

Full Length Research Paper

Cloning and expression of anthocyanidin synthase (ANS) gene from peel of mango (*Mangifera indica* Linn)

Zhao Zhichang*, Chen Yeyuan, Gao Aiping, Huang Jianfeng and Dang Zhiguo

Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences/Key Laboratory of Crop Gene Resources and Germplasm Enhancement in Southern China, Ministry of Agriculture, Danzhou 571737, P. R. China.

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Fruit coloring is a major source of anthocyanin and carotenoid in mango. The anthocyanidin synthase (ANS) is a key enzyme in the anthocyanin biosynthesis. The ANS gene was cloned from mango peel by homologous cloning method. This manuscript reported characterization of ANS from mango peel that comprised 1,262 bp full-length cDNA with open reading frame (ORF) of 1,056 bp and encoding a protein of 351 amino acids. The theoretical molecular weight of the deduced amino sequence of ANS was 39.8 kDa. It was found that the gene encoding for the protein had close relationship with mountain grape (*Vitis vinifera*), cocoa (*Theobroma cacao*), mulberry (*Morus alba*), litchi (*Litchi chinensis*), and other fruit trees through phylogenetic analysis. Expression of ANS was maximum in the green rather than the yellow and red fruit peel. Expression was down-regulated in mature fruit, there was no response to fruit coloration which was affected by anthocyanin.

Key words: Cloning, expression, anthocyanidin synthase (ANS), mango.

INTRODUCTION

The anthocyanins are one of the most widespread studied members of the tricyclic flavonoid family of secondary metabolites in plants (Grotewold, 2006). The anthocyanins contribute to some of the most important pigments in flowers and fruits for flower pollination or fruit and seed dispersal, and also the precursors of tannin oligomers present in tea and red wine, which have long-established biomedical properties, including inhibition of cell proliferation and antimutagenic, antimicrobial, anti-inflammatory, antioxidant and antihypertensive properties (Pool-Zobel et al., 1999; Harborne and Williams, 2000; Akihisa et al., 2003; Parejo et al., 2004). The structural genes that encode enzymes involved in the anthocyanin pathway and many regulatory genes for transcriptional

regulation of the structural genes have been cloned and characterized from a wide variety of plants (Mol et al., 1998; Holton and Cornish, 1995; Lesnick and Chandler, 1998).

Anthocyanidin synthase (ANS), an enzyme of the biosynthetic pathway to anthocyanin, catalyzes the reaction(s) from the colorless leucoanthocyanidins to the colored anthocyanidins. The ANS proteins belong to the 2-oxoglutarate iron-dependent oxygenases and were cloned first from *Perilla frutescens* (Saito et al., 1999). In a series of studies on recombinant ANS from *Arabidopsis thaliana* evidence was presented for the initial oxidation of the substrate at C-3 (Welford et al., 2001).

Furthermore, the ANS formed predominantly quercetin

*Corresponding author. E-mail: zhaozhichang2001@163.com or chenyy1962@126.com

and cis and trans-dihydroquercetin (DHQ) with cyanidin being a minor product only (Welford et al., 2001; Turnbull et al., 2003), and the product pattern from (2R, 3S, 4S) cis-leucocyanidin vs. that from (2R, 3S, 4R)-trans-leucocyanidin implied that cis-DHQ, trans-DHQ and cyanidin resulted mostly from the unnatural (2R, 3S, 4R)-trans-leucocyanidin (Turnbull et al., 2003). Moreover, co-crystallization of the ANS with Fe^{2+} , 2-oxoglutarate and racemic trans-DHQ or enantiomerically pure (2R, 3R)-DHQ as a substrate analogue in the absence or presence of molecular oxygen supported the stereoselective C-3 hydroxylation of the substrate and surprisingly revealed two molecules of the substrate analogue in the active site with (2R, 3R)-trans-DHQ closest to the iron atom, whereas either enantiomer was bound at the other location (Wilmouth et al., 2003).

