Insertion of a Retrotransposon into a *Flavonoid 3'-hydroxylase* Homolog Confers the Red Root Character in the Radish (*Raphanus sativus* L. var. *longipinnatus* L. H. Bailey)

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Flavonoid 3'-hydroxylase (F3'H) has an important role in determining anthocyanin patterns in plants. Here, we analyzed root coloration characteristics in the radish by investigating anthocyanidin and expression of *F3'H* genes in cultivars with purple, red, or white roots. Cyanidin was detected in the roots of the purple radish, while pelargonidin was found in the red radish; however, neither of these anthocyanidins was detected in white radish. We isolated the *RsF3'H* gene of the purple root radish and found that it showed a relatively higher level of expression compared to the *RsF3'H* of the red radish. Moreover, we identified a retrotransposon, *gypsy-Ty3*, in the first exon of the *F3'H* homolog in the red radish. These results suggested that the F3'H enzyme may determine cyanidin-based anthocyanin in the purple radish, and that the lack of F3'H function due to the retrotransposon insertion, contributed to pelargonidin-based anthocyanin accumulation in the red radish.

Key Words: cyanidin, *F3'H*, lack, pelargonidin, retrotransposon.

Introduction

The radish (*Raphanus sativus* L.) belongs to the Brassicaceae family and is an important vegetable that is grown worldwide. Some varieties of radish (*R. sativus* L. *sativus*) and Japanese radish (*R. sativus* L. var. *longipinnatus* L. H. Bailey) have red skin and/or flesh due to anthocyanin accumulation. The main anthocyanins in radishes are acylated cyanidin 3-sophoroside-5-glucosides or acylated pelargonidin 3-sophoroside-5-glucosides (Kato et al., 2013). Radish anthocyanins are brightly colored over a wide pH range and their multiple acylation with hydroxycinnamic acids contributes to their remarkable heat stability in acidic environments (Jing et al., 2012). Radish anthocyanins have been widely used as natural colorants and

for the health benefits of their antioxidant activities (Matsufuji et al., 2007; Rahman et al., 2006).

The genetics, biochemistry, and molecular biology of the anthocyanin biosynthetic pathway are well characterized (Grotewold, 2006; Koes et al., 2005). This pathway contains seven core enzymes, namely, chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-glucosyltransferase (3GT). These enzymes produce cyanidin-based anthocyanins in a chain catalysis process (Fig. 1). In the pathway, F3'H hydroxylates the 3'-position of the B ring of dihydrokaempferol, a pelargonidin precursor, to generate dihydroquercetin, a cyanidin precursor. Dihydroquercetin is an important intermediate in the biosynthesis of anthocyanins (Koes et al., 1994; Werck-Reichhart et al., 2002). Because a *F3'H* mutant would then result in the production of pelargonidin-based anthocyanin (Hoshino et al., 2003), *F3'H* plays an important role in production of cyanidin-based anthocyanin.

Recently, the expression of genes encoding enzymes for anthocyanin biosynthesis, including *phenylalanine*

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ammonia lyase (*PAL*), *cinnamate 4-hydroxylase* (*C4H*), *4-coumarate-CoA ligase* (*4CL*), *CHS*, *CHI*, *F3H*, *DFR*, and *ANS*, was investigated in white and red radishes (Park et al., 2011). However, little attention has been paid to the relationship between *F3'H* expression and root color in radishes.

In this study, to determine the cause of the red root character, we investigated the types of anthocyanidins in purple, red, and white root radishes. Moreover, to elucidate the molecular basis of the red root character, we investigated the role of the radish *F3'H* gene in determining radish root color.

Materials and Methods

Plant materials

Radish cultivars (*R. sativus* L. var. *longipinnatus* L. H. Bailey) of purple root 'Karaine-aka' (Watanabe seed Co., Ltd., Miyagi, Japan), red root 'Chouan-aomaru-

koshin' (Takii & Co., Ltd., Kyoto, Japan) and 'Benikururi 521' (Matsunaga Seed Co., Ltd, Aichi, Japan), and white root 'Taibyosoubutori' (Takii & Co., Ltd.) were used in the present study (Fig. 2A–C, E). White root pungent radish 'Susanoo' (Shimane University, Matsue, Japan), which was developed from the Japanese wild radish (*R. sativus* L. f. *raphanistroides* Makino) by mass selection, was also used in this study (Fig. 2D). These cultivars were maintained in the experimental field of Shimane University. Their root skin and flesh were collected for High Performance Liquid Chromatography (HPLC) analysis. For molecular analysis, root skin, flesh, and leaves were collected and immediately frozen in liquid nitrogen and stored at -80°C until extraction of total RNA and genomic (g) DNA.

