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Metabolomics and differential gene expression in anthocyanin chemo-varietal forms of *Perilla frutescens*

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Abstract

We have investigated metabolite profiles and gene expression in two chemo-varietal forms, red and green forms, of *Perilla frutescens* var. *crispa*. Striking difference in anthocyanin content was observed between the red and green forms. Anthocyanin, mainly malonylshisonin, was highly accumulated in the leaves of the red form but not in the green form. Less obvious differences were also observed in the stems. However, there was no remarkable difference in the contents and patterns of flavones and primary metabolites such as inorganic anions, organic anions and amino acids. These results suggest that only the regulation of anthocyanin production, but not that of other metabolites, differs in red and green forms. Microscopic observation and immunohistochemical studies indicated that the epidermal cells of leaves and stems are the sites of accumulation of anthocyanins and localization of anthocyanidin synthase protein. By differential display of mRNA from the leaves of red and green forms, we could identify several genes encoding anthocyanin-biosynthetic enzymes and presumptive regulatory proteins. The possible regulatory network leading to differential anthocyanin accumulation in a form-specific manner is discussed.

Keywords: Metabolomics; Metabolic profiling; Flavonoids; Differential display; Anthocyanidin synthase; *Perilla frutescens*

1. Introduction

Plant metabolomics is a newly emerged field in the post-genome era (Fiehn, 2002; Hall et al., 2002). The aim of plant metabolomics is to understand metabolic or other physiological phenotypes through global genome-related technology. In this new science, non-targeted metabolite profiling and linkage of these profiles to genotypes or transcript profiles are regarded as the key issues. The metabolite profiling can be performed by high-throughput analytical methods such as gas chromatography–mass spectrometry, high-performance liquid chromatography (LC)–mass spectrometry or Fourier transform ion cyclotron mass spectrometry. Genetic profiling is generally achieved by extensive expression profiling using techniques such as DNA array or differential display expression analysis.

Perilla frutescens Britton var. *crispa* Thunb. (perilla) belonging to the family Labiatae is a nice model to investigate how a genotype defines a metabolic phenotype (metabolotype), because two chemo-varietal forms regarding anthocyanin are available (Saito and Yamazaki, 2002). These chemo-varietal forms are believed to be defined genetically. ''Aka-jiso'' (red perilla) is a form of *P. frutescens* exhibiting dark red or purple color of leaf and stem during all developmental stages of the plant, and it is widely used as a red food coloring and as a traditional medicine in Japan, China and other Asian countries. There is another form, ''Ao-jiso'' (green perilla), which contains only a trace amount of anthocyanin in its leaves and stems in striking contrast to red perilla. The main anthocyanin pigment of red perilla is reported to be a cyanindin-type compound malonylshisonin (**32**)

Abbreviations: LC, high-performance liquid chromatography; ESI, electrospray ionization; LC/PDA/MS, high-performance liquid chromatography coupled with photodiode array detection and mass spectrometry; CE, capillary electrophoresis; ANS, anthocyanidin synthase; PBS, phosphate buffered saline; FITC, fluorecein isothiocyanate.

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[cyanidin 3-*O*-(6″-*O*-(*E*)-*p*-coumarol)-β-D-glucopyranoside-5-*O*-(6‴-*O*-malonyl)-β-D-glucopyranoside] (Kondo et al., 1989). Furthermore, related anthocyanins and flavones were isolated and identified from red perilla (Ishikura, 1981; Yoshida et al. 1990, 1997). It was also reported that the main polyphenolic compound rosmarinic acid accumulated slightly more in green perilla than in red perilla (Okuda et al., 1986). However, no extensive metabolite comparing has been carried out between anthocyanin chemotypes of *P. frutescens* so far. For gene expression analysis of perilla, we reported on several anthocyanin-related genes that were isolated in our recent study (Gong et al., 1997, 1999a,b; Saito et al., 1999; Yamazaki et al., 1999; Kitada et al., 2001). Structural genes encoding biosynthetic enzymes and a *Myb*-related regulatory gene are differentially expressed in a red form-specific manner. However, more detailed and extensive analysis of gene expression and also linkage of the gene expression profile to the metabolite profile are needed for better understanding the relations between gene expression and metabolic phenotype in the chemotypes of perilla.

In the present study, as the first trail of a metabolomics approach of an anthocyanin-chemotypes, we investigated metabolite profiling by high-performance liquid chromatography coupled with photodiode array detection and mass spectrometry (LC/PDA/MS), capillary electrophoresis (CE) for anion analysis and LC for amino acid analysis. In addition, we elucidated cell-specific anthocyanin accumulation and localization of anthocyanidin synthase (ANS). The extensive analysis of gene expression using mRNA differential display of two chemo-varietal forms of *P. frutescens* has also been conducted.

