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Gene Note

Isolation and characterization of a cDNA encoding polyketide reductase in *Lotus japonicus*

Norimoto Shimada[1], Takashi Nakatsuka[2], Masahiro Nishihara[2], Saburo Yamamura[2], Shin-ichi Ayabe[1], Toshio Aoki[1,*]

[1] Department of Applied Biological Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan; [2] Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan
* E-mail: taoki@brs.nihon-u.ac.jp Tel: +81-466-84-3703 Fax: +81-466-84-3353

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Abstract Leguminous plants have a unique pathway for production of 5-deoxy-type flavonoids and isoflavonoids that is distinct from the general flavonoid pathways. 5-Deoxy(iso)flavonoids are believed to play important eco-physiological roles as antimicrobial compounds and symbiotic signals toward rhizobia. The branching point of the 5-deoxyflavonoid pathway is the formation of the deoxy-type chalcone (isoliquiritigenin), which is catalyzed by the co-action of chalcone synthase and polyketide reductase (PKR). In the course of the comparative genomics of legume-specific genes, we cloned a putative cDNA for PKR (*cPKR1*) of a model legume *Lotus japonicus* (Regel) K. Larsen. Genomic Southern analysis showed that *L. japonicus* has a gene family composed of two to four paralogous *PKR* genes. The overexpression of *cPKR*1 in a red-flowered cultivar of petunia, "Polo Red Target", reduced anthocyanin accumulation and caused the formation of isoliquiritigenin and its putative derivatives. These results suggested that *PKR1* encodes a PKR that functions *in planta*.

Key words: Chalcone, 5-deoxyflavonoid, isoflavonoid, *Lotus japonicus*, polyketide reductase.

Leguminous plants have unique 5-deoxy-type flavonoids and isoflavonoids, some of which are believed to play significant roles in plant-microbe interactions (Aoki et al. 2000). 5-Deoxyisoflavonoids, in particular pterocarpans and isoflavans, are typical phytoalexins of many legumes and are active in the defense response against pathogenic organisms (Harborne 1999). Some 5-deoxy(iso)flavonoids, such as 7,4′-dihydroxyflavone and daidzein, are specific signal molecules that activate the *nod* genes of symbiotic rhizobia to trigger a series of processes for root nodulation and symbiotic nitrogen fixation (van Rhijn and Vanderleyden 1995).

The legume-specific 5-deoxyflavonoid pathway branches at an early step, where chalcone synthase (CHS) makes the C_6-C_3-C_6 skeleton from 4-coumaroyl-CoA and three molecules of malonyl-CoA (Figure 1). While CHS produces 4,2′,4′,6′-tetrahydroxychalcone (also termed 6′-hydroxychalcone or naringenin chalcone) in the general flavonoid pathway, CHS and polyketide reductase (PKR) together produce 4,2′,4′-trihydroxy-chalcone (6′-deoxychalcone or isoliquiritigenin) in the legume-specific 5-deoxyflavonoid pathway. PKR, which is synonymous with chalcone reductase, is a member of the aldo-keto reductases that encompass a large superfamily of NADP(H)-dependent oxidoreductases (Hyndman et al. 2003). The biochemical characterization and cDNA cloning of PKR from several leguminous plants have been reported (Welle and Griesbach 1988; Welle et al. 1991; Ballance and Dixon 1995; Sankawa et al. 1995; Tropf et al. 1995; Akashi et al. 1996; Hayashi et al. 1996). The substrate of PKR is not the chalcone molecule but an unstable intermediate of the CHS reaction (Ayabe et al. 1988; Oguro et al. 2004). cDNAs for PKR were also overexpressed in two non-leguminous plants, petunia and tobacco, and the changes in flavonoid constituents were investigated (Davies et al. 1998; Joung et al. 2003).

Two leguminous species, *Lotus japonicus* (Regel) K. Larsen and *Medicago truncatula* Gaertn., are subjects of the whole-genome sequencing programs after Arabidopsis and rice because of the agricultural and biological importance of the family Fabaceae (Udvardi et al. 2005). In *L. japonicus*, the treatment with reduced glutathione induces a coordinate increase in the transcription levels of genes encoding enzymes involved in the biosynthesis, leading to the accumulation of a

Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; ORF, open reading frame; PKR, polyketide reductase; RACE, rapid amplification of cDNA ends

The nucleotide sequence of *cPKR1* has been deposited in the DDBJ/EMBL/GenBank database under the accession number AB263016.
This article can be found at http://www.jspcmb.jp

