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ORIGINAL PAPER

Cloning and expression analyses of the anthocyanin biosynthetic genes in mulberry plants

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Abstract Anthocyanins are natural food colorants produced by plants that play important roles in their growth and development. Mulberry fruits are rich in anthocyanins, which are the most important active components of mulberry and have many potentially beneficial effects on human health. The study of anthocyanin biosynthesis will bring benefits for quality improvement and industrial exploration of mulberry fruits. In the present study, nine putative genes involved in anthocyanin biosynthesis in mulberry plants were identified and cloned. Sequence analysis revealed that the mulberry anthocyanin biosynthetic genes were conserved and had counterparts in other plants. Spatial transcriptional analysis showed detectable expression of eight of these genes in different tissues. The results of expression and UPLC analyses in two mulberry cultivars with differently colored fruit indicated that anthocyanin concentrations correlated with the expression levels of genes associated with anthocyanin biosynthesis including *CHS1*, *CHI*, *F3H1*, *F3′H1*, and *ANS* during the fruit ripening process. The present studies provide insight into anthocyanin biosynthesis in mulberry plants and may facilitate genetic engineering for improvement of the anthocyanin content in mulberry fruit.

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Abbreviations

CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
F3′5′H	Flavonoid 3′,5′-hydroxylase
DFR	Dihydroflavonol reductase
ANS	Anthocyanin synthase
DAA	Days after anthesis
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
UPLC	Ultra performance liquid chromatography

Introduction

Mulberry plants (*Morus* L.) are well known for their use as forage for silkworms (*Bombyx mori* L.), and also have important economic and medical uses. Mulberry fruit has been exploited industrially in many countries (Sánchez 2000; Singhal et al. 2010) to take advantages of its delicious taste and beneficial activity for human health (Wu et al. 2013). Anthocyanins are the most abundant nutrient components in mulberry fruits, which also have important biological activities. Liu et al. (2004) measured the total anthocyanin content in 31 mulberry cultivars and revealed that mulberry anthocyanins had broad prospects as natural food colorants in the food industry. Over 11 anthocyanins have been identified in mulberry and the main components are cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (Du et al. 2008; Dugo et al. 2001; Lee et al. 2004). Characterization of anthocyanin biosynthetic genes will facilitate

the genetic improvement of anthocyanins in mulberry. However, little information regarding anthocyanin biosynthetic genes is currently available for this species.

Anthocyanins are a group of water-soluble pigments found in plants. They are found predominantly in the epidermal cells of flowers and fruits and contribute to their brilliant red, purple, and blue colors (Sadilova et al. 2006; Stintzing et al. 2002). Anthocyanins also play important roles in plant growth, development, and defense, acting as major antioxidants in the scavenging of free radicals (Ravindra and Narayan 2003; Lev-Yadun and Gould 2009). In addition, studies reveal that anthocyanins are beneficial to human health in the treatment of inflammation, cancer, and cardiovascular diseases (de Pascual-Teresa and Sanchez-Ballesta 2008).

The biosynthetic pathway of anthocyanins has been well documented in many plants (Holton and Cornish 1995; Koes et al. 2005). A schematic representation is shown in Supplementary Fig. 1. Chalcone synthase (CHS) and chalcone isomerase (CHI) catalyze the formation of the intermediate product naringenin from upstream metabolites. Thereafter, flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) catalyze a series of hydroxylation reactions to produce different kinds of dihydroflavonols. These hydroxylation reactions are essential to the formation of different kinds of anthocyanins, which produce various colors. After the hydroxylation reactions, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to leucoanthocyanins using NADPH as a cofactor. Then, anthocyanin synthase (ANS) catalyzes the conversion of colorless leucoanthocyanins to colored anthocyanins. The connections between anthocyanin contents and the expression of anthocyanin biosynthetic genes have become a topic of intensive research. Boss et al. (1996) investigated the expression profile of genes involved in anthocyanin biosynthesis in five white and three red grape cultivars, and their results revealed that the majority of anthocyanin biosynthesis genes had higher levels of expression in red cultivars than in white ones. In apples, five anthocyanin biosynthesis genes were found to be coordinately expressed during the development of red coloration in the skin and their transcriptional levels showed a positive relationship with anthocyanin concentration (Honda et al. 2002). Salvatierra et al. (2010) investigated the expression of anthocyanin biosynthetic genes in strawberry fruits and found that the transcriptional levels of these genes increased during fruit ripening and the levels of expression were much higher in red fruit than in white fruit.

The complete sequence of the mulberry genome is now available (He et al. 2013). This provides an opportunity for the characterization of genes involved in anthocyanin biosynthesis. Here, nine putative anthocyanin biosynthetic genes were identified in the mulberry genome. The sequence features of these genes not only provide more data for plant comparative analysis but also facilitate future identification of candidate genes from closely related species of the family Moraceae. Their expression patterns were analyzed in two mulberry cultivars with differently colored fruit, and the results indicated that the transcription levels of genes in most anthocyanin biosynthetic steps are associated with anthocyanin content in the mulberry fruit. The information provided here will expand our knowledge of anthocyanin biosynthesis and could be incorporated into anthocyanin improvement strategies for mulberry fruit.