2. Results and discussion

2.1. Metabolite profiling in red and green forms of *P. frutescens*

The metabolites in green and red forms of *P. frutescens* were profiled by LC/DAD/MS, CE for anion analysis and LC for amino acid analysis. The acidic MeOH–H_2O extracts from leaves and stems were analyzed by HPLC/PDA/electrospray ionization (ESI)–MS. Nearly 50 peaks were detected in this analysis (Table 1). Metabolite identification was made by UV–visible absorption spectra and mass fragmentation by tandem MS analysis in comparison with the authentic compounds and the reported data (cited in Table 1). In the leaves of the red form, a number of anthocyanin pigments such as malonylshisonin (**32**), shisonin (perillanin) (**28**), *cis*-isomer of malonylshisonin (**26**), cyanidin 3-*O*-caffeoylglucoside-5-*O*-malonylglucoside (**24**), cyanidin 3-*O*-caffeoylglucoside-5-*O*-glucoside (**21**) and peonidin 3-*O*-malonylglucoside-5-*O*-*p*-coumarylglucoside (**36**) were detected (Fig. 1A). Among them malonylshisonin (**32**) is the main anthocyanin representing ca. 70% of total anthocyanins in red leaves. In striking contrast, green perilla leaves did not accumulate these anthocyanins; only trace levels of anthocyanins were detected. The stems of both red and green forms contained a similar pattern of anthocyanins to that in red leaves, although much less than in red leaves. The amount of total anthocyanin in the stem of green perilla was only 20% of that in the stems of red perilla. In *P. frutescens*, the major aglycone in anthocyanins is cyanidin. Trace amounts of peonidin derivatives (**36**, **38**) were also found in red form leaves. These results indicate that the accumulation of anthocyanin is strictly specific to the red form, and this form-specificity is more distinct in leaves than in stems.

Other flavonoids besides anthocyanins were detected, but were present at the almost same levels in both forms (Fig. 1B, Table 1). Flavones, luteolin 7-*O*-diglucuronide (**15**) and apigenin 7-*O*-diglucuronide (**22**), were accumulated more in red form leaves than in green form leaves. Apigenin 7-*O*-caffeoylglucoside (**8**), scutellarein 7-*O*-diglucuronide (**14**) and apigenin 7-*O*-glucuronide (**37**) accumulated at the same level in red and green forms. Luteolin 7-*O*-glucoside (**19**) and scutellarin (**27**) accumulated slightly more in the green form than in the red form. These results indicate that the regulation of production of flavones is different from that of anthocyanin production in the two forms of perilla. Rosmarinic acid (**39**) accumulated both in red and green forms. The amount of accumulated rosmarinic acid in green form leaves was slightly higher than that in red form leaves. This tendency is same as reported by Okuda et al. (1986) and might be caused by the competition for the common intermediate, 4-coumaroyl-CoA, between biosyntheses of rosmaric acid and flavonoids.

The contents of anions, such as nitrate, sulfate, phosphate, citrate, malate and other organic acids, were analyzed using CE. Additionally, the contents of amino acids (asparic acid, threonine, serine, asparagine, glycine, alanine, valine, arginine, histidine, tryptophane) were determined by using an HPLC system with post-column derivatization for fluorescence detection. However, no obvious difference was detected in these compounds between red and green forms (data not shown). The genetic control of volatile components, cyclic monoterpenoids, in *Perilla frutescens* had been precisely studied (Nishizawa et al., 1992) and it has been clarified the genetic control of monoterpene biosynthesis is independent from anthocyanin production (Fig. 2).

These results suggest that flavonoid patterns, particularly anthocyanins, are different between the red and green forms of *P. frutescens*, but most primary metabolites do not differ between the two forms.

Table 1
The profiling of acidic MeOH-H_2O extracts of *Perilla frutescens* by HPLC/PDA/ESI-MS