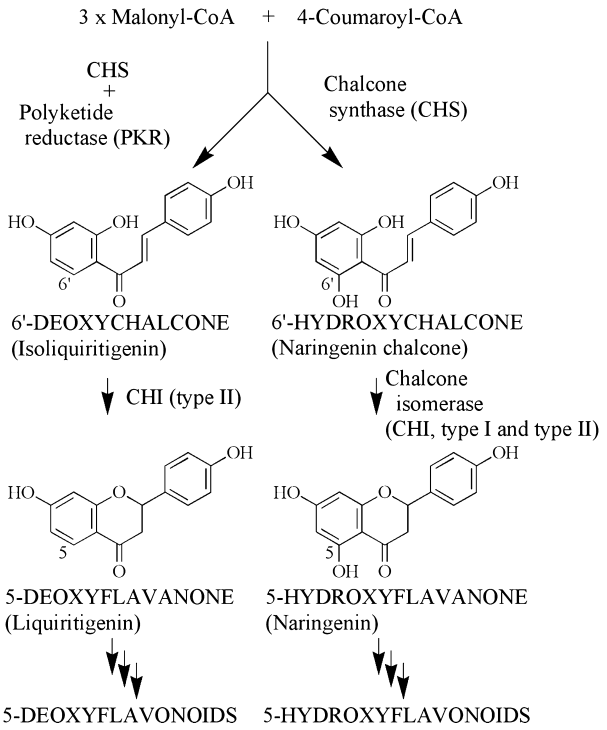

Figure 1. General and legume-specific flavonoid pathways. PKR co-acts with CHS to produce 6′-deoxychalcone (isoliquiritigenin), a common intermediate for 5-deoxyflavonoids.

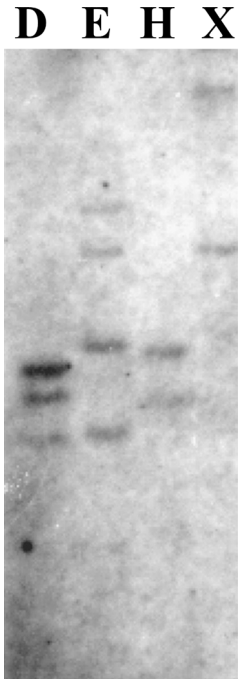

Figure 2. Genomic Southern blot analysis. Genomic DNA was digested with *Dra*I (D), *Eco*RI (E), *Hin*dIII (H) or *Xba*I (X), and subjected to hybridization using DIG-labeled *cPKR1* as a probe. The hybridization and stringency wash were performed under high stringency conditions.

5-deoxyisoflavonoid phytoalexin, vestitol (Shimada et al. 2000; Shimada et al. 2003). Here we report the cDNA cloning of *L. japonicus* PKR and identification of its function by over-expression in petunia as an approach for the comprehensive functional genomics of the biosynthetic genes in the 5-deoxyflavonoid pathway using a model system.

Degenerate primers were designed based on the amino acid sequences of the highly conserved regions of PKRs: PKR/S1 (5′-ACNTGYAARAARGAYAC-3′) and PKR/AS1 (5′-ARCATRTCRTTYTCCAT-3′). The degenerate PCR using the template cDNA prepared from the whole plant of *L. japonicus* Gifu B-129 (Shimada et al. 2003) gave a primer-specific product of ca. 600 bp. Rapid amplification of cDNA ends (RACE) was performed using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's manual. The specific primers for 3′-RACE and 5′-RACE were 5′-AAATCTGCTCTCTGTTGCCAATA-3′ and 5′-GATATTGGCAACAGAGAGCAGAT-3′, respectively. The 5′-RACE yielded a cDNA fragment, and the 3′-RACE yielded two fragments with 98% identity of the nucleotide sequences. The full ORF of a cDNA (*cPKR1*) was obtained by PCR using the primers, 5′-TCTCTACGGATCCCAAACTTTCAATACTAT-3′ and 5′-CAAAGCTCGAGTTTTCAGGATAAGATTCA-3′, which included the *Bam*HI and *Xho*I sites (underlined), respectively. The ORF of *cPKR1* (DDBJ/EMBL/GenBank accession no. AB263016) was 948 bp long, and the deduced amino acid sequence was 86%, 90%, and 88% identical to those of *Glycine max* (X55730), *Glycyrrhiza echinata* (D83718), and *Medicago sativa* (X82367) chalcone reductase, respectively.