Materials and methods

Mulberry materials

The mulberry *Morus notabilis* C. K. Schneid. used for the present study was grown in Sichuan province, China. The fruit color of *M. notabilis* changed from green to white during fruit ripening. Lateral roots, 1-year-old shoots, leaves, male flowers, female flowers, and fruit were collected for RNA extraction. The mulberry cultivars *M. atropurpurea* Roxb. ‘Da10’ and *M. alba* ‘Zhenzhubai’ were maintained at the Mulberry Germplasm Nursery in Southwest University. During ripening, ‘Da10’ fruits changed from green to red and finally purple, and ‘Zhenzhubai’ fruits changed from green to white (Supplementary Fig. 2). Fruit samples for RNA and anthocyanin extraction were collected at four stages: I (green, 15 days after anthesis, daa), II (intermediate green, 25 daa), III (semi-mature, 40 daa) and IV (mature, 50 daa). All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Identification and cloning of mulberry anthocyanin biosynthetic genes

The genome sequence of *M. notabilis* was downloaded from the Morus Genome Database (http://morus.swu.edu.cn/morusdb/). A total of 27,085 high-confidence protein-coding loci were identified in the mulberry genome by combining results from homology-based and de novo gene prediction methods. The amino acid sequences of genes involved in anthocyanin biosynthesis were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and then used as queries for blast searches against the mulberry protein database. Gene-specific primers were designed to amplify the anthocyanin biosynthetic genes using mixed cDNA of six mulberry tissues (roots, shoots, leaves, male flowers, female flowers, and fruit) as a template. For a list of primers used in this study see Supplementary Table 1. The purified PCR products were TA cloned into the pMD19-T

simple vector (Takara, Otsu, Japan) and the sequences were confirmed by sequencing.

DNA extraction and Southern blotting

Buds of *M. notabilis* and two mulberry cultivars were powdered in liquid nitrogen and the DNA was extracted using a CTAB method (Lodhi et al. 1994). After digestion with RNase, the DNA was re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The DNA quality and concentration were measured using a ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For Southern blotting, each 15 μg of genomic DNA was digested with restriction endonuclease and the resultant fragments were resolved on a 0.8 % (w/v) TAE agarose gel. The bands were transferred to nylon membranes (Roche, Indianapolis, IN, USA). Probes for Southern blotting were designed based on the genome sequence of *M. notabilis* (For *CHS*, the sequence identity of *MnCHS1* and *MnCHS2* was too high to distinguish them, so the same probe was used for both; Supplementary Table 2). The probes were labeled and signals were detected with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After being hybridized with the DIG-labeled probes, membranes were washed twice in 2 × SSC containing 0.1 % SDS for 15 min at room temperature, and then washed twice in 0.1 × SSC containing 0.1 % SDS for 15 min at 65 °C. Ready-to-use CSPD (Roche, Indianapolis, IN, USA) was used to detect the hybridization bands and the signals were observed using a chemiluminescence imaging system (Clinx, Shanghai, China).

RNA extraction and qRT-PCR

Total RNA from six different tissues was extracted using RNAiso Plus (Takara) according to the manufacturer's instructions. The RNA quality and concentration were measured using a ND-1000 UV spectrophotometer (Nanodrop Technologies). First-strand cDNA was synthesized using 3 μg of total RNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA) in a 25 μl reaction system. For quantitative real-time reverse transcriptional PCR (qRT-PCR), each reaction was prepared according to the manufacturer's instructions using SYBR® Premix Ex Taq™ II (Takara) and 2 μl of diluted cDNA as a template. The qRT-PCR reactions were conducted on the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mulberry ribosomal protein L15 (RPL) gene was used as a control to normalize the relative expression of target genes. The relative expression was defined as $2^{-[\text{Ct (target gene)} - \text{Ct (control gene)}]} \times 1{,}000$. For a list of gene-specific primers used for real-time RT-PCR see Supplementary Table 3.

Extraction and quantification of anthocyanins

Anthocyanins were extracted from mulberry fruits as described in Pang et al. (2007) with minor modifications. Briefly, frozen samples were powdered with liquid nitrogen, and 5 ml of extraction buffer (methanol: 0.1 % HCl) was added to 0.5 g ground powder. After 1 h of sonication, the homogenized samples were gently shaken overnight. The samples were centrifuged at 5,000 × *g* for 10 min and the supernatants were gathered. Thereafter, 1 ml of ddH_2O was added to 1 ml of supernatant, followed by the addition of 1 ml chloroform to remove chlorophyll. One milliliter of supernatant was evaporated using a concentrator plus (Eppendorf, Hamburg, Germany) and dissolved in 1 ml of buffer (acetonitrile: ddH_2O: 0.1 M HCl = 1:7:2). The extracts were filtered by 0.2 μm PTFE membrane and stored at −80 °C until use. A Waters Acquity UPLC system (Waters, Milford, MA, USA) was used to analyze the anthocyanin levels. Separations were performed on an Acquity UPLC ® BEH C18 column (1.7 μm, 1.0 × 100 mm) at 30 °C. A 7–11 % acetonitrile gradient in 0.07 % H_3PO_4 was used for elution and the flow rate was 1.4 ml/min. Fractions were monitored at 520 nm. Components were identified by comparison of the retention times of the eluting peaks to those of commercial standards (Sigma, St. Louis, MO, USA) under the same conditions. Dose-dependent calibration curves of the standards were used to determine the concentrations of the components.

