Dihydroflavonol 4-reductase cDNA from non-Anthocyanin-Producing Species in the Caryophyllales

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Two types of red pigment, anthocyanins and betacyanins, never occur together in the same plant. Although anthocyanins are widely distributed in higher plants as flower and fruit pigments, betacyanins have replaced anthocyanins in the Caryophyllales. We isolated cDNAs encoding dihydroflavonol 4-reductase (DFR), which is the first enzyme committed to anthocyanin biosynthesis in the flavonoid pathway, from Spinacia oleracea and Phytolacca americana, plants that belong to the Caryophyllales. The deduced amino acid sequence of Spinacia DFR and Phytolacca DFR revealed a high degree of homology with DFRs of anthocyanin-producing plants. The DFR of carnation, an exception in the Caryophyllales that synthesizes anthocyanins, showed the highest level of identity. In the phylogenetic tree, Spinacia DFR and Phytolacca DFR clustered with the DFRs of anthocyanin-synthesizing dicots. Recombinant Spinacia and Phytolacca DFRs expressed in Escherichia coli convert dihydroflavonol to leucoanthocyanidin. The expression and function of DFR in spinach and pokeweed are discussed in relation to the molecular evolution of red pigment biosynthesis in higher plants.

Keywords: Anthocyanin biosynthesis — Caryophyllales — Dihydroflavonol 4-reductase — Phytolacca americana — Spinacia oleracea.

Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; DFR, dihydroflavonol 4-reductase; DMACA, dimethylaminocinnamaldehyde; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; TLC, thin layer chromatography.

The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL, GenBank under accession numbers AB109016 (Spinacia DFR) and AB128768 (Phytolacca DFR).

Introduction

Flower colors are mainly due to three types of pigment: flavonoids, betalains and carotenoids. Anthocyanins, a type of flavonoid, are responsible for most pinks, reds, mauves and blues in flowers and fruits, where they serve as visual signals to recruit pollinators and seed dispersers. Blue flower color is due to intermolecular co-pigmentation involving anthocyanins and other flavonoids, flavones or flavonols (Mol et al. 1998, Harborne and Williams 2000). The biosynthetic pathway of flavonoids is probably one of the best-studied examples of secondary metabolism in higher plants and, with a few exceptions, flavonoid biosynthetic genes have been cloned and analyzed (Holton and Cornish 1995, Forkmann and Martens 2001, Shirley 2001). Chalcone synthase catalyzes the first and key regulatory step of flavonoid biosynthesis, which involves the stepwise condensation of three acetyl units from malonyl-coenzyme A (CoA) with the coumaroyl moiety of 4-coumaroyl-CoA derived from the phenylpropanoid pathway to give rise to the C15 flavonoid skeleton, naringenin chalcone. Further substitutions following isomerization of naringenin chalcone lead to the formation of flavones, flavonols and anthocyanins. Dihydroflavonol 4-reductase (DFR) is the first committed enzyme of anthocyanin biosynthesis in the flavonoid pathway and catalyzes the NADPH-dependent reduction of dihydroflavonols into leucoanthocyanidins (Fig. 1). Leucoanthocyanidins are converted into colored anthocyanidins by anthocyanidin synthase (ANS). The subsequent stabilization of anthocyanidins by glucosylation and acylation leads to a variety of anthocyanins. The DFR genes of various plant species have been cloned (Beld et al. 1989, Shirley et al. 1992, Helariutta et al. 1993, Sparvoli et al. 1994, Bernhardt et al. 1998) and the regulation of DFR gene expression has also been studied in several species (Dooner et al. 1991, Martin et al. 1991, Quattrocchio et al. 1993). DFR has been well characterized as the regulatory enzyme of anthocyanin biosynthesis. DFR contributes to controlling the range of flower color (Tanaka et al. 1998). Flower pigmentation designs based on flecks and sectors are controlled by mutation of DFR with transposable elements (Inagaki et al. 1999, Ishi et al. 2002).

