A cDNA clone encoding a putative dihydroflavonol 4-reductase gene has been isolated from a strawberry (Fragaria × ananassa cv Chandler) DNA subtractive library. Northern analysis showed that the corresponding gene is predominantly expressed in fruit, where it is first detected during elongation (green stages) and then declines and sharply increases when the initial fruit ripening events occur, at the time of initiation of anthocyanin accumulation. The transcript can be induced in unripe green fruit by removing the achenes, and this induction can be partially inhibited by treatment of de-achened fruit with naphthylacetic acid, indicating that the expression of this gene is under hormonal control. We propose that the putative dihydroflavonol 4-reductase gene in strawberry plays a main role in the biosynthesis of anthocyanin during color development at the late stages of fruit ripening; during the first stages the expression of this gene could be related to the accumulation of condensed tannins.

Flavonoids are a large family of secondary metabolites widespread among plants and involved in many processes. Anthocyanins, one class of flavonoid pigments, are the main pigments in flowers and fruits, where they serve as insect and animal attractants. Anthocyanin biosynthesis has been extensively investigated in several plant species. The structural and regulatory genes of this metabolic pathway have been cloned and their spatial and temporal expression studied (Koes et al., 1994; Holton and Cornish, 1995).

Strawberry (Fragaria spp.) is a commercially important, nonclimacteric soft fruit in which color is a crucial quality factor. Its pigments have been partly characterized and include anthocyanins, pelargonidin, cyanidin, and proanthocyanins (Macheix et al., 1990). However, the anthocyanin-synthesis pathway in strawberry is poorly understood, particularly at the molecular level. Anthocyanins accumulate during the ripening process, which is accompanied by a decrease in firmness and chlorophyll content (Perkin-Veazie, 1995). During this process there is an induction of the activities of PAL, the first enzyme of the general phenylpropanoid pathway, and UDP-Glc:flavonoid O-glucosyltransferase, the last enzyme of this pathway.

DFR catalyzes the last common step in the flavonoid-biosynthesis pathway leading to anthocyanins and proanthocyanidins (condensed tannins). In an NADPH-dependent reaction the enzyme reduces dihydroflavonols to 3,4-cis-leucoanthocyanidins, the immediate precursors of the anthocyanidins. DFR genes have been isolated from several higher plants (Beld et al., 1989; Kristiansen and Rohde, 1991; Helariutta et al., 1993; Bongue-Bartelsman et al., 1994; Sparvoli et al., 1994; Rosati et al., 1997). The crucial role of this gene in anthocyanin biosynthesis has been demonstrated by transformation of petunia with a heterologous DFR gene, which led to novel or increased flower pigmentation (Meyer et al., 1987; Helariutta et al., 1993; Tanaka et al., 1995).

In this paper we report the cloning and characterization of a strawberry (Fragaria × ananassa cv Chandler) cDNA gene (njjs24) encoding an amino acid sequence that shows a high homology to DFR from higher plants. In an NADPH-dependent reaction the enzyme reduces dihydroflavonols to 3,4-cis-leucoanthocyanidins, the immediate precursors of the anthocyanidins. DFR genes have been isolated from several higher plants (Beld et al., 1989; Kristiansen and Rohde, 1991; Helariutta et al., 1993; Bongue-Bartelsman et al., 1994; Sparvoli et al., 1994; Rosati et al., 1997). The crucial role of this gene in anthocyanin biosynthesis has been demonstrated by transformation of petunia with a heterologous DFR gene, which led to novel or increased flower pigmentation (Meyer et al., 1987; Helariutta et al., 1993; Tanaka et al., 1995).

In this paper we report the cloning and characterization of a strawberry (Fragaria × ananassa cv Chandler) cDNA gene (njjs24) encoding an amino acid sequence that shows a high homology to DFR from higher plants. In this first time, to our knowledge, that a predominantly fruit-expressed, putative DFR gene has been cloned from a soft fruit such as strawberry. Studies of the expression pattern of this gene have been carried out. The information provided here could be useful for genetic transformation programs aimed at changing fruit color by modifying anthocyanin synthesis.

**MATERIALS AND METHODS**

Strawberry (Fragaria × ananassa cv Chandler) fruit was harvested at different developmental stages: small-sized green fruits (G1), middle-sized green fruits (G2), full-sized
green fruits (G3), white fruits with green achenes (W1), white fruits with red achenes (W2), turning-stage fruits (T), and full-ripe red fruits (R).