Anthocyanidins by HPLC analysis

The anthocyanidin was extracted using the methods of Mizuta et al. (2009). The raw root skins and flesh (ca 0.5 g) mentioned above were soaked in 50% CH_3COOH in H_2O overnight. The crude extracts were concentrated to small amounts and hydrolyzed with 4 mL of 2N hydrochloric acid at 100°C for 1 h. The hydrolysates were adsorbed on a Sep-pak C18 cartridge (Nihon Waters K. K., Tokyo, Japan). The cartridge was washed to eliminate the water-soluble or hydrophilic contaminants, and then the anthocyanidins were eluted by 50% CH_3COOH in H_2O . A small portion of the eluate was passed through a Millipore filter, and the filtrates were analyzed with some standards by HPLC. As these standards for the assessment of anthocyanidin, commercially available cyanidin and pelargonidin (Extrasynthese, Genay, France) were used.

The analysis for anthocyanidins was performed using a GL science HPLC system (GL-7410, GL-7420, GL-7432, GL-7452A, GL-7480; GL sciences Inc., Tokyo, Japan) equipped with a degasser (BG-240; FLOM Inc., Tokyo, Japan) using an Inertsil ODS-4 column (4.6×150 mm; GL sciences Inc.), a column temperature of 40°C , and monitoring at 520 nm. The solvent system had a ratio of 55% solvent A [H_3PO_4 - H_2O (1.5 : 98.5, v/v)] to 45% solvent B [H_3PO_4 -

Fig. 1. Anthocyanin biosynthetic pathway. Enzyme abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavonone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; 3GT, flavonoid 3-glucosyltransferase.

Fig. 2. Photographs of the five radish varieties used in this study: (A) 'Karaine-aka', (B) 'Chouan-aomaru-koshin', (C) 'Benikururi 521', (D) 'Susanoo', and (E) 'Taibyosoubutori'. Bars indicate 10 cm.

CH₃COOH-CH₃CN-H₂O (1.5 : 20 : 25 : 53.5, v/v)] run for 25 min with a flow rate of 1.0 mL·min⁻¹.

Isolation of the F3'H gene from mRNA of the purple root radish 'Karaine-aka'

Total RNA was extracted from root skin of 'Karaine-aka' using the hot borate extraction method (Wan and Wilkins, 1994). The RNA extract was treated with DNase I (TaKaRa Bio, Shiga, Japan) to remove contaminating gDNA. cDNA was synthesized from the DNase-treated total RNA (1 µg) using ReverTra-Ace (Toyobo, Osaka, Japan).

To isolate the *F3'H* gene from the purple radish 'Karaine-aka', PCR amplification was carried out using root skin cDNA and with the following primers: forward, 5'-ATGACTAATCTTTACCTCACAATCC-3' and reverse, 5'-TTAAGCCGACCCGAGTCCGTAAGCACTC-3'; these primers were designed to amplify the sequence from the start codon to stop codon in *Brassica napus F3'H* (DQ324379). The PCR mixture (10 µL) contained 1X Ex-Taq buffer, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U Ex-Taq (TaKaRa Bio), and 2.5 ng cDNA template. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min 30 s; and final extension at 72°C for 3 min. The amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, MI, USA) and *E. coli* HST08 Premium Competent Cells (TaKaRa Bio); they were sequenced using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130 genetic analyzer (Applied Biosystems), following plasmid DNA extraction by a FastGene Plasmid Mini kit (Nippon Genetics Co., Tokyo, Japan). The nucleotide sequences and deduced amino acid sequences of the isolated radish *F3'H* mRNA were aligned by the BLASTx function in Genetyx ver. 11 (Software Development Co., Tokyo, Japan).

To confirm the sequence in the reverse primer region of the isolated *F3'H* gene, rapid amplification of cDNA ends (RACE) PCR was performed using the cDNA template produced from the root skin RNA with the 3'-Full RACE Core Set (TaKaRa Bio). To obtain forward primer region sequence information, the 5' upstream region of *F3'H* was identified by inverse PCR (Ochman et al., 1988) in 'Karaine-aka' gDNA extracted from leaves using a modified CTAB method (Kobayashi et al., 1998). We used the restriction enzyme *Eco*RI to digest 500 ng gDNA and the digested fragments were self-ligated with T4 ligase (Promega). Inverse PCR was performed in 10 µL reaction mixtures containing 1 µL self-ligated gDNA, 1X buffer, 200 µM dNTPs, 0.25 U Ex-Taq (TaKaRa Bio), and the following primers: first PCR, forward 5'-TTCTAACGCTATAGCTCACC-3' and reverse 5'-CTGTCTGAACATGTTTAAATCTTC-

3'; second PCR, forward 5'-GTATATTGAGTTATTCCG AATTA-3' and reverse 5'-ATCGTGAACCTTCAAGA ACT-3'. The reaction conditions of the nested PCR were as follows: preheating at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (1st PCR)/51°C (2nd PCR) for 30 s, and extension at 72°C for 3 min; and final extension at 72°C for 5 min. The amplified fragments were sequenced as described above.