Results

Identification and cloning of the anthocyanin biosynthetic genes in mulberry plants

As shown in Supplementary Table 4, we identified nine putative anthocyanin biosynthetic genes in *M. notabilis* by bioinformatic methods, including two *CHS* genes, two *F3H* genes, two *F3′H* genes, and one gene each of *CHI*, *DFR*, and *ANS*. A *F3′5′H* gene was not found in the mulberry genome. Prominent among these predicted genes, two *CHS* genes were located near each other on scaffold508 and two *F3′H* genes were located near each other on scaffold255. Gene-specific primers were designed based on the genomic sequences and used to clone the cDNAs of these genes. Except for *ANS*, transcripts of eight mulberry anthocyanin biosynthetic genes were detected and further cloned for sequence analysis. The transcript of *ANS* was not detected in *M. notabilis* and its genomic sequence was analyzed. The genomic structures of the cloned genes are illustrated

Table 1 Comparison of deduced amino acid sequences of predicted mulberry anthocyanin biosynthetic genes and their counterparts in other plants

Gene	Protein (species)	Swissprot ID	Alignment length	Identity (%)	*E* value
MnCHS1	CHSY (*Hypericum androsaemum*)	Q9FUB7	389	93	0.0
	CHS1 (*Camellia sinensis*)	P48386	388	92	0.0
	CHS2 (*C. sinensis*)	P48387	399	91	0.0
	CHS1 (*Citrus sinensis*)	Q9XJ58	388	92	0.0
MnCHS2	CHSY (*H. androsaemum*)	Q9FUB7	389	92	0.0
	CHS1 (*C. sinensis*)	P48386	388	91	0.0
	CHS2(*C. sinensis*)	P48387	399	91	0.0
	CHS1 (*C. sinensis*)	Q9XJ58	388	91	0.0
MnCHI	CFI (*Pyrus communis*)	A5HBK6	218	77	2e-115
	CFI2 (*Fragaria* × *ananassa*)	Q4AE12	217	76	2e-112
	CFI (*Elaeagnus umbellata*)	O65333	217	76	1e-111
	CFI1 (*Fragaria* × *ananassa*)	Q4AE11	217	76	2e-111
MnF3H1	F3H (*Vitis vinifera*)	P41090	366	85	0.0
	F3H (*Petunia* × *hybrida*)	Q07353	366	83	0.0
	F3H (*Matthiola incana*)	Q05965	364	82	0.0
	F3H (*Malus* × *domestica*)	Q06942	366	83	0.0
MnF3H2	F3H (*Malus* × *domestica*)	Q06942	344	36	1e-68
	F3H (*Petroselinum crispum*)	Q7XZQ7	337	36	3e-68
	F3H (*Petunia* × *hybrida*)	Q07353	332	38	2e-67
	F3H (*Callistephus chinensis*)	Q05963	316	38	2e-67
MnF3′H1	F3′H (*Arabidopsis thaliana*)	Q9SD85	512	67	0.0
	F3′H (*Petunia* × *hybrida*)	Q9SBQ9	511	71	0.0
MnF3′H2	F3′H (*A. thaliana*)	Q9SD85	510	65	0.0
	F3′H (*Petunia* × *hybrida*)	Q9SBQ9	470	71	0.0
MnDFR	DFR (*Malus* × *domestica*)	Q9XES5	327	75	0.0
	DFR (*Pyrus communis*)	Q84KP0	330	74	0.0
	DFR (*Vitis vinifera*)	P51110	335	71	0.0
	DFR (*Dianthus caryophyllus*)	P51104	326	65	4e-168
MnANS	ANS (*Petunia* × *hybrida*)	P51092	346	83	0.0
	ANS (*Malus* × *domestica*)	P51091	347	80	0.0
	ANS (*A. thaliana*)	Q96323	343	80	0.0
	ANS (*Perilla frutescens*)	O04274	352	78	0.0

in Supplementary Fig. 3. The structural features of these genes are conserved with their counterparts in other plants (Almeida et al. 2007).

Characterization of anthocyanin biosynthetic genes in mulberry

The deduced amino acid sequences of the nine predicted mulberry anthocyanin biosynthetic genes were blast searched against the SwissProt database. The sequence identities among eight of these mulberry genes ranged from 65 to 93 % (Table 1). The results of multiple sequence alignment indicated that the catalytic domains and active sites were conserved in these mulberry proteins. For example, both MnCHSs showed two conserved phenylalanine residues and the active site cysteine, histidine, and asparagine residues were also conserved. These sites are essential for substrate specificity (Supplementary Fig. 4) (Ferrer et al. 1999). Both the conserved substrate preference residues (Supplementary Fig. 5) and phylogenetic analysis with other plant CHIs (Supplementary Fig. 6) indicated that MnCHI was a type-I CHI, which are capable of isomerizing 6′-hydroxychalcone to 5-hydroxyflavanone (Shimada et al. 2003). MnF3H was found to have conserved histidine, aspartic acid, and arginine residues involved in iron binding (histidine, aspartic acid) and 2OG binding (arginine), which is important for 2-oxoglutarate-dependent dioxygenase activity (Supplementary Fig. 7) (Lukačin and Britsch 1997). Sequence alignment revealed that the mulberry MnDFR contained a conserved motif, VTGASGYIGSWLVMRLLERDY, for NADP binding (Fig. 1); (Johnson et al. 1999). In Fig. 1, underlined amino