**DFR cDNA Cloning**

The putative DFR cDNA from strawberry was isolated from a cDNA subtractive library by the magnet-assisted subtraction technique-PCR-Southern blot differential screening method previously described (Medina-Escobar et al., 1997b).

**RNA Isolation and Northern Analysis**

Total RNA from fruits at the stages indicated and from roots, leaves, flowers, and stolons was isolated according to the method of Manning (1991). Northern analysis was as described by Medina-Escobar et al. (1997a, 1997b). A cDNA corresponding to 18S rRNA was used as a control for equal loading of the RNA samples. The probes were radiolabeled to a specific activity of approximately $10^8$ cpm µg$^{-1}$ using a commercial random-priming kit (Pharmacia).

**Auxin Treatment**

Achenea from two sets of G2-stage strawberry fruits were carefully removed in the growing plant using the tip of a scalpel blade. One set of de-achenched fruits was treated with the synthetic auxin NAA in a lanolin paste at 1 mm NAA in 1% (v/v) DMSO. The other set (the control group) was treated with the same paste without NAA. Both treatments were applied over the whole fruit surface. Fruits were harvested 0, 24, 48, 72, and 96 h after treatment and immediately frozen in liquid N2 and stored at $-80^\circ$C.

**DNA Isolation and Southern Analysis**

Strawberry genomic DNA was extracted from achenes removed from W1-stage strawberry fruits as described by Medina-Escobar et al. (1997a). The DNA (4 µg) was digested with the restriction enzymes BamHI, BglII, EcoRI, and HindIII. Southern analysis was performed according to the method of Medina-Escobar et al. (1997a).

**DNA Sequencing and Analysis of Sequences**

DNA was sequenced by the dideoxy chain-termination method using T3, T7, and specific primers within the cDNA insert. An automated DNA sequencer (Prism, Perkin-Elmer) and a sequencing kit (Perkin-Elmer) were used to generate the sequence data. Sequences were analyzed using the TFASTA, FASTA, BESTFIT, COMPARE, and DOTPLOT programs from the Genetics Computer Group (Madison, WI) package (version 9.0; December 1996; Devereux et al., 1984) and compared with the GenBank (release 100, April 1997), EMBL (release 50.0, March 1997), PIR (release 52.0, March 1997), and SWISSPROT (release 34.0, November 1996) databases.

**RESULTS**

**Sequence Analysis of the njjs24 cDNA Clone and the Encoded Protein**

The cDNA corresponding to clone njjs24 was sequenced to completion. The nucleotide and the predicted amino acid sequences are shown in Figure 1. The cDNA insert is 1320 bp long and presents an open reading frame that encodes a protein of 341 amino acid residues, with a predicted molecular mass of 38.9 kDa and a pI of 6.78. Two possible initiation codons are identified in the njjs24 cDNA sequence (nucleotide positions 15 and 87; Fig. 1). DOT PLOT comparison (data not shown) between the njjs24 cDNA and that of higher plant DFRs allowed us to propose the first ATG as the initiation codon for the strawberry njjs24 predicted protein. Moreover, this initiation codon is more closely related to the consensus sequence of initiation codons of higher plants, ATGCT (Gallie, 1993).

The clone has a 3’-untranslated region of 283 bp, including the stop codon and 31 residues of the poly(A)$^+$ tail. A consensus plant polyadenylation sequence, AATAAA, is also found in this region (nucleotide position 1255) to an upstream distance (26 nucleotides) of the cleavage and polyadenylation site in the range of the consensus distance (10–30 nucleotides) for eukaryotic genes. In addition, 5’ upstream of this polyadenylation site there is a TAG stop codon that is preferentially utilized in higher plants (Gallie, 1993).

Sequence analysis and comparison with sequences in the databank revealed high identity of this strawberry nucleotide sequence (data not shown) and the deduced amino acid sequence of higher-plant DFR genes previously cloned (Fig. 2). The higher values for identity and similarity were to DFR proteins from Rosa hybrida (83.6 and 87.6%, respectively), Vitis vinifera (74.2 and 81.4%, respectively), Arabidopsis thaliana (72.3 and 78.1%, respectively), Dianthus caryophyllus (70.6 and 77.7%, respectively), and Antirrhinum majus (68.9 and 77.6%, respectively). In addition, the strawberry njjs24 deduced protein showed significant homology to other DFRs from Gerbera hybrida, Medicago sativa, Petunia hybrida, Lycopersicon esculentum, Gentiana triflora, Forsythia × intermedia, Callistephus chinensis, Hordeum vulgare, Oryza sativa, Ipomea purpurea, and Zea mays (with identities and similarities ranging from 64.0 to 69.0% and 76.0 to 78.0%, respectively).