Clearly, additional data are required to define the substrate specificity of ANS. Mango (*Mangifera indica* Linn) is one of the delicious seasonal fruits grow in the tropics, and cultivated in many regions of India and now distributed wide across the world in many continents. Mango fruit is one of the most popular, nutritionally rich fruits with unique flavor, fragrance, taste, and health promoting qualities making it a common ingredient in new functional foods often labeled "The king of the fruits." Internally, it has juicy flesh features, orange-yellow in color with numerous soft fibrils radiating from the husk. Its flavor is pleasant and rich, and tastes sweet with mild tartness. Outer skin of mango is smooth and green in unripe, but turns into golden yellow, bright yellow or orange-red when ripen depending upon the cultivar.

In the present study, we reported the isolation of cDNA clone coding for an ANS gene from mango. This is the first report on ANS gene from mango peel, these studies add to the understanding of the biosynthesis of anthocyanin in tropical fruit, with significant practical implications for enhancing the specific biomedical anthocyanin contents in mango by genetic engineering.

MATERIALS AND METHODS

Plant materials

Mango (*M. indica* Linn) cv "Guifei" cultivar was grown at Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences in Danzhou, China. Before the commercially mature stage, green, yellow and red peels were taken from at least 20-30 fruit at a month interval throughout the growing season. Fresh fruit samples were taken to laboratory and immediately frozen in liquid nitrogen and stored at -80°C.

cDNA of ANS cloning and expression

Total RNA was isolated from mango peel sample according to the method of Wan and Wilkins (1994). Reverse transcription was achieved with oligo-d (T)₁₈ primers by using 3 µg of total RNA from peel of mango litchi and the primeScript II 1st strand cDNA synthesis kit (TaKaRa). To isolate partial cDNA clone, two oligonucleotides designed by on the basis of conserved amino acid

sequences of several available ANSs were used: forward primer (5' AAGTACATCCATCCAGTCATYTT '3); reverse primer (5' GAATCCAAGGATCCYGAGAAYGA 3'). The PCR reaction was carried out using the following conditions: 94°C, 3 min (1 cycle); 94°C, 30 s; 55°C, 30 s; 72°C, 1 min (30 cycles); 72°C, 5 min (1 cycle). Product of 280 bp, whose identity was confirmed by sequencing was amplified. The 3'RACE and 5'RACE of ANS gene was gotten according to 3'-Full RACE Core Set ver.2.0 and 5'-Full RACE Kit (TaKaRa in Dalian, China). For the analysis of ANS expression in three phages of mango peel, RT-PCR was performed for 30 cycles to determine the linearity of the PCR. The thermal cycling parameters used for the RT-PCR for all genes were as follows: 94°C for 30 s, 56°C for 30 s, 72°C for 100 s; followed by 72°C for 10 min. The cDNA was amplified from 30 ng of total RNA, using specific primers sets for, ANS-F 5' ATTATGGCAGTGTTATCAATCGGGT'3, ANS-R 5' GGGAGGAGCGTGGAAAGTCGTCGTA'3. As a positive control, *actin7* fragment was amplified under the same RT-PCR conditions, using the primer pair: *Actin7-F* 5'AATGGAAGTGGAAATGGTCAAGGC'3, *Actin7-R5'* TGCCAGATCTTCTCCATGTCATCCCA'3.

Sequence analysis

Nucleotide and amino acid sequence comparisons were performed using the LASERGENE DNA software package (DNASTar, Madison, WI). Sequence similarity searching was performed using the BLASTN and BLASTX, nucleic and protein databases at NCBI (<http://www.ncbi.nlm.nih.gov>). The phylogenetic analysis of ANS from mango and other species was carried out by alignment with the bioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The phylogenetic and molecular evolutionary analysis used MEGA (molecular evolutionary genetics analysis) version 5.0 (Kumar et al., 2004).

RESULTS

Isolation of the anthocyanidin synthase (ANS) gene from mango

To obtain ANS from mango, the 280 bp cDNA ANS fragments were isolated with two oligonucleotides primers by RT-PCR. The entire length of the cDNA ANS was gotten with the rapid amplification of cDNA ends (RACE) reactions. The 920 and 530 bp cDNA ANS fragments were gotten by the 3'-RACE and 5'-RACE reactions.