Peak No.	Compound	R_t (min)	λ_{max} (nm)	ESI-MS (m/z)	Fragment[a] (m/z)	Red perilla		Green perilla		References
						Leaf	Stem	Leaf	Stem	
1	Unknown	18.23	167, 357			+/–				
2	Unknown	19.65	229, 287	613.04		+/–		+		
3	Unknown	20.13	317	292.30	275.11, 218.11	+/–		+/–		
4	Unknown	20.91	285	501.25	471.18, 486.14, 387.04	+/–				
5	Unknown	21.03	248, 327	322.32		+/–	+/–	+	+/–	
6	Unknown	21.91	277	525.20		+/–		+/–		
7	Unknown	23.01	310	277.22	147.02	+/–				
8	Apigenin 7-*O*-caffeoylglucoside	24.45	272, 329	595.19 $[M+H]^+$		++	++	++	+	Ishikura, 1981
9	Cyanidin 3-*O*-glucoside-5-*O*-malonylglucoside	24.55	323, 518*	697.09 $[M]^+$		+				
10	Unknown	25.35	298	263.19	147.05, 116.97	+/–		+/–		
11	Anthocyanin der.	26.11	282, 519*			+/–				
12	Unknown	26.27	292	277.21	147.09	+	+/–	+/–		
13	Anthocyanin der.	26.96	284, 508*	365.22	202.91	+/–				
14	Scutellarein 7-*O*-diglucuronide	27.35	284, 332	639.10 $[M+H]^+$		+		+		
15	Luteolin 7-*O*-diglucuronide	28.04	255, 347	639.15 $[M+H]^+$	287.37 $[Lt+H]^+$	+++	+/–	+		Yoshida et al., 1997
16	Anthocyanin der.	28.43	323, 507*	240.96		++				
17	Unknown	28.99	315	797.28	489.08, 635.13	+	+/–		+/–	
18	Unknown	29.96	331	625.18		+/–		+/–		
19	Luteolin 7-*O*-glucoside[b]	30.49	251, 340	449.11 $[M+H]^+$	287.41 $[Lt+H]^+$	+	+/–	++	+/–	Ishikura, 1981
20	Anthocyanin der.	30.82	281, 521*	739.17		+				
21	Cyanidin 3-*O*-caffeoylglucoside-5-*O*-glucoside	31.66	269, 332, 519*	773.20 $[M]^+$		+	+/–	+/–	+/–	Yoshida et al. 1990
22	Apigenin 7-*O*-diglucuronide	31.81	268, 325	623.15 $[M+H]^+$	271.38 $[Ap+H]^+$	++	+	++		Yoshida et al., 1997
23	Anthocyanin der.	31.90	317, 522*	883.22	287.38 $[Cy]^+$·, 839.10	+				
24	Cyanidin 3-*O*-caffeoylglucoside-5-*O*-malonylglucoside	32.71	281, 325, 520*	859.15 $[M]^+$	287.36 $[Cy]^+$ 535.20 $[Cy+Glc+Mal]^+$ 611.12 $[Cy+Glc+Caf]^+$	++	+	+/–	+/–	Yoshida et al., 1990
25	Unknown	33.00	299, 327	666.14				+/–		
26	Malonyl-*cis*-shisonin	33.44	280, 526*	843.18 $[M]^+$	287.40 $[Cy]^+$ 535.23 $[Cy+Glc+Mal]^+$ 595.27 $[Cy+Glc+Cou]^+$	++	+/–		+/–	Yoshida et al., 1990
27	Scutellarin	34.52	282, 335	463.12 $[M+H]^+$	287.38 $[Lt+H]^+$			++		
28	Shisonin	34.61	281, 314, 520*	757.18 $[M]^+$	287.37 $[Cy]^+$ 595.24 $[Cy+Glc+Cou]^+$	+++	+	+/–	+	Yoshida et al., 1990
29	Shisonin der.[c]	35.05	281, 519*	787.20 $[M]^+$	287.36 $[Cy]^+$ 625.16 $[Cy+Glc+Cou+OMe]^+$		+/–			
30	Unknown	35.54	313	773.21	443.23, 729.10	+/–	+/–			
31	Unknown	35.83	310	813.22	505.20, 384.36	+/–				
32	Malonylshisonin	36.35	281, 312, 522*	843.18 $[M]^+$	287.37 $[Cy]^+$ 535.20 $[Cy+Glc+Mal]^+$	+++	++	+	+	Kondo et al., 1989

(*continued on next page*)

Table 1 (*continued*)