Although anthocyanins are widely distributed in higher plants as flower and fruit pigments, in the Caryophyllales, excluding the families Caryophyllaceae and Molluginaceae, betacyanins have replaced anthocyanins. The occurrence of anthocyanins in these plants has not been reported. Therefore, these two types of red pigment, anthocyanins and betacyanins, never occur together in a plant. Nothing is known about the mutual exclusion of anthocyanins and betacyanins in higher plants. This curious phenomenon has been examined from genetic and evolutionary perspectives. Nevertheless, while the structural and regulatory genes of anthocyanin biosynthesis are now well known, the biosynthesis of betacyanins is poorly understood. The evolutionary mechanism of the mutual exclusion of these two pathways is still a mystery (Stafford 1994).

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We focused on the potential for anthocyanin biosynthesis from dihydroflavonols to anthocyanins might be blocked in the Caryophyllales. To address the evolutionary significance of the biosynthetic pathways of pigments, we examined why anthocyanins are not synthesized in the Caryophyllales. To address its phylogenetic relationships with other DFRs, DFR sequences from various plant species were aligned using Clustal W (Thompson et al. 1994). A phylogenetic tree of the full-length DFRs was constructed by the neighbor-joining method (Saitou and Nei 1987) using PHYLIP (J. Felsenstein, Phylogeny Inference Package ver. 3.5c, University of Washington, Seattle, WA, U.S.A.). The results are shown in Fig. 3 with bootstrap values. Monocot and dicot DFRs were clearly separated in the tree. Spinacia DFR and Phytolacca DFR clustered with the dicot DFRs of other anthocyanin-synthesizing plants. The tree also indicated that DFRs of non-anthocyanin-producing species were not distantly related to other DFRs and were most closely related to carnation DFR.

### Results

#### Identification and isolation of DFR

We attempted to detect DFR homologs using nested PCR with Spinacia and Phytolacca genomic DNA as the template. The nucleotide sequences of DFRs from different plants were aligned to find the most highly conserved regions to design two sets of degenerate primers. DNA fragments with an expected size of about 0.2 kb were amplified by nested PCR. Moreover, DNA fragments of the same size were also amplified by reverse transcription–PCR (RT–PCR) with mRNA isolated from spinach and pokeweed. Nucleotide sequence analysis of the products of PCR with genomic DNA and cDNA revealed that both amplified fragments were identical and the nucleotide sequence showed 60–75% identity to the corresponding region of published sequences of other DFRs. These observations suggested that DFR orthologs were expressed in spinach and pokeweed. Then, we attempted to isolate the full-length DFR cDNAs using 3′- and 5′-rapid amplification of cDNA ends (RACE) from the Caryophyllales. mRNA isolated from spinach and pokeweed was subjected to 3′- and 5′-RACE. The degenerate primers for nested PCR were also used for 3′- and 5′-RACE. The full lengths of Spinacia DFR cDNA (1.2 kb) and Phytolacca DFR cDNA (1.3 kb) were isolated by PCR using primers designed from the sequences of the 3′- and 5′-RACE products and subjected to sequence analysis. The Spinacia DFR cDNA and Phytolacca DFR cDNA contained a 1,026 bp open reading frame encoding a protein of about 37 kDa and a 1,020 bp open reading frame encoding a protein of about 36 kDa. The deduced amino acid sequence of Spinacia DFR and Phytolacca DFR shared 78% and 82% identity with the carnation DFR sequence, 76% and 72% with grape, 73% and 74% with Arabidopsis, 71% and 66% with petunia, and 62% and 64% with corn, respectively (Fig. 2). The deduced amino acid sequences of the DFRs, including Spinacia and Phytolacca DFR, were well conserved, except at both ends of the sequences. A putative NADPH binding region at the N-terminal, which is likely part of the co-factor binding site (Lacombe et al. 1997), was also conserved in the Caryophyllales. We focused on the potential for anthocyanin biosynthesis in the Caryophyllales, and first examined whether these plants possess DFR genes. Here, we report the isolation and functional identification of DFR genes from Spinacia oleracea and Phytolacca americana. We show significant sequence identity of DFR of the Caryophyllales to those from anthocyanin-producing plants and discuss the phylogenetic relationships of DFR in higher plants.
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**Fig. 2**  Comparison of the amino acid sequences of DFRs. Using published DFR sequences of flowering plants: spinach, *S. oleracea*, AB109016; pokeweed, *P. americana*, AB128768; carnation, *Dianthus caryophyllus*, Z67983; Arabidopsis, *Arabidopsis thaliana*, M86359; grape, *Vitis vinifera*, X75964; petunia, *Petunia hybrida*, X15537; corn, *Zea mays*, Y16040. Identical amino acid residues are marked with asterisks. The amino acid residues strictly conserved in the mammalian 3β-hydroxysteroid dehydrogenase/DFR superfamily (Baker and Blasco 1992, Lacombe et al. 1997) are marked with black arrows above the sequences. The gap common to the DFRs of spinach and carnation is indicated by a black circle. The boxed region is a putative NADPH binding region (Lacombe et al. 1997). The region predicted to be related to substrate specificity (Johnson et al. 1999, Johnson et al. 2001) is underlined.
**DFR assay**

