3. Phylogenetic analyses

CHS and PKR are members of plant polyketide synthase and aldo/keto reductase superfamilies, respectively. IFR and PTR are in the SDR family, which includes two enzymes of the lignan pathway, PCBER and PLR. The phylogenetic relationships of the orthologous and paralogous genes related to CHS, PKR, IFR, and PTR were investigated based on the coding sequences (Fig. 4).

Most of the established and putative CHS genes of L. japonicus and other leguminous CHS genes constituted a monophyletic group (Fig. 4A). The topology of the legume-specific clade was consistent with the phylogeny of Papilionoidea.34 It is thus likely that the duplication processes that generated the paralogous CHS genes in L. japonicus occurred after the divergence of the legume clades. The phylogenetic tree also suggests the existence of a non-leguminous type of CHS, CHS1, of L. japonicus (Fig. 4A), but the origin and actual catalytic function of the CHS1 protein are unknown.

The PKR genes of legumes fell into two subclades (Fig. 4B). PKR1, PKR2, and PKR4 of L. japonicus were grouped with PKR/CHR genes of other legumes previously identified, whereas PKR5 and PKR6 were in another branch with several genes of G. max and M.
truncatula annotated as ‘CHR-like’. Because PKR5 showed catalytic activity, as described earlier, it is possible that other CHR-like genes in this clade may encode active PKR. In the evolutionary processes, the ancestral PKR was possibly duplicated once before the speciation of Papilionoideae, and the subsequent duplication caused the paralogous genes in the two subclades.

A recent study showed that four reductases of L. japonicus, which are significantly similar to IFR, PCBER, and PLR, have PTR activity. The phylogenetic tree of these reductases showed that the PTR genes of L. japonicus (PTR1–PTR4) constitute a monophyletic clade with the PCBER of Populus trichocarpa (Fig. 4C). The putative IFRs of L. japonicus were likely to encode active IFR proteins because they were positioned in a monophyletic clade with other functionally characterized IFR genes. Both PCBER and PLR are involved in lignan biosynthesis, but the PTR/PCBER clade is closer to the IFR clade than the PLR clade. No orthologous gene similar to PCBER or PLR has so far been found in L. japonicus.

3.4. Expression of vestitol biosynthesis genes

We previously showed that treatment of L. japonicus seedlings with GSH induced the production of vestitol and the accumulation of transcripts from type II CHIs, CHI1 and CHI3, and I2H genes. To examine the expression of each paralogous gene in GSH-treated seedlings comprehensively, semi-quantitative RT–PCR analysis was performed. Among the 17 genes examined, the transcripts of 11 genes, i.e. PKR1, PKR2, PKR6, IF S1, IF S2, I2H, IFR1, IFR2, VR2 and PTR3, were markedly elevated 10 h after GSH treatment (Fig. 5A). A slight increase of PKR4 and VR1 transcripts during 10 h of elicitation and their decrease in 20 h after GSH treatment were also obvious. In contrast, those of PKR5, PTR1, PTR2, and PTR4 were constitutively expressed and not substantially affected by GSH.

The RT–PCR analysis also showed that transcripts of the genes were detectable in almost all tissues investigated. The expression patterns were rather complex, but some genes clearly showed organ specificity (Fig. 5B). Characteristically, no transcripts of any PKR paralogs, HID, I2H, IFR1, VR2, and PTR2 genes in flowers were observed, and neither the transcripts of PTR paralogs in nodules. The PKR transcripts were accumulated in both aerial (stems and leaves) and underground (roots and nodules) parts, but the expression level of PKR paralogs was higher in underground parts. In contrast, the expression of all PTR genes was mainly observed in aerial parts, and only the PTR4 transcript was detected.
in roots. Also, the expression of some paralogous genes was different among the organs: e.g. IFS1 was expressed in all organs, whereas IFS2 was highly expressed in leaves and weakly in underground parts; and the expression of VR1 was ubiquitous in all tissues investigated, but expression of VR2 was limited to leaves, roots, and stems. On the other hand, the expression patterns of paralogous genes, such as CHI1 and CHI3 and PKR1 and PKR2, were almost the same, suggesting their functional redundancy.

4. Discussion

Structural analysis of the genes involved in the legume-specific 5-deoxyisoflavonoid biosynthesis of L. japonicus revealed that six genes of the 10 investigated compose multigene families and form gene clusters in many cases. The origin of multigene families is attributed to gene duplication. The paralogous genes of L. japonicus involved in the flavonoid and triterpenoid biosynthesis were suggested to have acquired new functions different from the ancestral types as the result of accumulated nucleotide substitutions during their molecular evolution. The gene cluster of HID and carboxylesterase-like genes (Fig. 2D) also implies the evolution of HID through local gene duplication of an ancestral carboxylesterase-like gene. These findings support the idea that gene duplication is a major driving force for the evolution of novel metabolic pathways. Considering that enzymes with new functions might have been established through such evolutionary processes, we expected that candidates for enzymes and genes could be narrowed down by the combination of the predicted reaction mode of the target enzyme and the phylogenetic relationship. Actually, the genes for PTR were found in the SDR family, which includes PCBER, IFR, and PLR.