Peak No.	Compound	R_t (min)	λ_{max} (nm)	ESI-MS (m/z)	Fragment[a] (m/z)	Red perilla		Green perilla		References
						Leaf	Stem	Leaf	Stem	
					595.21 $[Cy+Glc+Cou]^+$					
33	Anthocyanin der.	36.87	281, 313, 522*			+	+		+/−	
34	Anthocyanin der.	38.38	300, 513*	899.19	591.03, 547.04	+				
35	Unknown	38.56	250, 328	741.26		+	+/−	+	+/−	
36	Peonidin 3-*O*-malonylglucoside-5-*O*-*p*-coumarolglucoside	39.07	287, 313, 519*	857.21 $[M]^+$	301.27 $[Pn]^+$	+	+/−			
					609.20 $[Pn+Glc+Cou]^+$					
					549.16 $[Pn+Glc+Mal]^+$					
37	Apigenin 7-*O*-glucuronide	39.50	251, 328	447.18 $[M]^+$		+	+	+	+	Yoshida et al., 1997
38	Peonidin 3-*O*-malonylglucoside-5-*O*-*p*-coumarylglucoside	39.79	284, 522*	857.20 $[M]^+$		+	+/−			
39	Rosmarinic acid	40.47	329	360.97	163.13	+++	+++	++++	+++	Okuda et al., 1986
40	Unknown	42.09	317	1699.03	1451.01, 1203.06, 1143.02	+		+		
41	Anthocyanin der.	43.05	289, 529*			+/−				
42	Unknown	44.15	287	583.09		+/−		+/−		
43	Unknown	46.10	323					+		
44	Unknown	47.65	309	945.32	797.15, 619.18	+				
45	Unknown	48.94	312	1031.33	987.15, 839.12	+				
46	Unknown	50.95	264, 329			+/−	+/−	+	+/−	
47	Unknown	54.76	253, 337			+/−	+/−	+	+/−	
48	Unknown	57.67	258, 337			+/−	+/−	+/−	+/−	
49	Unknown	59.76	255, 370					+/−		

The relative peak areas of PDA chromatogram were shown as +/−(×1), + (×10), ++ (×10^2), +++ (×10^3), ++++ (×10^4). Lt, luteolinidin; Ap, apigenidin; Cy, cyanidin; Glc, glucose; Cou, *p*-coumaroyl moiety; Caf, cafferoyl moiety; Mal, malonyl moiety; OMe, methoxyl moiety; Pn, peonidin; der., derivative; *characteristic UV–visible absorbance for anthocyanin pigments.

[a] Detected in mass and/or tandem mass data.

[b] The structure was not confirmed.

[c] One hydroxyl group might be methylated.