The properties of DFR recombinant proteins expressed in *Escherichia coli* were analyzed. The open reading frames were subcloned into pET-28a expression vectors, which were then introduced into *E. coli* BL21 (DE3) cells. Cell extracts from transformed *E. coli* were incubated with dihydroquercetin in the presence of the cofactor NADPH. The products were converted to anthocyanidin under acidic conditions with HCl and subjected to thin layer chromatography (TLC). The products of the *Spinacia* DFR and *Phytolacca* DFR reactions were detected as a red spot with the same *Rf* value as authentic cyanidin on TLC (Fig. 4). Cyanidin was also detected in the reaction using recombinant *Perilla* DFR expressed in *E. coli*. These results indicated that the recombinant *Spinacia* and *Phytolacca* DFR proteins converted dihydroquercetin into leucocyanidin.

Although a DFR enzyme assay using crude extracts from plant cells was performed, no DFR activity was detected by the assays using crude extracts from mature leaves and seedlings of spinach and young leaves of pokeweed (data not shown).

**Expression profiles of DFR**

To gain an overview of the expression profiles of DFR, we adopted semi-quantitative RT–PCR with total RNA prepared from various tissues and/or organs including seedlings at different stages of development and whole plants of spinach (Fig. 5A). The results showed that the transcripts of *Spinacia DFR* were more or less detected in most samples tested (Fig. 5A, lower panel). A higher level of *Spinacia DFR* transcript was detected in seedlings (Fig. 5A, upper panel). The maximal accumulation of transcripts was observed in seedlings at stage 3 (second set of foliage leaves appears). As shown in Fig. 5B, Northern blot analysis revealed that the probe hybridized with a 1.2 kb transcript and that its expression was the strongest during stage 3 then declining as the third set of foliage leaves developed (stage 4). Under the condition used, no transcripts were detected by Northern blot analysis of mRNA isolated from leaves, stems or roots of mature plants (data not shown).
We next examined the accumulation of proanthocyanidins (condensed tannins) in spinach seedlings, in which DFR was expressed transiently, because anthocyanin and proanthocyanidin synthesis share the same sequence of reactions to the common intermediate, leucoanthocyanidins. Seedlings at different stages of development of spinach were stained with DMACA.

**Fig. 5** RT-PCR analysis and Northern hybridization. (A) Total RNA extracted from leaves, stems, roots and seedlings at stage 1 (cotyledons appear), stage 2 (foliage leaves appear), stage 3 (second set of foliage leaves appears), stage 4 (third set of foliage leaves appears) was used for the semi-quantitative RT-PCR analysis. PCRs were carried out for 30 or 40 cycles using gene-specific primers. (B) Northern blot analysis of the expression of *Spinacia DFR* in seedlings. Aliquots of 2 µg of mRNA from seedlings (stage 1–4) were subjected to Northern hybridization.

**Fig. 6** Histochemical analysis of proanthocyanidin accumulation. (A) DMACA staining showing proanthocyanidin accumulation in stem (center panel) and root (right panel) of *L. japonicus*. (B) Spinach seedlings of different stages (1–4) of development stained with DMACA (upper panels). Bottom panels show enlarged images of stem and root of stained seedling.
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which specifically reacts with proanthocyanidins and its precursors and visualizes their compounds as blue staining (McMurrough and McDowell 1978). Fig. 6 shows spinach seedlings and *Lotus japonicus*, which is known to accumulate proanthocyanidins, stained with DMACA. In contrast to the DMACA reaction of *L. japonicus*, showing a dark blue–purple coloration on stems or a spotty pattern of coloration on roots (Fig. 6A), spinach seedlings at stage 1 through stage 4 were uniformly DMACA negative (Fig. 6B).