The isolated ANS cDNA had a 1,262 bp full-length with ORF of 1,056 bp, and encoding a protein of 351 amino acids (Figure 1). The theoretical molecular weight and isoelectric point of the deduced amino sequence of ANS were 39.8 kDa and 5.68, respectively.

Sequence analysis of anthocyanidin synthase (ANS) from mango

A search for amino acid sequence homology revealed high sequence identity (*Arabidopsis thaliana*, GenBank number NM_118417.1, *Vitis vinifera*, GenBank Accession number ABV82967.1, *Pyrus pyrifolia*, GenBank

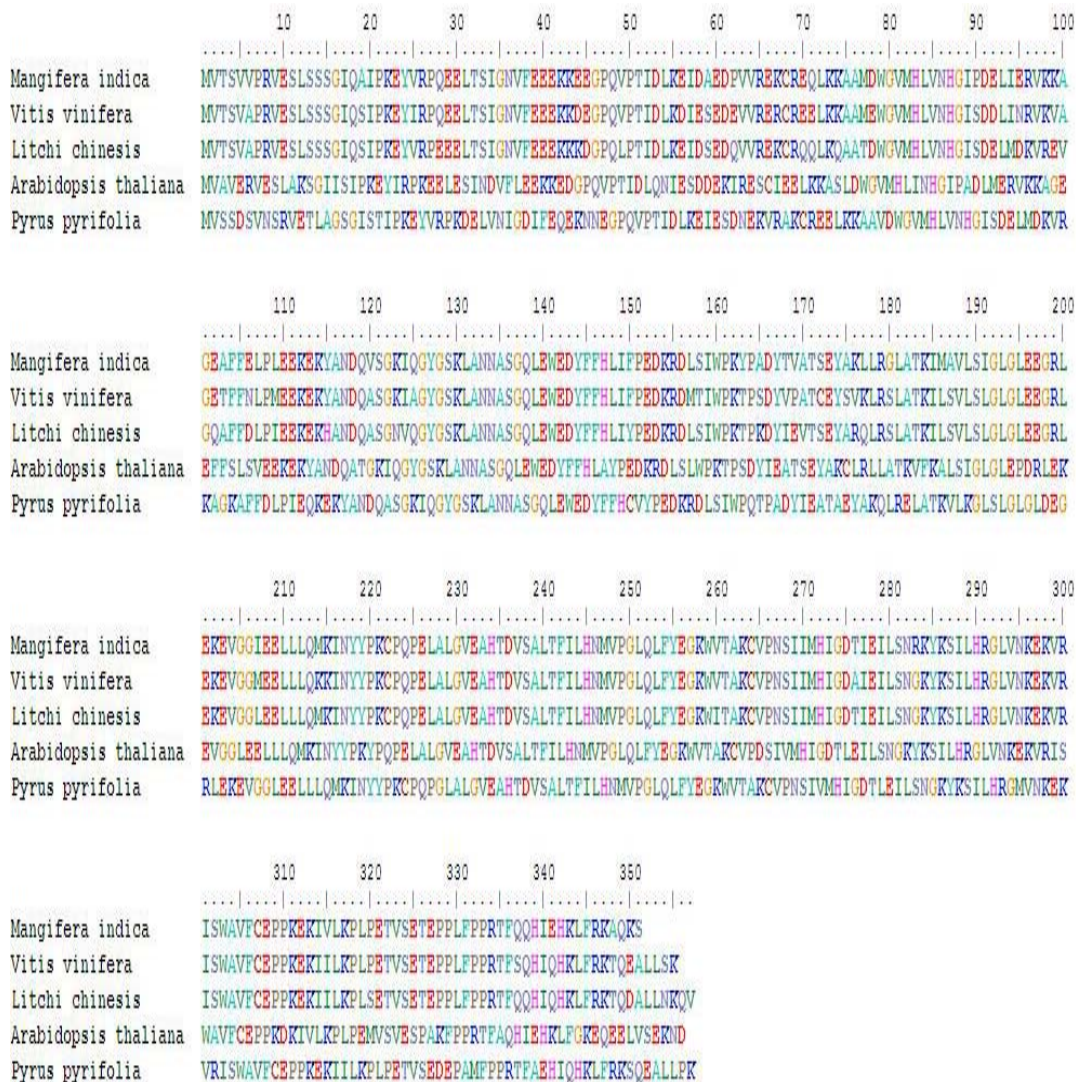


Figure 1. Amino acid sequence of ANS comparison between mango and other species. Sequence analysis of amino acid region proposed to determine specificity.

Accession number ADP09379.1, *Litchi chinensis* GenBank Accession number HQ402913.1) between ANS and its closest sequence homolog (Figure 1). An alignment of the deduced amino acid sequences of all these proteins indicated that ANS contain a conserved sequence with other plant in the same domain. Phylogenetic analysis was performed on ANS protein sequences. The tree confirms the conclusions that ANS of mango was most closely related to *Morus alba* (GenBank Accession number AEN55613.2), *V. vinifera* (GenBank Accession number ABV82967.1), and *Litchi chinensis* (GenBank Accession number HQ402913.1). ANS of mango from *Medicago truncatula* (GenBank Accession number ABU40983.1), and *Anthurium andraeanum* (GenBank Accession number ABK76317.1) was more distant related in this work clusters. It formed two separate branches, one branch mainly included fruit and crops, for

example *Vitis*, *Litchi*, *Malus* and *Fragopyrum*, interesting, other branch mainly included flower of plants, for example, *Glycine max*, *M. truncatula* and *Ipomoea trifida* (Figure 2).

Expression of ANS and measurement of anthocyanin different stages in mango peel

Expression of ANS was the highest in the green stage, and the lowest in the yellow stage (Figure 3). A close correlation was also observed between the ANS gene expression and enzyme activity (data not shown). In this study, ANS activity was detected, and changed over the growth period. Anthocyanin was measured in the three phenotypes for peel (green, yellow and red). The green stage examined contains lowest detectable amounts of