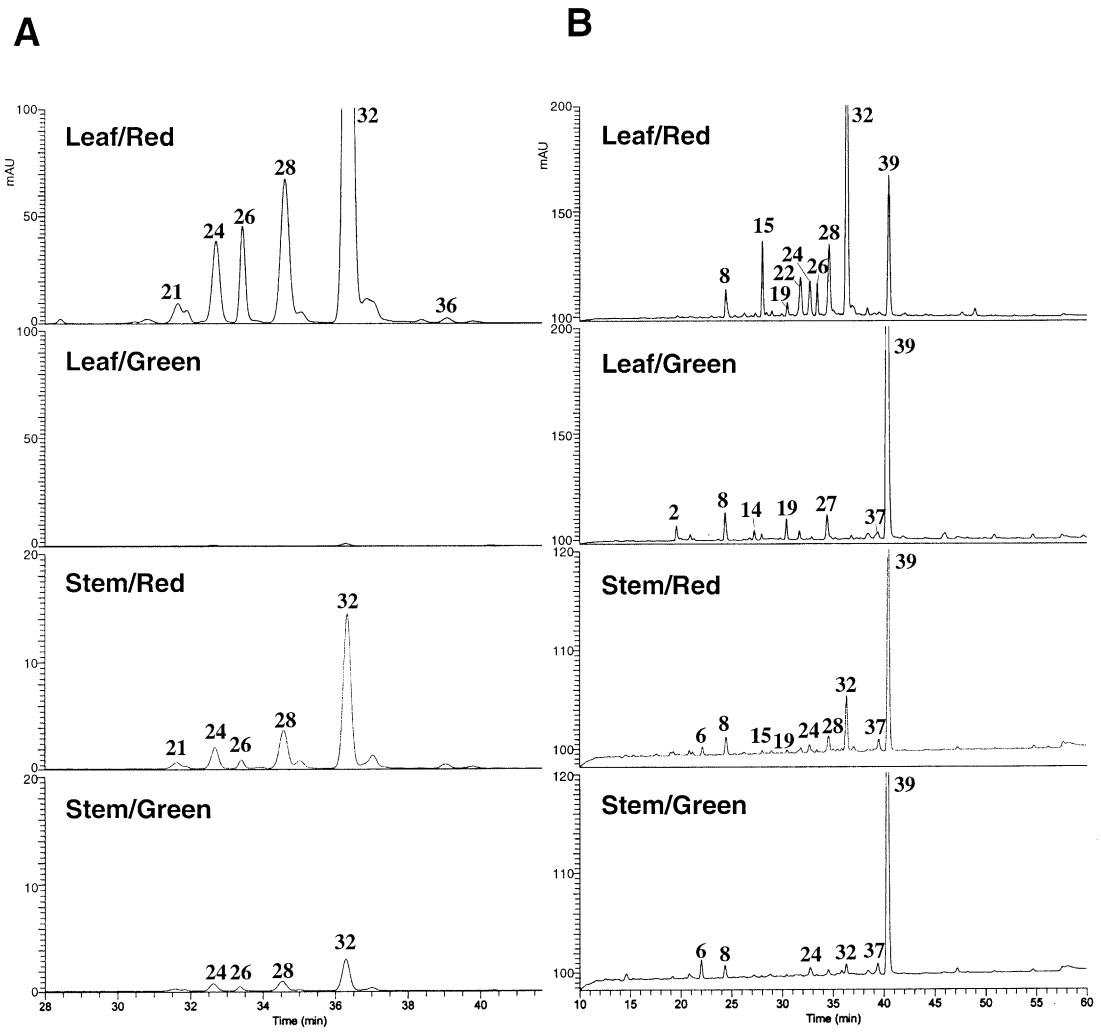

Fig. 1. HPLC/PDA/MS chromatograms of flavonoid-rich fractions from red and green perilla. (A) Chromatogram detected by PDA at 520 nm; (B) chromatogram of total scan by PDA (200–700 nm).

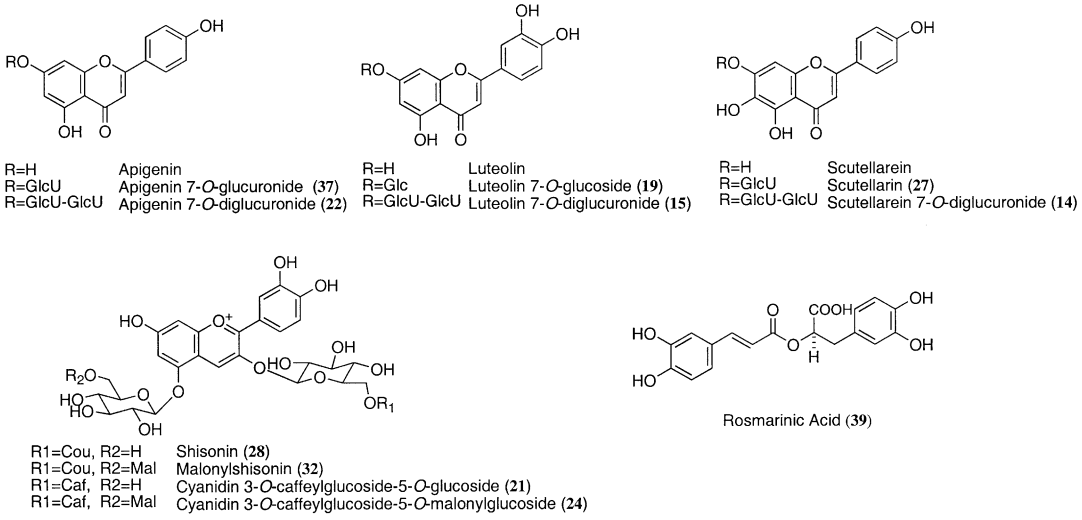

Fig. 2. Typical flavonoids and organic acid in perilla frutenscens.

2.2. Sites of anthocyanin accumulation and tissue-specific expression of anthocyanidin synthase

The sites of accumulation of anthocyanin were elucidated by microscopic analysis of transverse section of perilla leaf and stem (Figs. 3A, B). Anthocyanins are accumulated in a single layer of epidermal cells of leaf (both upper and lower epidermal cells) and stem of red perilla. No evident accumulation was found in leaves of green perilla (data not shown). Cell-specific accumulation of anthocyanidin synthase (ANS), the key enzyme for production of colored anthocyanidin, was also investigated by immuno-fluorescent histochemical analysis with antibodies raised against recombinant ANS (Saito et al., 1999) (Figs. 3C–E). The epidermal cells of the stem of red perilla are the sites of both ANS protein localization and anthocyanin accumulation (Fig. 3C). Fig. 3D is a negative control experiment indicating that the pre-absorbed antibodies to recombinant ANS protein did not show any signals in red perilla stem. The tissues of green perilla also showed no evident signals (Fig. 3E). The immuno-fluorescent staining using leaves of red and green perilla gave similar results (data not shown). As reported previously (Saito et al., 1999), tissue-specific and form-specific accumulation of ANS protein analyzed by western blotting also correlated well with the pattern of anthocyanin production. These data indicate that epidermal cells of red perilla are the sites of biosynthesis and storage of anthocyanins.