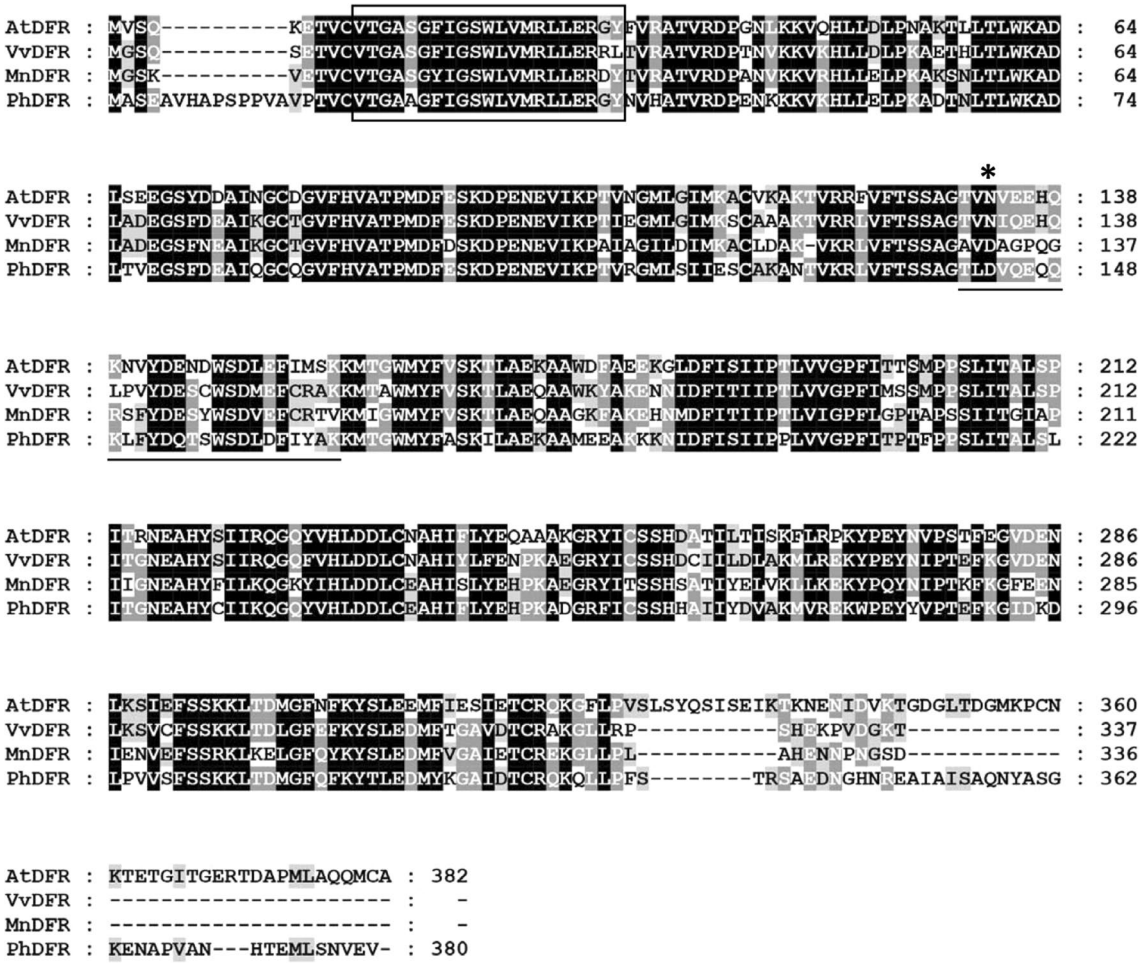

Fig. 1 Multiple sequence alignment of deduced MnDFR and other DFR proteins. The alignment was performed using CLUSTALX and the results were displayed using GENEDOC. Identical residues are highlighted in *black*. The *black box* shows the putative NADP-binding domain. The *underlined* amino acids indicate the region that determines substrate specificity and asterisks indicate residues key to the determination of substrate specificity. Proteins used for alignment are MnDFR from *M. notabilis* (KF438048), AtDFR from *A. thaliana* (P51102), VvDFR from *V. vinifera* (P51110) and PhDFR from *P. hybrida* (P14720)

acids indicate regions that determine substrate specificity. Those labeled aspartic acid/asparagine were found to directly determine the substrate specificity of DFR (Johnson et al. 2001). The MnDFR is an Asp-type DFR, which, like that of petunia, cannot convert dihydrokaempferol to leucopelargonidin (Johnson et al. 2001).

Southern blotting and transcriptional analyses of mulberry anthocyanin biosynthetic genes

The copy numbers of the anthocyanin biosynthetic genes in *M. notabilis* and two mulberry cultivars were investigated by Southern blotting. The results indicated that all of the identified genes existed in *M. notabilis* and two mulberry cultivars. Generally, there were less gene copies in *M. notabilis* than in 'Da10' or 'Zhenzhubai' and the hybridization patterns of 'Da10' were similar to those of 'Zhenzhubai', as shown in Fig. 2. Experiments were carried out to confirm the expression levels of the anthocyanin biosynthetic genes in six tissues of *M. notabilis* using qRT-PCR. Two *MnCHS* genes, *MnCHS1* and *MnCHS2*, showed different expression patterns. As shown in Fig. 3a, b, the *MnCHS1* gene was expressed exclusively in male flowers and its transcriptional level was much higher than that of *MnCHS2*, which was mainly expressed in roots and female flowers. The *MnCHI* gene was expressed at higher levels in roots (Fig. 3c). The expression levels of the two *MnF3H* genes were tissue-complementary; *MnF3H1* was expressed mainly in roots and male flowers, while *MnF3H2* was highly expressed in shoots, leaves, and female flowers (Fig. 3d, e). For the two *MnF3′H* genes, the expression of *MnF3′H2* was restricted to roots, while *MnF3′H1* was broadly expressed in several tissues (Fig. 3f, g). DFR was found to catalyze the reduction of dihydroflavonols to leucoanthocyanins (Johnson et al. 2001),