An N-terminal NADP-binding domain, previously proposed as the dinucleotide-binding fold of NADP(H) and NAD(H)-dependent reductases and dehydrogenases (Lacombe et al., 1997), is also present in the strawberry putative DFR protein (Fig. 1).

**Tissue and Developmental Gene Expression**

The spatial and temporal expression pattern of this strawberry putative DFR gene has been studied. Total RNA isolated from fruits (receptacle plus achenes) at different development stages and from roots, leaves, flowers, and stolons were analyzed by northern hybridization (Fig. 3). A transcript of about 1.3 kb was specifically detected in fruits,
showing two peaks during the developmental stages. Transcripts slightly increased in fruits during the elongation/green stages and then declined in the W1 stage. A second increase in the transcript level was observed in the W2 stage when the initial ripening events began. The high expression level continued throughout the T and R stages. Accumulation of anthocyanins occurs parallel to ripening in strawberry fruits (Perkin-Veazie, 1995). We evaluated the anthocyanin content of ripening fruits of cv Chandler, which were undetectable during the green and W1 stages but rapidly accumulated in the T and R stages (results not shown), reaching a value of approximately 32 μmol fruit⁻¹ at the R stage. This increase correlated well with the second increase of the njjs24 gene expression observed from the W2 to the R stage.

Because achenes are the true fruits embedded in the epidermal layer of the receptacle in strawberry (Perkin-Veazie, 1995), expression of the njjs24 gene was separately followed in the achenes and the receptacle (de-achened fruit; Fig. 4). Transcripts were present in the receptacle but were not detectable in achenes (data not shown).

Studies of the effect of auxin in njjs24 gene expression were performed in de-achened green strawberry fruits supplied with external NAA in a lanolin paste. A clear increase in the expression of this gene was observed in de-achened G2 fruits. However, a reduction in the expression of this gene was observed in de-achened G2 fruits treated with NAA, and practically no expression was found in normal G2 fruits (with achenes; Fig. 5). The inability to completely revert njjs24 gene expression by NAA in G2 de-achened fruit has also been observed in other ripening-related genes in strawberry (Medina-Escobar et al., 1997a).

Figure 1. Nucleotide and deduced amino acid sequence from strawberry cDNA clone njjs24 (accession no. AF029685). Initiation and stop codons are shown in bold. The putative polyadenylation sequence is underlined. Asterisks (*) indicate amino acid residues conserved in the hydroxysteroid dehydrogenase/DFR superfamily (Baker and Blasco, 1992). The box located in the N-terminal region shows the putative NADP-binding domain. A second box shows the stretch thought to define the substrate specificity of the DFR enzyme (Beld et al., 1989).

Figure 2. Comparison of the amino acid sequences of the putative strawberry DFR with that of higher plants. Identical and conservative amino acids found in all DFR sequences are shown in black; those in four or more sequences are shown in gray. The amino acid region determining the substrate specificity is underlined. The accession numbers are X15536 for A. majus (ampallid); X15537 for P. hybrida (phybrida); AF029685 for F. ananassa (dhfrt7); D85102 for R. hybrida (rosa); X75964 for V. vinifera (vvinifera); P51102 for A. thaliana (athaliana); and Z67983 for D. caryophyllus (dcaryophyl). Alignment was performed using the GenDoc program (version 2.0.004, 1997) by K.B. Nicholas and H.B. Nicholas, Jr.
and was explained by an incomplete penetration and/or degradation in the receptacle of the NAA.

Southern-Blot Analysis

Strawberry genomic DNA was digested with the restriction enzymes BamHI, BglII, EcoRI, and HindIII and analyzed by Southern-blot hybridization using the same probe previously utilized in northern-blot studies (Fig. 6). Several hybridization fragments above 5.4 kb were detected in the BamHI, BglII, and EcoRI digestions. No restriction sites were found in the strawberry njs24 cDNA sequence for these enzymes. Furthermore, only a restriction site was found in the njs24 CDNA sequence for HindIII; therefore, at least two hybridization fragments were expected. However, six hybridization fragments of sizes ranging between 1.5 and 7.6 kb were detected in the HindIII digestion. The long fragments observed can be due either to a small multigenic family or to the ploidy level of the strawberry cultivar (octoploid).