Genomic Southern blot analysis using a DIG-labeled *cPKR1* fragment (97 to 704 bp region from start codon) as a probe was carried out as described previously (Shimada et al., 2003). This probe has two restriction sites for *Hin*dIII and no restriction sites for *Dra*I, *Eco*RI or *Xba*I. The hybridization was performed at 42°C overnight, and the stringency wash was 0.1×SSC and 0.1% SDS at 68°C, which was expected to detect homologous sequences of more than 90% identity. The results showed four bands from the *Eco*RI-digested DNA, three from the *Dra*I-digested, and two from the *Hin*dIII or *Xba*I-digested, respectively, suggesting the presence of two to four *PKR*-related genes (Figure 2). Thus, although two cDNA fragments have been obtained by the 3′-RACE, *L. japonicus* should have one or two more *PKR*-like genes with closely related (>90%) sequences. In general, nucleotide sequence identities higher than 90% means that the protein products have the same or very similar catalytic functions. The multiple *PKR* genes in *L. japonicus* may be redundant, or each paralogous gene may show a specific expression pattern. The organ-specificity and elicitor-induction of the *PKR* gene expression will be reported elsewhere.

To identify the function of *PKR1*, transgenic petunia plants were produced. A red-flowered petunia was used as the host for the *in planta* identification of the *PKR1* gene because of the high frequency of regeneration and abundant flavonoid (anthocyanin) accumulation in the petal. *cPKR1* was subcloned into the *Bam*HI-*Xho*I sites of the pBIS221S vector at the downstream of the CaMV 35S promoter (Kiba et al. 2005), and the resultant vector was then integrated with the binary vector pEKB to give pEKBPKRBar. *Petunia hybrida* Vilm. "Polo Red Target" was transformed with pEKBPKRBar by a standard *Agrobacterium*-mediated leaf-disc transformation method (Mori et al. 2004) using the *A. tumefaciens* strain EHA101. Five independently regenerated petunia lines were grown to flowering. The flower colors of two regenerated lines, no. 6 and no. 8, were obviously changed from red to variegated red (Figure 3A). No other visual changes were found in the two lines compared to the wild type plants. Northern analysis of the total RNA isolated from leaves showed the accumulation of *PKR1* transcripts in the regenerated lines no. 6 and no. 8 (Figure 3B). The transcript accumulation in the petals of the two transgenic petunia lines was also confirmed by RT-PCR analysis (data not shown). The *PKR1* transcripts were also detected in two other regenerated lines, but they were not investigated further because of no obvious change of the flower color.

Flavonoids in petals of the wild type and the transgenic lines were analyzed with an HPLC-photodiode array spectrophotometry system (JASCO, Tokyo, Japan). Flavonoids were extracted with three volumes of 0.1% HCl in methanol overnight at 4°C and separated on a CAPCELL PAK C18 MG column (4.6×150 mm; Shiseido, Tokyo, Japan) at the flow rate of 1.2 ml min^{-1} at 40°C. The mobile phase was a linear gradient of acetonitrile in phosphoric acid/H_2O (pH 3.0), 0% to 40% in 40 min. The analysis with the HPLC chromatogram at 530 nm showed that the anthocyanin contents of the petals of the PKR transgenic lines no. 6 and no. 8 were ca. 30% and 50% of those of the wild type (data not shown). The chromatogram at 385 nm showed five transgenic-specific compounds, **a** to **e** (Figure 3C). The compound **e** exhibited a retention time (39.1 min) and UV-visible spectrum (λ_{max} at 239 and 371 nm) identical to those of the authentic isoliquiritigenin, and the identity was further confirmed by co-chromatography (Figure 3C, D). The other transgenic-specific compounds also showed the chalcone-like spectra, and, in particular, the λ_{max} (239 and 371 nm) of the compounds **b** to **d** were nearly identical to that of isoliquiritigenin (data not shown). These results showed the accumulation of isoliquiritigenin in the petunia "Polo Red Target" expressing *cPKR1* although other transgenic-specific products were not identified. The *PKR1* gene of *L. japonicus* was thus demonstrated to encodes an active PKR protein that functions *in planta*.