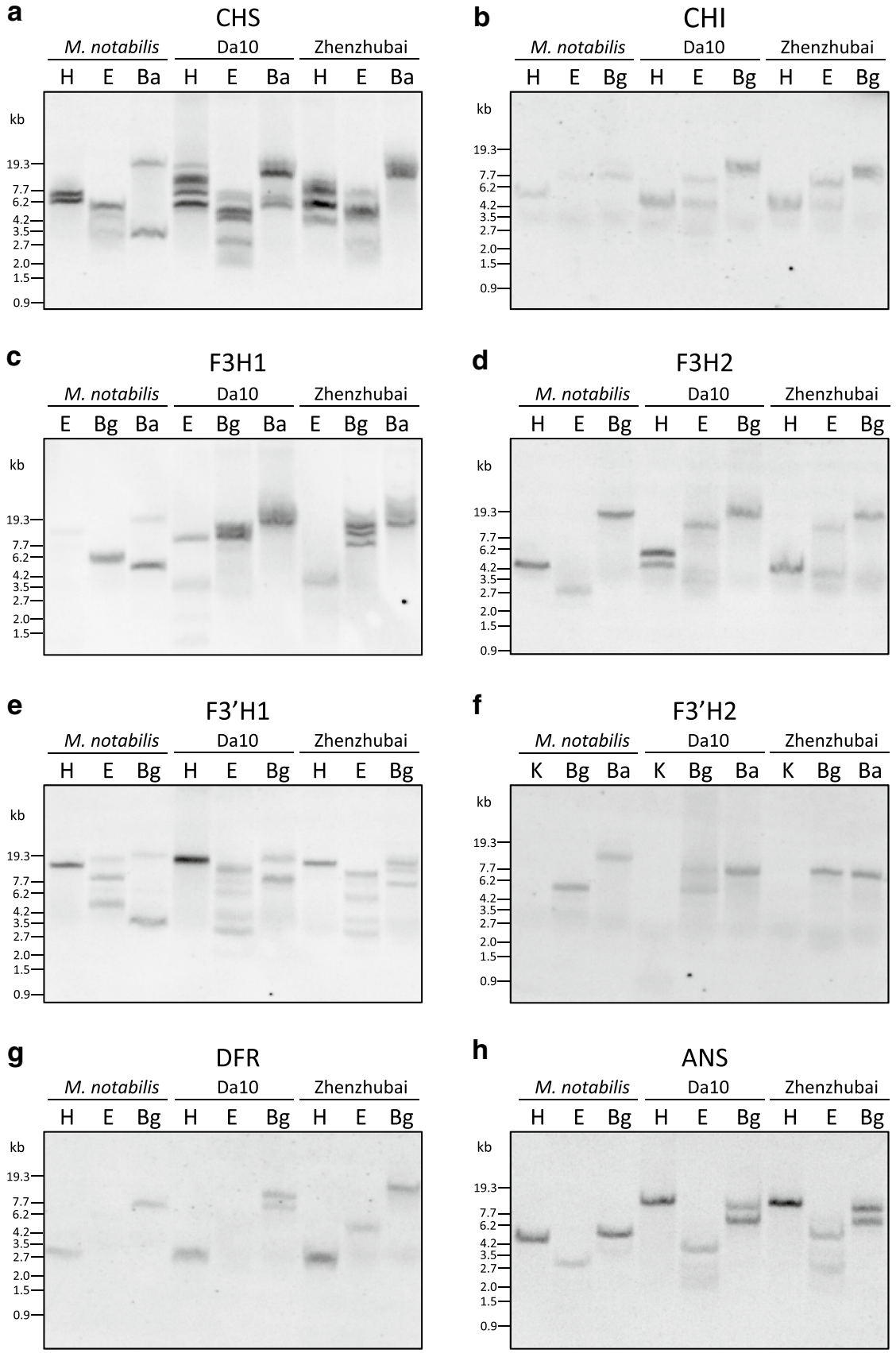

Fig. 2 Southern blotting analysis of anthocyanin biosynthetic genes in *M. notabilis* and two mulberry cultivars. *Letters* on the *top* represent restriction enzymes used to digest genomic DNA. *H Hin*dIII, *E Eco*RI, *Bg Bgl*II, *Ba Bam*HI and *K Kpn*I