The phylogenetic tree implies that PTR genes should have been derived from the ancestral PCBER genes (Fig. 4C).

We present a comprehensive expression analysis of the genes involved in 5-deoxyisoflavonoid biosynthesis. Together with the previous finding on the GSH-induced transcription of type II CHI and I2H genes, all single genes and most of the paralogous genes encoding the biosynthetic enzymes as far as identified were shown to be synchronously up-regulated by the GSH treatment (Fig. 5A), which has been thought to mimic the signal of defense response, guaranteeing the elicitor responsive synthesis of vestitol. The coordinate increase of the transcripts suggests a common set of transcription factors involved in the regulation of this pathway, and the GSH-treatment will serve as a potential experimental system to analyze the transcriptional regulation of the 5-deoxyisoflavonoid genes. Although no significant cis elements common to the GSH-dependent genes has been predicted by in silico analysis, they will be obtained by further experiments of promoter analysis and used to identify the corresponding transcription factors. An interesting observation is the constitutive expression of PTR paralogs, except for PTR3, which was apparently up-regulated on GSH treatment. The role of PTR3 in vestitol biosynthesis has been considered to be insignificant, because it possesses low specific activity and no enantioselectivity. If the final step of vestitol synthesis is performed by enantiospecific PTR1 and PTR2 with high specific activity, this step is out of the regulation asserted by elicitor and not a rate-limiting step of phytoalexin biosynthesis.

The semiquantitative RT–PCR analyses also revealed the organ specific expression of each paralogous gene (Fig. 5B). Weak or no expression of PTR paralogs and the high expression levels of the other up-stream genes in underground parts imply that 5-deoxyisoflavonoids may be produced in symbiotic roots and nodules. Functions of flavonoids as positive and negative regulators in nodule development have been discussed. The observation that transcripts of some genes, i.e. PKR paralogs and HID, were not detected in flowers may conform the notion that the normal L. japonicus flower tissue producing flavonols and anthocyanins does not synthesize 5-deoxyisoflavonoids. However, the expression of genes may not simply reflect the flavonoid compositions, and more detailed and comprehensive examination of the transcripts and metabolites of the 5-deoxyisoflavonoid pathway in specific tissues and cells and during nodule organogenesis would be expected in the future.
On the other hand, the nearly identical expression patterns of some paralogous genes, such as PKR1 and PKR2, and CHI5 (Fig. 5B), strongly suggested that these paralogous genes are functionally redundant. They are likely to have been maintained during the legume evolution under selection pressure. A previously presented genetic model on the molecular evolution of genes showed that genetic redundancy is common in some cases, contrary to the widespread view that a redundant gene would be abolished by accumulated deleterious mutations. According to the model, redundant genes can be stable, provided they have pleiotropic functions or they are expressed in specific spatiotemporal patterns. Our expression analysis showed that some duplicated genes, e.g. PKR4, IFS2, VR2, PTR3, and PTR4, have acquired different expression patterns (Fig. 5A and B). IFS paralogs of soybean were also reported to show different expression patterns in different tissues, and in response to nodulation and defense signal. Alternatively, one of the two completely redundant genes will become extinct, but it may take a long time, provided that the mutation rates of the two genes are not very different.

The fact that the legume-specific 5-deoxyisoflavonoid pathway is at present widely distributed among Papilionoideae strongly suggests its substantial contributions to the fitness of leguminous plants, that is, 5-deoxyisoflavonoids have significant ecological and physiological functions. The roles of the individual biosynthetic genes will be clarified in part by dissection of the spatiotemporal patterns of gene expression and the distribution of 5-deoxyisoflavonoids, namely, by transcriptomics and metabolomics. In general, these ‘omics’ approaches are profoundly dependent on gene annotations. The results of this study provide the correct annotations for the legume-specific 5-deoxyisoflavonoid genes. On the other hand, reverse genetic approaches such as RNA interference or TILLING will enable us to test the significance of a gene, a biosynthetic step, or a pathway. RNA interference is applicable to redundant genes. On the other hand, reverse genetic approaches are profoundly dependent on gene annotations. The results of this study provide the correct annotations for the legume-specific 5-deoxyisoflavonoid genes. On the other hand, reverse genetic approaches such as RNA interference or TILLING will enable us to test the significance of a gene, a biosynthetic step, or a pathway. RNA interference is applicable to redundant genes if the consensus sequences common to all the paralogous genes are known, single genes, e.g. HI4, OMT, HID, and 12H of L. japonicus, can be the targets for knocking out by TILLING. The present study will also offer useful information for these functional genomic studies of leguminous plants.

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