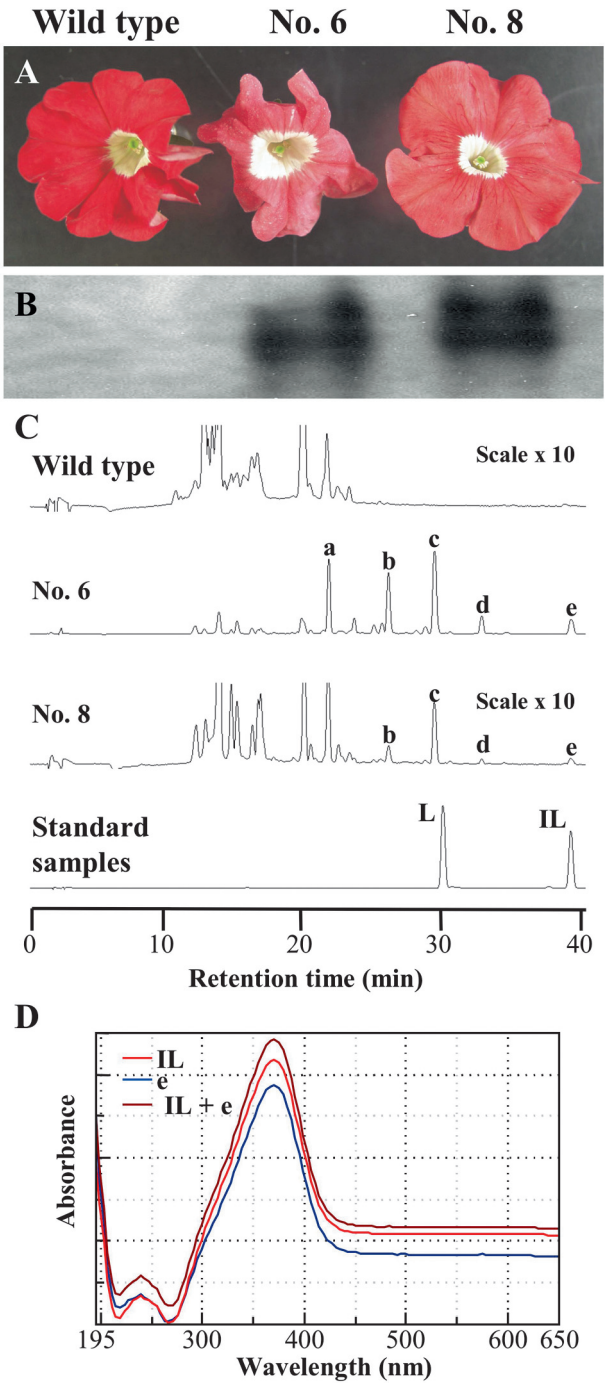

Figure 3. Analysis of *PKR*-transgenic petunia. (A) Phenotype of flowers from wild type and *cPKR1*-transgenic petunia "Polo Red Target". (B) Northern blot analysis showing the transcript accumulation of *PKR1* in leaves of transgenic lines no. 6 and no. 8. (C) HPLC chromatograms of the wild type and the transgenic plants. (D) UV-visible spectra of authentic isoliquiritigenin (red), the compound *e* (blue) and a mixture of nearly equal amount of authentic isoliquiritigenin and the compound *e* (brawn). IL, isoliquiritigenin; L, liquiritigenin.

In leguminous plants, isoliquiritigenin is converted to liquiritigenin (5-deoxyflavanone) by legume-specific

type II chalcone isomerase (CHI, see Figure 1) (Shimada et al. 2003). On the other hand, general type I CHI in non-leguminous plants such as petunia is not capable of this catalytic activity but only able to isomerize naringenin chalcone to naringenin. Reduced anthocyanin accumulation in the transgenic red-flowered petunia (Figure 3A) can be attributed to the restricted supply of the intermediate naringenin. The absence of detectable liquiritigenin or other 5-deoxyflavonoids in the petal of the transgenic petunia indicates the absence of type II CHI activity (Figure 3C). Although liquiritigenin was detected in tobacco overexpressing *PKR* cDNA from *Pueraria* (Joung et al. 2003), the acid treatment in the analytical procedure to liberate aglycones might cause the artificial conversion of isoliquiritigenin to liquiritigenin. When *P. hybrida* "Mitchell" was transformed with a cDNA for the PKR of *M. sativa* (CHR7), isoliquiritigenin was not detectable in the petals, but butein (3,4,2′,4′-tetrahydroxychalcone) and its glucosides were accumulated (Davies et al. 1998). "Mitchell" has white flowers and is homozygously recessive for five genes involved in anthocyanin biosynthesis, while the "Polo Red Target" used in this study has red flowers. The transgenic flowers of "Mitchell" were yellowish in color, which was attributed to the production of butein glucosides. Butein has two hydroxyl groups at the 3- and 4-positions on the B-ring. Since CHS generally produces the chalcones with mono hydroxyl on the B-ring, i.e., naringenin chalcone or isoliquiritigenin, additional hydroxylation must have occurred in the petals of the transgenic "Mitchell". The accumulation of isoliquiritigenin in the petals of the transgenic "Polo Red Target" may be due to insufficient hydroxylation activity present in " Mitchell" petals.

The structural analysis and linkage mapping of *PKR* genes in *L. japonicus* are in progress. To the best of our knowledge, the distribution of *PKR* is almost exclusive to Fabaceae and probably common to leguminous plants, particularly Papilionoideae. The genomic information of *PKR* genes will serve as a means for the comparative genomics of leguminous plants.

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