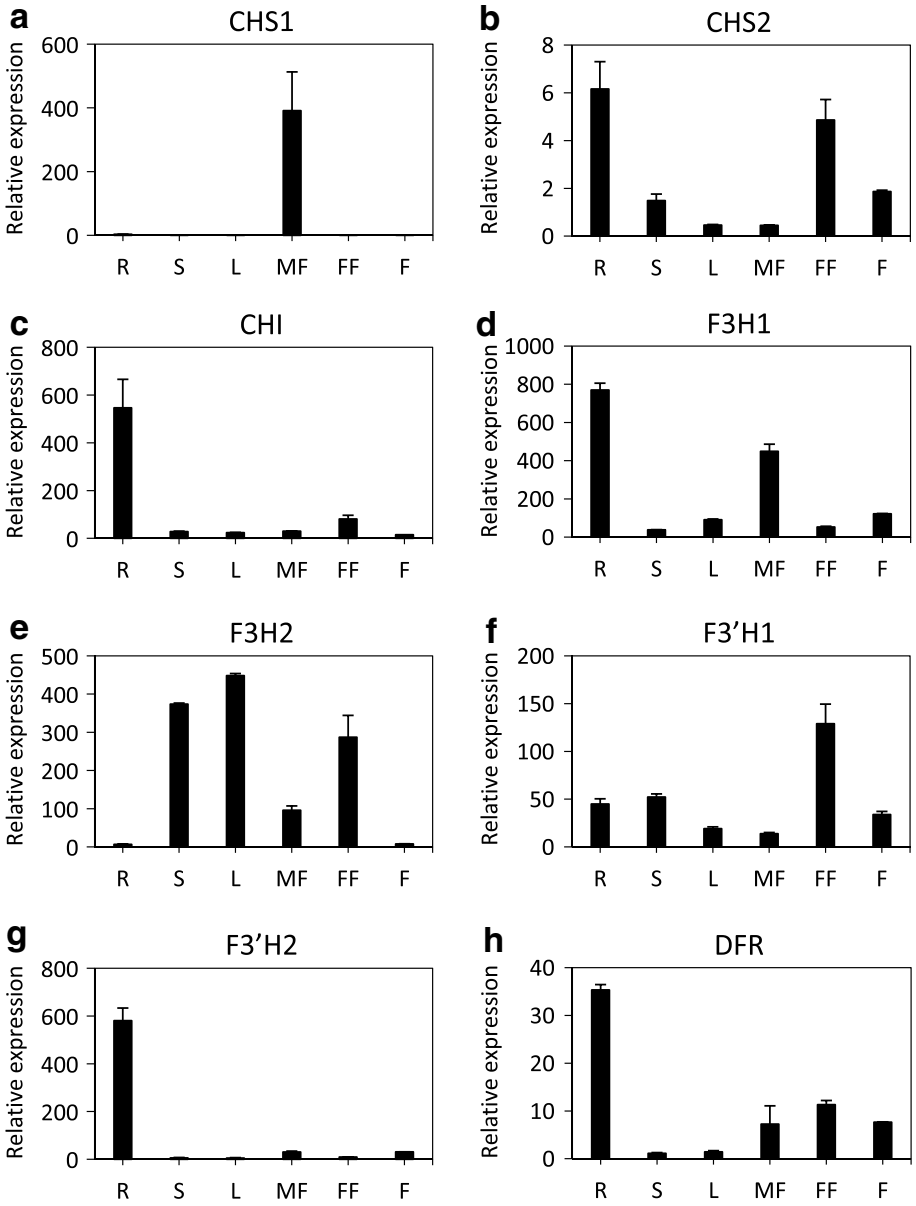

Fig. 3 Spatial transcriptional analysis of genes associated with anthocyanin biosynthesis in *M. notabilis* using qRT-PCR. Six tissues were used: *R* root, *S* stem, *L* leaf, *MF* male flowers, *FF* female flowers, and *F* fruit. Relative gene expression levels were normalized against *RPL* transcript levels. Values represent the average ± SD of three biological replicates

which are direct precursors of anthocyanins. In *M. notabilis*, the *MnDFR* gene was more abundantly expressed in roots than in flowers or fruit (Fig. 3h). ANS is a key enzyme in anthocyanin biosynthesis that catalyzes the conversion of leucoanthocyanin to anthocyanin. No *MnANS* transcripts were detected in any of the six types of tissues. Also, no anthocyanin was measured in *M. notabilis* (data not shown).

Accumulation of anthocyanin during fruit development in two mulberry cultivars

The types and concentrations of anthocyanins in the fruits of two mulberry cultivars with differently colored fruit were determined using a Waters Acquity UPLC system. By comparing the retention times to standards, the two compounds present in the fruits of 'Da10' were determined to be cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (Fig. 4a). In the 'Da10' fruit, the concentrations of the two anthocyanins were increased significantly in stage IV as compared with stage III and the degree of cyanidin-3-*O*-glucoside increase was much higher than that of cyanidin-3-*O*-rutinoside (Fig. 4b). No anthocyanin was detected in 'Zhenzhubai' fruits at any stage.

Expression of genes associated with anthocyanin biosynthesis during fruit development in two mulberry cultivars

The expression patterns of the nine mulberry genes associated with anthocyanin biosynthesis were assessed in the

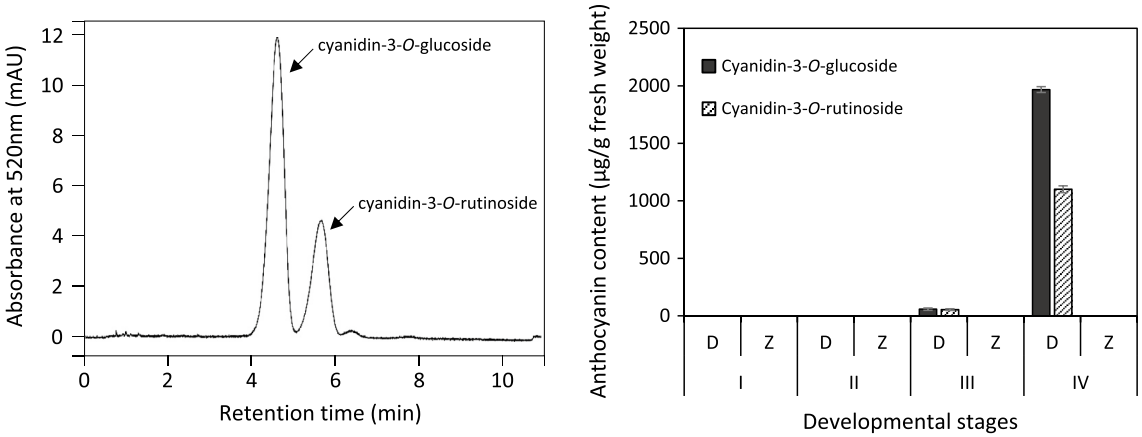

Fig. 4 Identification and quantification of anthocyanins in the fruits of two mulberry cultivars during fruit ripening. **a** Identification of the two main anthocyanins in 'Da10' fruits using UPLC. **b** Changes in the concentration of anthocyanin (μg/g fresh weight) during fruit ripening in two mulberry cultivars. D and Z represent 'Da10' and 'Zhenzhubai', respectively