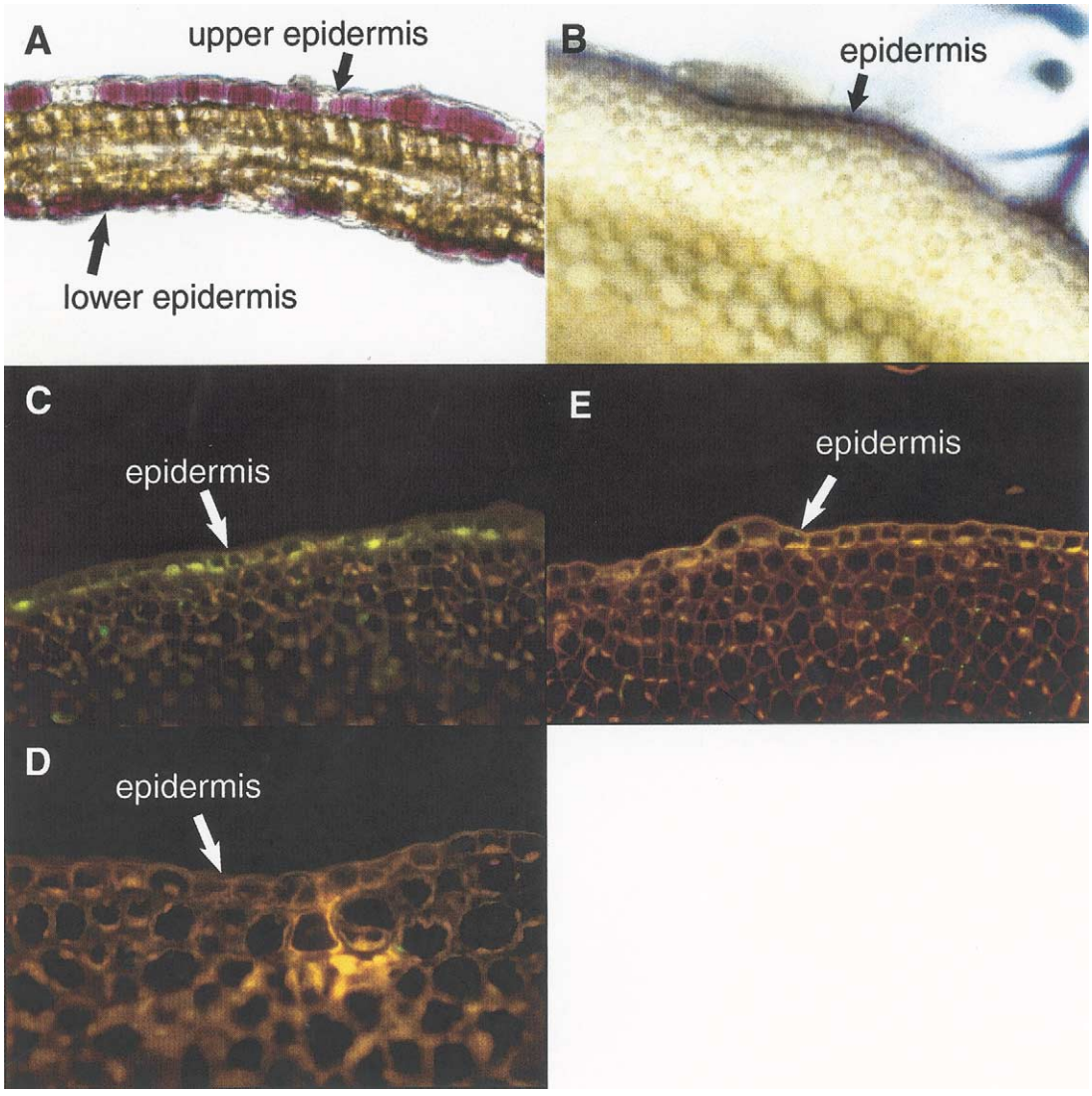

Fig. 3. The sites of accumulation of anthocyanins and ANS protein. (A) Leaf transverse section of red perilla under bright field; (B) Stem transverse section of red perilla under bright field; (C) stem transverse section of red perilla stained immnohistochemically with the antibodies against recombinant ANS protein. Immuno-fluorescent signals were visualized using anti rabbit IgG-FITC conjugate; (D) negative control of red perilla stem stained with pre-absorbed antibodies to ANS; (E) stem transverse section of green perilla stained with the anti-ANS antibodies.