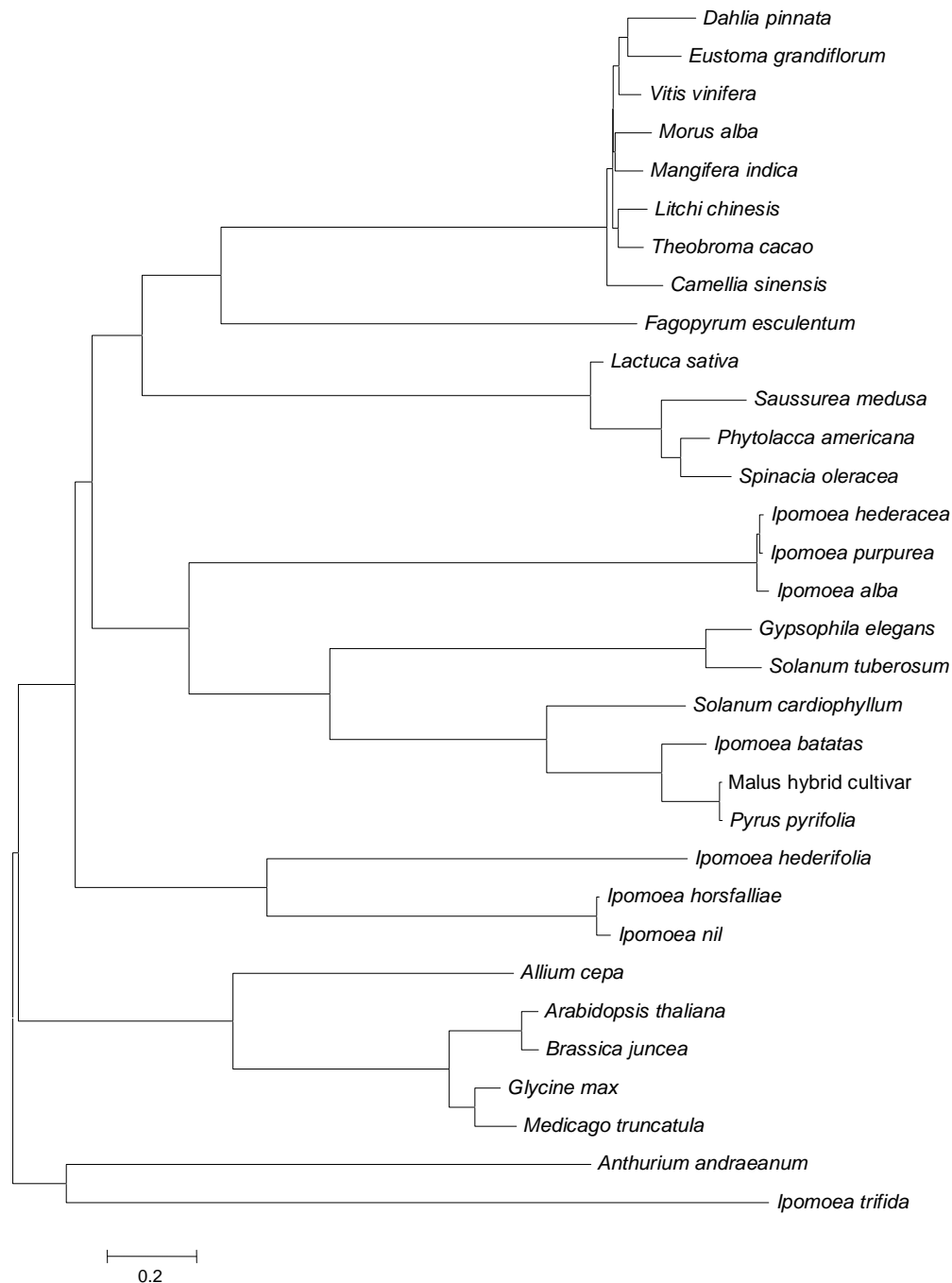


Figure 2. Phylogenetic relationship of ANS. The tree was constructed using MEGA (version 5.0). *Vitis vinifera* (ABV82967.1), *Gypsophila elegans* (AAP13054.1), *Glycine max* (AAR26525.1), *Ipomoea batatas* (ACT31916.1), *Arabidopsis thaliana* (NM_118417.1), *Anthurium andraeanum* (ABK76317.1), *Camellia sinensis* (AAV88087.1), *Allium cepa* (ABR24157.1), *Ipomoea batatas* (ABM63373.1), *Dahlia pinnata* (BAJ21536.1), *Brassica juncea* (ACH58398.1), *Ipomoea hederifolia* (BAK78919.1), *Solanum tuberosum* (AEJ90548.1), *Fagopyrum esculentum* (ADT63066.1), *Pyrus pyrifolia* (ADP09379.1), *Ipomoea nil* (BAB71811.1), *Malus hybrid cultivar* (ACP30363.1), *Solanum tuberosum* (ADP37440.1), *Eustoma grandiflorum* (BAJ08929.2), *Ipomoea trifida* (ACF59729.1), *Lactuca sativa* (BAJ10383.1), *Saussurea medusa* (AAS48200.1), *Ipomoea batatas* (ADE08370.1), *Theobroma cacao* (ADD51356.1), *Ipomoea purpurea* (AAP82030.1), *Glycine max* (ABY51685.1), *Medicago truncatula* (ABU40983.1), *Allium cepa* (ABM66367.1), *Phytolacca americana* (BAE54521.1), *Spinacia oleracea* (BAE54520.1), *Brassica juncea* (ACH58397.1), *Solanum cardiophyllum* (AEJ90547.1), *Ipomoea horsfalliae* (ACS71531.1), *Ipomoea alba* (AAP82018.1), *Morus alba* (AEN55613.2) and *Litchi chinensis* (HQ402913.1).