**Discussion**

The diversity of particular secondary metabolites of higher plants has been used as a character in chemotaxonomy. Anthocyanins and betacyanins, two types of red pigment, never occur together in plants. However, the evolutionary mechanism of the mutual exclusion of these two pathways is still a mystery (Stafford 1994). We isolated structural genes encoding DFR from spinach and pokeweed, plants belonging to the Caryophyllales. Comparison of the deduced amino acid sequences of *Spinacia* DFR and *Phytolacca* DFR with published sequences revealed a high degree of homology (62–82% identity) with the previously characterized DFRs of anthocyanin-producing plants (Fig. 2). The DFR from carnation, which is an exception in the Caryophyllales that synthesizes anthocyanins, showed the highest level of identity with *Spinacia* DFR (78%) and *Phytolacca* DFR (82%). Phylogenetic analysis using the deduced amino acid sequences of DFRs also indicated that *Spinacia* DFR and *Phytolacca* DFR were most similar to carnation DFR. In the phylogenetic tree, *Spinacia* DFR and *Phytolacca* DFR clustered with the DFRs of anthocyanin-synthesizing dicots (Fig. 3). This suggests that the DFRs of betacyanin-producing plants were derived from a common ancestor of the DFRs of anthocyanin-producing plants. This indicates that *Spinacia* DFR and *Phytolacca* DFR have the highly conserved structure of the DFRs of anthocyanin-producing plants.

The amino acid residues that are strictly conserved in the mammalian 3β-hydroxysteroid dehydrogenase/DFR superfamily (Baker and Blasco 1992, Lacombe et al. 1997) were also found in DFRs of non-anthocyanin-producing species. An amino acid is missing in the region corresponding to the dicot third exon in the *Spinacia* and *Phytolacca* DFRs and in the DFR of carnation, which might be unique characters of the Caryophyllales (Fig. 2).

There is diversity of DFR substrate specificity in some plant species (Forkmann and Ruhnau 1987). The study of substrate specificity of recombinant DFR enzymes of six plant species has shown that dihydroquercetin was reduced by enzyme extracts from yeast expressing DFR from all species (Martens et al. 2002). Therefore, we used dihydroquercetin as substrate of DFR for enzyme assay in this study.

DFR activity has been demonstrated in vitro using crude extracts of many plant species (Stafford and Lester 1982) and in vivo in transgenic plants (Johnson et al. 2001). Recently, the functional expression of DFR enzymes using yeast (Martens et al. 2002) and *E. coli* (Peters and Constabel 2002) expression systems has been reported. The biochemical study revealed that recombinant DFRs of non-anthocyanin-producing species expressed in *E. coli* convert dihydroquercetin into leucocyanidin (Fig. 4). In spinach, common flavonol glycosides, quercetin 3-O-galactoside and kaempferol 3-O-glucoside have been detected. Also in pokeweed, flavonol glucosides, kaempferol 3-O-diglycoside and quercetin 3-O-rhamnosylglucoside have been detected (Iwashina 2001). This implies the occurrence of dihydroquercetin and dihydrokaempferol, which are substrates of DFR in spinach and pokeweed. *Spinacia* DFR and *Phytolacca* DFR might contribute to the reduction of dihydroflavonols in plants.

Nevertheless, no DFR activity was detected in crude extracts of mature leaves and seedlings of spinach and young leaves of pokeweed under the same conditions as used for heterologously expressed enzyme extracts (data not shown). Furthermore, the accumulation of DFR transcripts was not detected in any organs of mature spinach using Northern blot analysis. The comparison of DFR expression using Northern hybridization showed that the expression of DFR in spinach seedlings was much weaker than in an anthocyanin-producing suspension culture of grape cells (data not shown). These results suggest that insufficient expression or suppression of DFR results in the inability of spinach to biosynthesize anthocyanin. In white grapes, the transcription of UDP-glucose flavonoid glucosyl transferase, which catalyzes the last step in anthocyanin biosynthesis, is suppressed by mutation of a regulatory gene, which leads to the lack of color (Boss et al. 1996). The expression of DFR in spinach needs to be investigated with a special focus on the promoter region and transcription factors.