fruits of two mulberry cultivars. Based on these expression patterns in two mulberry cultivars, we classified genes with similar pattern into one type. Nine mulberry genes were then divided into four types, as shown in Fig. 5. The genes in type I included *CHS1*, *CHI*, *F3H1*, *F3′H1*, and *ANS*. They were expressed increasingly during fruit ripening in the 'Da10' cultivar, but only trace expression of these genes was observed in the 'Zhenzhubai' cultivar. The gene *CHS2*, whose expression levels were higher in the 'Zhenzhubai' cultivar than in the 'Da10' cultivar during fruit ripening, was classified as type II. The *F3H2* and *F3′H2* genes were classified as type III. The transcriptional levels of these two genes were much lower than those of *F3H1* or *F3′H1* in both cultivars. The *DFR* gene was classified as type IV because of its distinct expression pattern. *DFR* expression was lower in 'Da10' fruits than in 'Zhenzhubai' fruits during ripening.

Discussion

Mulberry trees are cultivated worldwide. Their leaves are used as forage for silkworms in sericulture. People are also interested in mulberry fruits because of their delicious taste and considerable nutritional value. They are harvested for making jam, juice, wine, and food coloring. Mulberry fruits have been exploited industrially in many countries (Sánchez 2000; Singhal et al. 2010). The improvement of mulberry fruit quality is important for the use of mulberry fruits so genes that control fruit quality are of immediate interest. Anthocyanins are important to fruit quality, and research into anthocyanin biosynthesis in mulberry plants may facilitate improvement of the quality of mulberry fruits.

The completion of mulberry genome sequencing has provided researchers with an opportunity to identify genes involved in anthocyanin biosynthesis. In the present study, we identified nine putative anthocyanin biosynthetic genes that encoded enzymes controlling the committed steps in anthocyanin biosynthesis. However, no *F3′5′H* gene was identified in the mulberry genome. The *F3′5′H* gene encodes a cytochrome P450 enzyme that catalyzes the 3′,5′-hydroxylation of dihydroflavonols (Holton et al. 1993). The products of this reaction are the precursors of delphinidin, which is essential for the formation of blue colors in flowers (de Vetten et al. 1999). Previous studies have identified several varieties of anthocyanins in mulberry plants, including cyanidin, pelargonidin, and petunidin derivatives (Dugo et al. 2001). The absence of the *F3′5′H* gene in mulberry plants may account for the composition of anthocyanins in mulberry plants. The two anthocyanins identified in our experiments were cyanidin derivatives and these results are consistent with earlier reports (Dugo et al. 2001). The substrate specificity of DFR determines the type of anthocyanins produced (Johnson et al. 2001). Sequence analysis indicated that MnDFR belongs to the Asp-type DFRs, which are unable to convert dihydrokaempferol to leucopelargonidin. This would explain why cyanidin derivatives are the most abundant anthocyanins in mulberry plants.

Two *CHS*, two *F3H*, and two *F3′H* genes were identified in the mulberry genome. Each of these genes was found to have different spatial and temporal expression patterns. Similar phenomena have been observed in other plants. For example, in strawberries, one member of the *FaCHS* family was found to be expressed in petals and transcripts of the other were found to be abundant in fruit (Almeida et al. 2007). Jeong et al. (2006) cloned four *F3′H* genes in grape (*F3′h1* and *F3′h2* are alleles of one locus, and *F3′h3* and *F3′h4* are alleles of another) and found that the transcriptional levels of both sets of alleles (*F3′h1–F3′h2*;