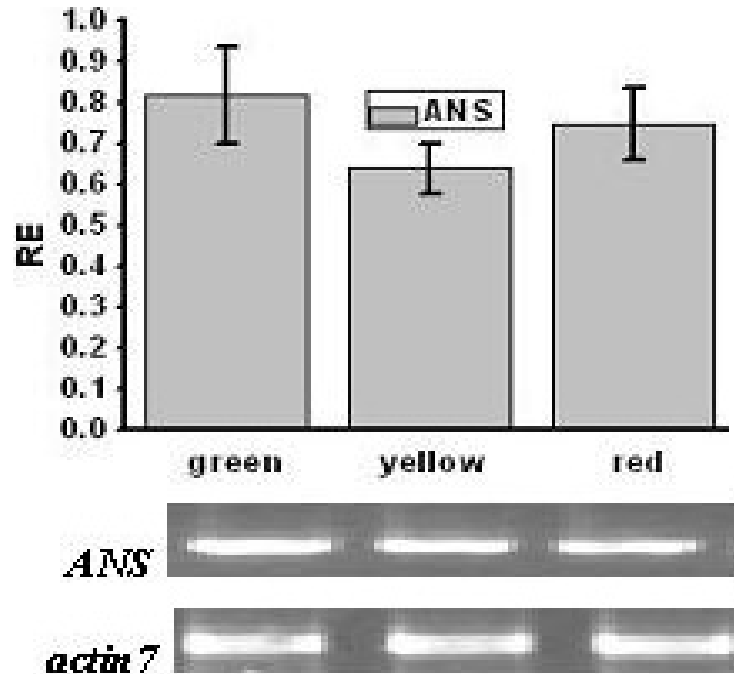


Figure 3. Expression of ANS was measured by RT-PCR at three developmental stages. Expression of ANS was the highest in the green stage, and the lowest in the yellow stage. Values are the average \pm SD of three replicant.

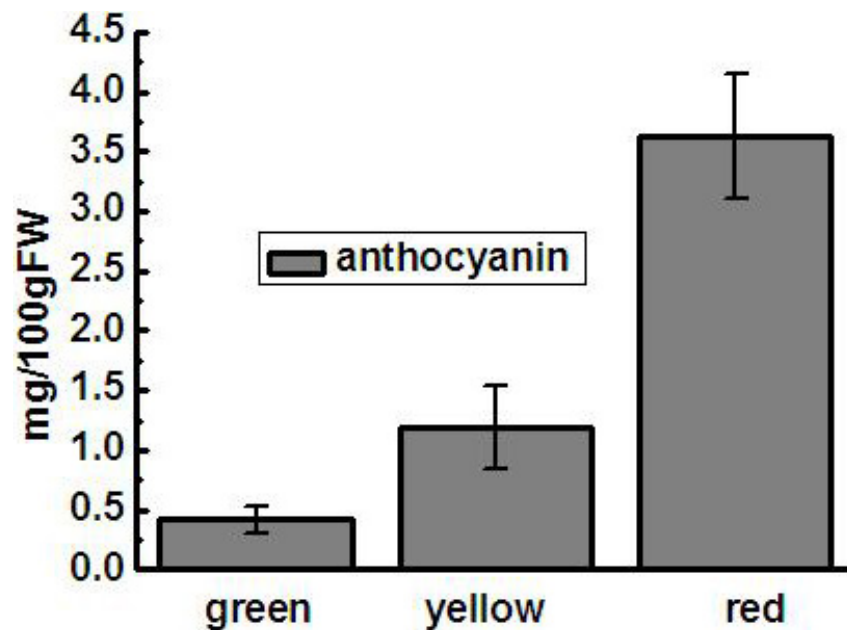


Figure 4. Total anthocyanin concentration in the three stages of mango peel.

anthocyanin in the peel. The accumulation of anthocyanin in the peel started at the yellow stage and was 1.12 mg/100 g. The anthocyanin was 8-fold higher in red peel as compared to green peel (Figure 4).