DISCUSSION

Although there is no functional proof of the role of the njs24 gene product, we propose that this gene encodes a DFR enzyme. The high level of homology to the DFR sequences from the databank, combined with fruit-specific and ripening-related expression, supports this assignment.
Furthermore, the presence of an N-terminal NADP-binding domain, common to other reductases, adds further support to the njjs24 gene showed the highest sequence homology with both the nucleotide and the amino acid level to that of R. hybrida, which is understandable since strawberry belongs to the Rosaceae family. Next in homology level was the DFR from V. vinifera, the fruit of which, like that of strawberry, is considered nonclimacteric.

DFR is encoded either by a small multigene family (Beld et al., 1989; Helariutta et al., 1993) or by only one gene (Kristiansen and Rohde, 1991; Bongue-Bartelsman et al., 1994; Sparvoli et al., 1994; Rosati et al., 1997). In strawberry, the hybridization pattern suggests that DFR is probably encoded by a small family of genes. The clear differences in band intensity among the DFR-hybridizing fragments suggest the presence of other genes with different homology to njjs24, but the confidence level for statements concerning gene number is reduced when the source of the DNA is an octoploid (such as Fragaria × ananassa cv Chandler). Also, the two peaks in expression observed during fruit development and ripening could be explained by the existence of two differentially regulated DFR genes. This issue remains to be further investigated.

The enzymatic activities of PAL and UDP-Glc-flavonoid O3-glucosyltransferase in the biosynthetic pathway leading to pelargonidin-3-glucoside (the major anthocyanin pigment of ripe strawberry) increase during strawberry fruit ripening (Perkin-Veazie, 1995). Parallels between PAL activity and anthocyanin content have specifically been reported in the strawberry cv Tillikum (Cheng and Breen, 1991). It is noteworthy that two peaks of PAL activity have been reported in strawberry fruits; one in green fruits and one in nearly ripe fruits (Cheng and Breen, 1991). Whereas the second activity peak has been associated with the anthocyanin accumulation that occurs in ripe fruits, the first peak has been explained by its involvement in the synthesis of other flavonoids (condensed tannins) and phenolics that take place during early fruit development (Macheix et al., 1990; Cheng and Breen, 1991).

The two peaks of DFR transcripts reported here in strawberry fruits during growth and ripening seem to be coincident to the PAL activity peaks previously reported in this fruit. Therefore, we propose that the first DFR expression increase is probably associated with the synthesis of condensed tannins in green fruits, and the ripening-associated increase functions in the biosynthesis of anthocyanins. Close association between DFR expression and condensed tannin accumulation has been reported in young V. vinifera berries (Boss et al., 1996) and in root cultures of bird’s foot trefoil (Bavage et al., 1997). There are also abundant reports in the literature showing that DFR gene expression is spatially and developmentally correlated with the anthocyanin accumulation pattern in different plant tissues (Beld et al., 1989; Jackson et al., 1992; Huits et al., 1994; Tanaka et al., 1995; Rosati et al., 1997).

It has been proposed that auxin stimulates expansion of the receptacle and inhibits ripening in strawberry fruit (Manning, 1993; Perkin-Veazie, 1995). Moreover, an induction pattern of mRNA population in immature de-achenched fruit was found to be reverted by the application of the synthetic auxin NAA to the de-achenched fruit receptacle, supporting a relationship between ripening-induced gene expression and the absence of auxin (Perkin-Veazie, 1995). Some unidentified fruit-specific strawberry genes have been shown to be under the control of auxins (Reddy and Poovaiah, 1990; Reddy et al., 1990). More recently, it has been reported that a fruit-specific strawberry pectate lyase gene, the expression of which was only induced in the W2 to R stages, was negatively regulated by auxins (Medina-Escobar et al., 1997a). Similarly, the expression of the fruit-specific strawberry DFR was clearly induced in de-achenched G2 fruit, and this induction was partially reverted by NAA application to de-achenched receptacles. These results indicate that DFR expression in strawberry is either directly or indirectly under the control of internal auxin, as has been proposed for other ripening-related strawberry genes (Reddy and Poovaiah, 1990; Reddy et al., 1990; Medina-Escobar et al., 1997a).

In summary, we have isolated and characterized a strawberry cDNA clone, njjs24, which corresponds to an mRNA probably encoding a DFR protein. Experiments with antisense RNA are in progress to elucidate the involvement of this strawberry DFR gene in phenylpropanoid metabolism during fruit development and ripening.

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