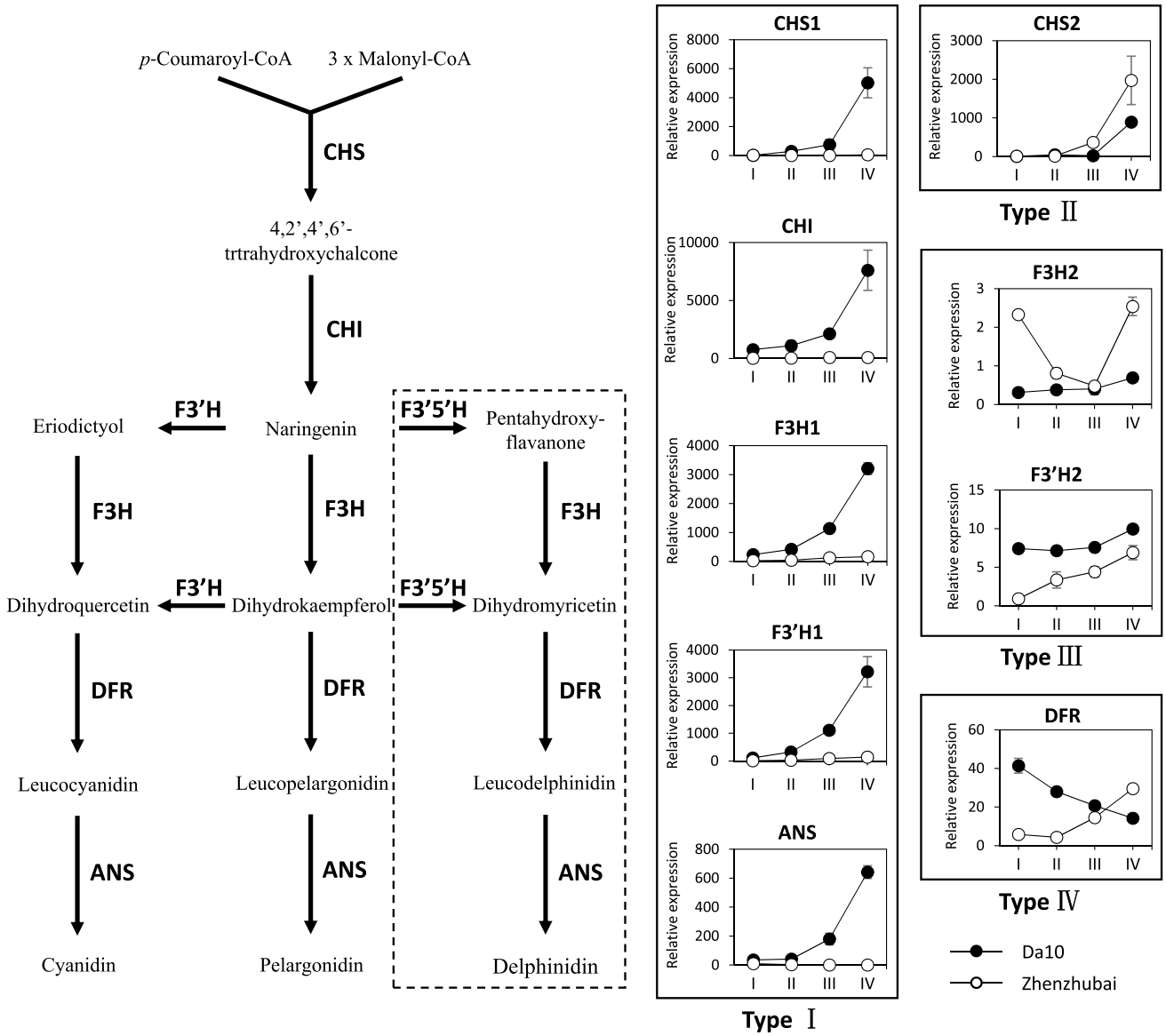

Fig. 5 Transcriptional analysis of genes associated with anthocyanin biosynthesis during the development of 'Da10' and 'Zhenzhubai' fruits. The fruits were collected at four developmental stages, as described in the "Materials and methods" section. *Black dots* represent the transcriptional levels of 'Da10' and *white dots* represent those of 'Zhenzhubai'. *Dashed lines* represent branch of delphinidin that was not synthesized in mulberry. The *y*-axis represents relative gene expression levels normalized against *RPL* transcript levels. Values represent the average ± SD of three biological replicates

F3′h3–F3′h4) were clearly different in seeds and skins during development. In sorghum, the expression levels of two *SbF3′H* genes were shown to respond to different stimuli. The expression of *SbF3′H1* was shown to be involved in the accumulation of light-specific anthocyanin, while *SbF3′H2* expression was involved in pathogen-specific 3-deoxyanthocyanin accumulation (Shih et al. 2006). Differences in the expression of members of the same gene subfamilies might be the cause of functional differences and specialization of anthocyanin biosynthesis. Mulberry *F3H1* and *F3′H1* may be related to anthocyanin accumulation during fruit ripening, and *F3H2* and *F3′H2* may have functions that are not yet documented.

The biosynthesis of anthocyanins is part of flavonoid biosynthesis process and most anthocyanin biosynthetic genes participate in the biosynthesis of other flavonoids such as flavonols, isoflavonoids and proanthocyanidins. The transcripts of eight anthocyanin biosynthetic genes were detected in *M. notabilis,* but anthocyanin was undetectable in this mulberry species. This inconsistency indicated that the genes expressed may contribute to the biosynthesis of other flavonoids rather than anthocyanins.

The connections between the expression patterns of mulberry anthocyanin biosynthetic genes and the anthocyanin concentrations in mulberry fruits were evaluated. During the development of 'Da10' fruits, the transcription levels of genes in most anthocyanin biosynthetic steps clearly increased. Conversely, in the white fruit cultivar 'Zhenzhubai', the expression levels of anthocyanin biosynthetic genes were relatively low, and no anthocyanin was found in its fruit. Considering the results in these two mulberry cultivars, we found that the transcriptional levels of genes in most anthocyanin biosynthetic steps were positively related with anthocyanin content.

In this study, we identified nine putative anthocyanin biosynthetic genes in *M. notabilis* genome. Sequence analyses revealed that these genes were conserved with their counterparts of other plants. We speculated that the absence of *F3′5′H* gene and the substrate specificity of DFR lead to the accumulation of cyanidin derivatives in mulberry. Transcriptional levels of identified genes and the amount of anthocyanins in the fruits of two mulberry cultivars were analyzed. The results indicated that anthocyanin concentrations correlated with the transcriptional levels of anthocyanin biosynthetic genes including *CHS1*, *CHI*, *F3H1*, *F3′H1*, and *ANS* during the fruit ripening process. The information provided here will expand our knowledge of the biosynthesis of anthocyanin in mulberry fruits. Artificially changing the gene transcriptional levels by genetic engineering may lead to variations in anthocyanin content. The data from this study of genes associated with anthocyanin biosynthesis will accelerate the agricultural improvement of mulberry fruits.

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