DISCUSSION

Some mango cultivars do not have anthocyanin in their shaded skin, however, anthocyanin was tested in “Guifei”

mango. Although several structural genes as well as some regulatory genes in the anthocyanin pathway of mango had been isolated, there are currently no reports on the ANS gene in anthocyanin pathway of mango. In this experiment, we initially investigated the sequence characterization, expression patterns of ANS from mango and analyzed the contents of total anthocyanin. The results indicated that the ANS is one of the key enzymes in anthocyanin-pigmentation pathway of mango, and it is responsible for the formation of anthocyanin in the outer mango peel. Molecular evolutionary trees deduced from amino acid sequences indicated that the enzymes in anthocyanin biosynthesis form a distinct group in the supper family of 2-oxoglutarate-dependent enzymes. ANS had close relationship with mountain grape (*V. vinifera*), cocoa (*Theobroma cacao*), mulberry (*M. alba*) litchi (*Litchi chinensis*), and other fruit trees through phylogenetic analysis. ANS, a key enzyme of the biosynthetic pathway to anthocyanin, catalyzes the reaction(s) from the colorless leucoanthocyanidin to the colored anthocyanidin. So the present research on isolation and characterization analysis of ANS from mango will provide an alternative to controlling the overall metabolic flux to the target products by appropriate genetic engineering strategies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

- Akihisa T, Tokuda H, Ukiya M, Iizuka M, Schneider S, Ogasawara K, Mukainaka T, Iwatsuki K, Suzuki T, Nishino H (2003). Chalcones, coumarins, and flavanones from the exudate of *Angelica keiskei* and their chemopreventive effects. *Cancer Lett.* 201:133-137.
- Grotewold E (2006). The genetics and biochemistry of floral pigments. *Annu. Rev. Plant Biol.* 57:761-780.
- Harborne JB, Williams CA (2000). Advances in flavonoid research since 1992. *Phytochem.* 55:481-504.
- Holton TA, Cornish EC (1995). Genetics and Biochemistry of anthocyanin biosynthesis. *Plant Cell* 7:1070-1083.
- Lesnick ML, Chandler VL (1998). Activation of the maize anthocyanin gene *a2* is mediated by an element conserved in many anthocyanin promoters. *Plant Physiol.* 117:437-445.
- Mol J, Grotewold E, Koes R (1998). How genes paint flowers and seeds. *Trends Plant Sci.* 3:212-217.
- Parejo I, Viladomat F, Bastida J, Schmeda-Hirschmann G, Burillo J, Codina C (2004). Bioguided isolation and identification of the nonvolatile antioxidant compounds from fennel (*Foeniculum vulgare* Mill.) waste. *J. Agric. Food Chem.* 52:1890-1897.
- Pool-Zobel BL, Bub A, Schröder N, Rechkemmer G (1999). Anthocyanins are potent antioxidants in model systems but do not reduce endogenous oxidative DNA damage in human colon cells. *Eur. J. Nutr.* 38:227-234.
- Saito K, Kobayashi M, Gong Z, Tanaka Y, Yamazaki M (1999). Direct evidence for anthocyanidin synthase as a 2-oxoglutarate-dependent oxygenase: molecular cloning and functional expression of cDNA from a red form of *Perilla frutescens*. *Plant J.* 17:181-189.
- Turnbull JJ, Nagle MJ, Seibel JF, Welford RWD, Grant GH, Schofield CJ (2003). The C-4 stereochemistry of leucocyanidin substrates for anthocyanidin synthase affects product selectivity. *Bioorg. Med. Chem. Lett.* 13:3853-3857.
- Welford RWD, Turnbull JJ, Claridge TDW, Schofield CJ, Prescott AG (2001). Evidence for oxidation at C-3 of the flavonoid C-ring during anthocyanin biosynthesis. *Chem. Commun.* 12:1828-1829.
- Wilmouth RC, Turnbull JJ, Welford RWD, Clifton IJ, Prescott AG, Schofield CJ (2002). Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. *Structure.* 10:93-103.