DFR expression in spinach was analyzed by semi-quantitative RT–PCR. It has been shown that *Spinacia* DFR was expressed transiently in seedlings at a specific stage during seedling development and the level of DFR expression was very low in mature spinach.

DFR is also involved in the biosynthetic pathway of proanthocyanidins (Fig. 1). Proanthocyanidins are synthesized from leucoanthocyanidins via (+)-flavan-3-ols (e.g. catechins) or (−)-epi-flavan-3-ols (e.g. epicatechins). Recent study has shown that the BANYULS gene in *Arabidopsis thaliana* encodes anthocyanidin reductase (ANR), which converts anthocyanidins to their corresponding 2,3-cis-flavan-3-ols (Xie et al. 2003). In the Aizoaceae, a family in the Caryophyllales, the occurrence of flavan-3-ols and proanthocyanidins has been reported (Kolodziéj 1984). Proanthocyanidins were also found in some other members of betacyanin-containing family (Stafford 1994). DMACA reacts specific flavonoids with *mete*-oriented di- or trihydroxy substituted benzene rings and a single bond at the 2,3-position in the A-ring: leucoanthocyanidins, flavan-3-ols and proanthocyanidin polymers (McMurrough and
McDowell 1978). The sensitivity of the DMACA stain (another aromatic aldehyde) is some four-fold greater than vanillin (Li et al. 1996). Staining of spinach seedlings with DMACA resulted in no distinguishing coloration of seedlings at stage 3 (second set of foliage leaves appears) in which transient expression of \( DFR \) was observed. These results suggest that transient expression of \( DFR \) in spinach seedlings is not enough to give rise to the accumulation of detectable amounts of proanthocyanidins.

Another possible explanation for the lack of anthocyanin synthesis in the Caryophyllales might be due to the loss of function or suppression of the ANS catalyzing the latter step to anthocyanidin, which would block anthocyanin production. Recent studies have provided some biochemical information regarding the reaction catalyzed by ANS (Nakajima et al. 2001, Saito et al. 1999). The conversion of leucoanthocyanidin by ANS yields not only anthocyanidin but also dihydroflavonol and flavonol (Turnbull et al. 2000). In vitro experiments using recombinant ANS have shown that the stereochemistry of substrate directly affects product selectivity (Turnbull et al. 2003). Studies are currently in progress to clone ANS cDNA from the Caryophyllales.

**Materials and Methods**

**Plant materials**

Seeds of the spinach variety ‘Active’ were purchased from Sakata Seed (Yokohama, Japan) and germinated at 25°C in the dark for 2 d. After germination, the seedlings were grown in a growth chamber under a 12 h photoperiod at 22°C. Suspension cultures were prepared from callus initiated from stem explants of \( P. \) americana \( L. \). Cells were subcultured every 7 d in Murashige and Skoog (1962) medium containing 3% sucrose and 5 \( \mu \)M 2,4-D as described previously (Sakuta et al. 1986).

**Preparation of genomic DNA and RNA**

Genomic DNA was extracted with extraction buffer \([0.3 \text{ M NaCl}, 0.05 \text{ M Tris–HCl pH 7.5}, 20 \text{ mM EDTA}, 0.5\% \text{ SDS}, 10 \text{ mM 2-mercaptoethanol}, 5\% (\text{v/v}) \text{ phenol} \] and phenol/chloroform. Total RNA was extracted from 2 g of frozen cells with extraction buffer \([0.1 \text{ M Tris–HCl pH 9.0}, 0.1 \text{ M NaCl}, 10 \text{ mM EDTA}, 0.5\% \text{ SDS}, 14 \text{ mM 2-mercaptoethanol} \) and phenol/chloroform and precipitated with LiCl (Ozeki et al. 1990). \( mRNAs \) were prepared using Oligotex-dT\( \_30 \) Super (Takara, Kyoto, Japan), as described in the supplier’s manual.