2.3. Profiling of genes specifically expressed in the red-form by mRNA differential display

The form-specific gene expression was profiled by the mRNA differential display technique (Liang and Pardee, 1992; Yamazaki and Saito, 2002; Yamazaki et al., 1997). The mRNAs extracted from the leaves of red and green forms were reverse-transcribed into cDNAs with a subset of specific one-base anchored oligo(dT) primers that recognize different fractions of the total poly(A)$^+$ RNA population. The resulting cDNA was amplified by polymerase chain reaction (PCR) with a combination of the same anchored oligo(dT) primer used in reverse transcription and a 13- or 10-mer primer of arbitrary sequence. With 144 primer combinations, 81 fragments were specifically amplified in mRNA from the red form out of ca. 6600 fragments displayed in total. These red-form specific fragments were isolated and re-amplified using the same primer combinations as in the first PCR, and subsequently cloned into plasmid vector. The partial sequences were determined and mRNA expression of these clones was investigated by northern blot analysis. Among these fragments, eight clones were selected and further investigated as red form specifically expressed fragments.

Table 2 summarizes the cDNA clones isolated by differential display. The deduced amino acid sequences of 3R4 and 3R6 exhibited homology with known flavonoid: UDP-glucose glucosyltransferases. By using recombinant protein expressed in yeast, the 3R4 cDNA was eventually confirmed to encode UDP-glucose: anthocyanin 5-*O*-glucosyltransferase (5-GT) (Yamazaki et al., 1999). The recombinant 3R6 protein, which lacks C-terminal region of 3R4, did not show 5-GT activity to cyanidin 3-*O*-glucoside. The 8R5 cDNA encodes ANS, and the enzymatic characters of recombinant proteins and cell-specific expression were determined (Saito et al., 1999). The partial sequence of F5RA1-16 was identical with the sequence of cDNA encoding dihydroflavonol reductase (DFR) (Gong et al., 1997). The clones, 2R3 and F3G1, were speculated to encode homologues of Myb and basic helix-loop-helix (bHLH) transcription factors, respectively, by their sequence comparison. These differential display results suggest that probably 1–0.1% of total transcripts are differentially expressed in a red-form specific manner. These differentially expressed genes are presumably responsible for the distinct metabolite patterns in the red and green forms.

2.4. Regulatory factors determining the red and green forms of *P. frutescens*

So far, we have studied the expression of the structural genes encoding catalytic enzymes involved in anthocyanin biosynthesis in red and green forms of *P. frutescens* (Gong et al., 1997; Saito et al., 1999; Yamazaki et al., 1999; Kitada et al., 2001). In those studies, it was shown that all structural genes examined except chalcone synthase are expressed specifically in the red form but not in the green form, and their expressions are induced coordinately by light illumination. These results suggest that specific regulatory factors are responsible for such form-specific gene expression (Saito and Yamazaki, 2002). We have isolated and characterized a constitutively expressed bHLH gene *Myc-rp* (Gong et al., 1999a), a light inducible *Myb*-like gene *Myb-p1* (Gong et al., 1999b) and a WD40 repeat protein gene *Pfwd* (Sompornpailin et al., 2002) from *P. frutescens*. These factors are involved in the regulation of anthocyanin biosynthesis but one not alone the definitive factor determining these chemo-varieties. The red-form specific expressed genes encoding *Myb* and bHLH transcription factors newly isolated by differential display are good candidates for being determinant factors of the chemo-varietal forms of *P. frutescens*.

Table 2
The red-form specifically expressed genes isolated by differential display

Clone	Primers	Size (kb)	Encoded protein	References
3R4	H-T_{11}A, H-AP3	1.4	5-*O*-Glucosyltransferase	Yamazaki et al., 1999
3R6[a]	H-T_{11}A, H-AP3	1.4	Glucosyltransferase homologue	Yamazaki et al., 1999
8R5	H-T_{11}G, H-AP8	1.4	Anthocyanidin synthase	Saito et al., 1999
F5RA1-16	H-T_{11}A, OPF-5	Partial	Dihydroflavonol reductase	Unpublished
2R3	H-T_{11}A, H-AP2	1.0	MYB homologue	Unpublished
F3G1	H-T_{11}G, OPF-3	2.0	bHLH factor	Yamazaki et al., in press
8R6	H-T_{11}G, H-AP8	1.4	Unknown	Unpublished
MYBC05	H-T_{11}G, *Myb* primer[b]	1.0	Likely MYB homologue	Yamazaki et al., in press

[a] 3R6 encodes 3R4 homologue which lacks 5-GT activity.
[b] MYBC05 was amplified by using one-base anchored oligo(dT) primer and a *Myb* specific primer designed from the conserved sequence in *Myb* repeat 3.