**Detection of \( DFR \) by PCR**

To detect conserved \( DFR \) homologs, two sets of primers were designed for nested PCR from the most conserved region of known \( DFR \) sequences (Beld et al. 1989, Shirley et al. 1992, Sparvoli et al. 1994, Tanaka et al. 1995). The primers for the first PCR were \( DFR-\_S1 \) \([5'-\text{AGAAGGAAAGCTTCATATTGGAATCTACATCCCCTGTGAGG-3'}\) and \( DFR-\_SP1 \) \([5'-\text{CAGATGCTACATTTGGAGAGAT-3'}\) respectively, with SuperScript II reverse transcriptase (Invitrogen) and used as the template for PCR. The PCR was performed with \( \text{primers} \) for first PCR of spinach, \( DFR-\_SP1 \) and \( GSP (5'-\text{CCAGTACATTTGGAGAGAT-3'}) \) for second PCR of spinach, \( DFR-\_SP2 \) and \( GSP-T3 (5'-\text{CCACAGGCGCATAGCTAC-3'}) \) for pokeweed, the set of \( PADFR-\_SP1 (5'-\text{GTCCGCAATAGCTACATTT-3'}) \) and \( DFR-\_ES-\_AS1 (5'-\text{CA(G/A)TGC(A/G)TGC(A/TG/C)A(A/G)(A/G)TC(A/G)TGC(A/TG/C)AC-3'}) \) and \( DFR-\_AS2 (5'-\text{CCAAGCGAGACTAATGCCATCCC-3'}) \) and \( GSP-T3 (5'-\text{CCAAACGCGACAGATCTACATTT-3'}) \) for 30 cycles at 94°C 15 s, 55°C 30 s, 70°C 45 s. A 900 bp PCR product from spinach and 400 and 600 bp PCR products from pokeweed were cloned in pT7Blue vector (Novagen, Madison, WI, U.S.A.) and sequenced. For 5'-RACE, single-stranded DNA was synthesized from 1 \( \mu \)g of the mRNA of spinach and pokeweed using a 5'-Full RACE Core Set (Takara) according to the manufacturer’s instructions. The primers for the reverse transcription reaction \( [\text{for spinach, SO5'-Full-DFR-RT (5'-}\text{GGAGCAGAGGAGGTGCAAGGAGAGAT-3'}) \] and \( DFR-\_RT (5'-\text{TTACATTGCTGTTTATC-3'}) \) were used. The first PCR was performed with \( \text{primers} \) \([\text{for spinach, SO5'-}\text{Full-DFR-S1 (5'-}\text{CCTGTGATGAAACTTGTG-3'}) \] and \( SO5'-\text{Full-DFR-AS1 (5'-}\text{TGGATTTCTTCAACATTGAC-3'}) \) for pokeweed, \( PA5'-\text{Full-DFR-S1 (5'-}\text{TATGATGAAATCCATGGAAG-3'}) \) and \( PA5'-\text{Full-DFR-AS1 (5'-}\text{CAGGTTTCTTGAGCTTCAACATTGAC-3'}) \) and a second PCR was performed with \( \text{primers} \) \([\text{for spinach, SO5'-}\text{Full-DFR-S2 (5'-}\text{TATGATGAAATCCATGGAAG-3'}) \] and \( PA5'-\text{Full-DFR-S2 (5'-}\text{ATATGGAATTTTGTCGATCG-3'}) \) and \( PA5'-\text{Full-DFR-AS2 (5'-}\text{TATGATGAAATCCATGGAAG-3'}) \) and \( PA5'-\text{Full-DFR-AS2 (5'-}\text{TATGATGAAATCCATGGAAG-3'}) \) using the same conditions as for 3'-RACE. Both 500 bp PCR products were cloned using the same method as for the 5'-RACE product and sequenced.

**DNA sequencing**

The nucleotide sequences of at least five clones were determined using a ThermoSequenase Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, U.S.A.) and a DSQ-2000L sequencer (Shimadzu, Kyoto, Japan) or using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience) and an ABI 373A sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

**Northern hybridization**

Aliquots of 2 \( \mu \)g of mRNA were subjected to electrophoresis in 0.8% agarose gels containing formaldehyde and blotted onto Hybond N \(^{-} \) membranes (Amersham Bioscience). The RNA blots were probed with \( ^{32}P\)-labeled \( DFR \) cDNA prepared using the Megaprime DNA Labeling System (Amersham Bioscience). The filters were hybridized according to the manufacturer’s protocol. The membranes were washed for 15 min in 2\( \times \) SSC/0.1\% SDS, twice for 15 min each time in 1\( \times \) SSC/0.1\% SDS at room temperature, and for 15 min in 0.1\( \times \) SSC/0.1\% SDS at 55°C for 1 h.
**Heterologous expression in E. coli**

For bacterial expression, the coding sequences of DFRs of the Caryophyllales were PCR amplified using Pyrobest DNA polymerase (Takara) from cDNA using primers [for Spinacia DFR, SOORF-DFR-S and SOORF-DFR-AS (5′-CGCATGTCGATTAATCAATAATGG-TATTAG-3′); for Phytolacca DFR, PAORF-DFR-S and PAORF-DFR-AS (5′-CGCATGTCGATTAATGGTTGTTCCATTACAT-3′)]. *Perilla DFR* was PCR amplified from the full-length DFR of a *Perilla frutescens* plasmid clone, pDFR-PF (Gong et al. 1997) using PFORF-DFR-S (5′-CCGGGATCCATGTCGATACGGAAACCCTGGC-3′) and PFORF-DFR-AS (5′-CGCATGTCGATCATATCTGCATTTCTTGTC-3′) primers. These were cloned into pET-28a bacterial expression vector (Novagen). The resulting plasmids were moved into E. coli strain BL21 (DE3). Cultures were grown at 28°C for 3 h in the presence of 1 mM IPTG, pelletted by centrifugation and resuspended in 0.1 M K-Pi (10% sucrose, pH 7.0). The cells were disrupted by sonication, the suspension centrifuged and the supernatant used directly for the DFR activity assay as a crude enzyme extract. The empty expression vector was used to transform the same E. coli strain and the crude enzyme extracts were obtained as controls.

**Preparation of crude extracts from plants**

Approximately 1 g of frozen cells was extracted in 3 ml of 0.2 M K-Pi pH 7.0, 0.2 g of dry polyvinylpyrrolidone and 0.1 g of Dowex 1 × 2. Samples were centrifuged and the supernatant fraction was de-salted on medium resin Sephadex G-25 PD-10 column (Amer- Dowex 1 × 2). The cell-free supernatant was precipitated by the addition of ammonium sulfate to 40% saturation, centrifuged and the supernatant used directly for the DFR activity assay as a crude enzyme extract. The crude enzyme extracts were obtained as controls.

**DFR enzyme assay and identification of products by TLC**

The reaction mixture (500 µl) containing 7.5 µM (+/-)-dihydro- quercetin, 2 mM NADPH, 1 U glucose-6-phosphate dehydrogenase, 6 mM glucose-6-phosphate and enzyme extract (100–300 µg) was incubated at 25°C for 4 h. The protein concentrations were determined by the Bradford method (Bradford 1976). The leucoanthocyanidins were extracted twice with ethyl acetate, which was then evaporated to dryness in vacuo. The residue was dissolved in 20 µl of n-butanol : HCl (95 : 5 v/v) and boiled for 5 min. The reaction products were applied to TLC cellulose plate (Funakoshi, Tokyo, Japan) and developed with acetic acid/HCl/water (30 : 3 : 10).

**Phylogenetic analysis**

Multiple sequence alignments were made using the program Clustal W (Thompson et al. 1994). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in PHYLIP.

**DMACA staining**

Seeds of spinach at different stages of development were harvested and placed directly into DMACA reagent [2% (w/v) DMACA in 3 M HCl/50% (w/v) methanol] for 16–18 h and rinsed three times with 70% (v/v) ethanol (Abrahams et al. 2002). As control, same samples were placed into the reagent without including DMACA [3 M HCl/50% (w/v) methanol].

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**References**


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