3. Experimental

3.1. Plant materials

The red and green forms of *Perilla frutescens* var. *crispa* were grown on rock wool with nutrient solution of Hyponex (5-10-5) in a plant growth room for 16 weeks with a photoperiod of 18 h light (4500 lux)/6 h dark at 25 °C.

3.2. HPLC/PDA/ESI-MS analysis

The ca. 1 g leaves and stems of red or green forms were extracted with 6 ml extraction solvent (MeOH–AcOH–H_2O = 9:1:10) per 1 g fresh weight of tissues at 4 °C overnight. The extracts were filtered through a 0.45 μm nylon filter and applied to HPLC/PDA/ESI-MS system consisted of a Finnigan LCQ DECA mass spectrometer (ThermoQuest, San Jose, CA) and an Agilent HPLC 1100 series (Agilent Technologies, Palo Alto, CA). HPLC was carried out on an ODS-A312 column (ϕ 6 mm×150 mm, YMC) at a flow rate of 0.5 ml/min. Elution gradient program was 0–60 min linear gradient from solvent A [CH_3CN–H_2O–TFA (7.5:92.5:0.1)] to solvent B [CH_3CN–H_2O–TFA (55:45:0.1)]. PDA was used for detection of UV-visible absorption in the range of 200–700 nm. Nitrogen was used as sheath gas for the positive-ion ESI-MS performed at capillary temperature and voltage of 320 °C and 5.0 kV, respectively. The tube lens offset was set at 10.0 V. Full scan mass spectra were acquired from 80 to 2000 m/z at 2 scans/s. Tandem MS analysis was carried out with helium as the collision gas. The normalized collision energy was set to 30%.

3.3. Differential display of mRNA and cDNA cloning

Differential display of mRNA was performed as described previously (Yamazaki et al., 1999) with some modifications. Poly(A)$^+$RNA isolated from the top young leaves of *P. frutescens* was reverse transcribed with subsets of specific one-base anchored oligo(dT) primers (H-T11G, H-11A, or H-T11C, GenHunter). The resulting cDNA was amplified with a combination of the same anchored oligo(dT) primer used in reverse transcription and a 13- or 10-mer primer of arbitrary sequence (one of H-AP1-8, GenHunter; OPD1-20 and OPF1-20, Operon) by a polymerase chain reaction (PCR). PCR conditions were same as those described previously (Yamazaki et al., 1999). Amplified DNA was fractionated by electrophoresis in a sequencing gel. The differentially appearing bands were cut out of the gel and DNA was eluted with H_2O and purified by ethanol precipitation. Re-amplification was carried out using eluted DNA as a template under the same PCR conditions. The re-amplified DNAs were cloned into pBluescriptII SK-, and used as probes for initial RNA blot analysis and cDNA screening. The cDNA cloning was carried out using a library constructed from red perilla as reported previously (Gong et al., 1997).

3.4. Localization of anthocyanin accumulation and anthocyanidin synthase protein

For bright field microscopic observation, the plant tissues were embedded in 5% agar and sliced (50–200 μm thickness) by using a microslicer (DTK-1000, D.S.K, Japan). Immuno-fluorescent histochemical staining was carried out as described previously (Yamazaki et al., 2002). The plant tissues were fixed in a fixative (3% formaldehyde, 50% ethanol and 5% acetic acid) and embedded into polyethylene glycol 1540. The tissue sections of 8 μm thickness were incubated with rabbit antibodies raised against the recombinant ANS (Saito et al., 1999). Antibodies were diluted 1:250 in phosphate buffered saline (PBS) (0.16 M NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 0.5% BSA. The plant sections on a slide were incubated with 150 μl of antibody dilutions overnight at 4 °C in a moist environment. For negative control, sections were also incubated with pre-immune sera and pre-absorbed antibodies to the purified recombinant ANS (Saito et al., 1999). For the absorption of antibodies, 40 μg ml^{-1} recombinant ANS were added to the antibody dilution and incubated overnight at 4 °C. After centrifugation of the antibody–antigen mixture at 17,700 g for 10 min, the supernatant was used as the pre-absorbed antibodies. For visualization of immuno-signals, sections washed with PBS were incubated with goat IgG anti-rabbit IgG-fluorecein isothiocyanate (FITC) conjugate for 1–2 h at room temperature. The sections were washed again with PBS and mounted with Vectashield (Vector Laboratories) and green fluorescence emitted by FITC was monitored under a fluorescence microscope (Olympus BX50) with a filter set (U-DM-FI/TR) for dual excitation (470–490 and 545–570 nm) and dual emission (505–535 and 580–620 nm).

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