To isolate the complete *F3'H* sequence from 'Karaine-aka', the amplifications were carried out with root skin cDNA and the following primers: forward 5'-ATGACTAATCTCTTCCTCACAATCC-3' and reverse 5'-GTTTAAACAGACCCCAAGCCC-3'. These primers were designed to amplify the sequence from the start codon to the stop codon. All amplified fragments were sequenced as described above.

Expression analysis of radish anthocyanin biosynthesis genes

RACE PCR was used to isolate the anthocyanin biosynthesis genes *CHS* (AF031922), *F3'H* (AB087211), *DFR* (KF280272), and *ANS* (KR262954) from the purple radish 'Karaine-aka' using a cDNA template produced from root skin RNA with the 3'-Full RACE Core Set (TaKaRa Bio).

Semi quantitative reverse transcription-PCR (semi qRT-PCR) and reverse transcription-quantitative PCR (RT-qPCR) were carried out to determine the relative expression levels of the anthocyanin biosynthesis genes in root skin and flesh from all varieties. In the semi qRT-PCR assay, each 20 µL reaction mixture contained 1X buffer, 200 µM dNTPs, 0.2 µM of each primer for radish *CHS* (DDBJ accession number LC202030, forward 5'-TCCAAGCGGAGTATCCTGACTAC-3' and reverse 5'-GCACATGTTAGGGTTCTCTTTCAA-3'), *F3'H* (DDBJ accession number LC202031, forward 5'-TGATCTAACCCTCG GACTCA-3' and reverse 5'-TCTGGAACGTGGCTATTGAT-3'), *F3'H* (forward 5'-GCCGAACAGTTCTTGAAAGT-3' and reverse 5'-CTGTCTGAACATGTTTAAATCTTC-3'), *DFR* (DDBJ accession number LC202032; forward 5'-ACCGGATGGATGTATTTCATGTGC-3' and reverse 5'-ATGATGGAGTAATGTGCCTCGTT-3'), and *ANS* (DDBJ accession number LC202033; forward 5'-GAGCCTGACCGTCTAGAGAAAGA-3' and reverse 5'-CAAACCAGGAACCATGTTGTGTA-3'), 0.5 U Ex-Taq (TaKaRa Bio), and 1 µL 40X diluted cDNA templates. The PCR conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C (*F3'H*), 60°C (radish *CHS*, *F3'H*, *DFR*, and *ANS*), and 61°C (*ACTIN*) for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 5 min. The amplified PCR products were separated by electrophoresis on a 1% agarose gel (Nippon Genetics Co.) in 0.5X TBE buffer at 100 V for 30 min. For semi qRT-PCR, the amplified products were nor-

malized against radish *ACTIN* (FY430005; forward 5'-CGATGGTGAGGACATTCAAC-3' and reverse 5'-TCACCAGAGTCGAGCACAATA-3').

Expression of *F3'H* in root skin and flesh was assayed by RT-qPCR assay using a 20 µL reaction mix containing 1 µL of 10X diluted cDNA templates, 10 µL of SYBR Premix Ex-Taq II (TaKaRa Bio), and 0.25 µM of 26S ribosomal RNA (26S) primer (Park et al., 2011; forward 5'-AACACCCTTTGTGGGTCTAGGT-3' and reverse 5'-GCCCTCGACCTATTCTCAAACCT-3') and the above *F3'H* primers. Each RT-qPCR assay was conducted using a Thermal Cycler Dice Real-Time System (TaKaRa Bio) with an initial denaturation of 30 s at 95°C, followed by 50 cycles of denaturation for 5 s at 95°C, annealing for 10 s at 58°C (*F3'H*)/56°C (26S), and elongation for 20 s at 72°C. Three technical replicates were analyzed for each cDNA sample. Quantitation was performed by using the difference in the threshold cycle values (ΔC_t) between the two samples (the target gene and the reference gene) to calculate the relative amounts of the template present. The value of the relative expression was calculated as the standard using the maximum value for the *F3'H* gene, and the mean and standard error (SE) were calculated from the value of the relative expression (Cheon et al., 2011).

Isolation of the radish *F3'H* homolog and insertion sequence from red radish gDNA

The radish *F3'H* gene in 'Karaine-aka' gDNA was isolated by PCR amplification of the region between the 5' upstream to 3' UTR, and was performed in 20 µL reaction mixtures containing 20 ng gDNA, 1X buffer, 200 µM dNTPs, 0.5 U PrimeSTAR GXL polymerase (TaKaRa Bio), and 0.2 µM of each primer. The primers were designed using the 5' upstream and 3' UTR sequences (forward 5'-CTAACCGAGATATGCATGGT-3' and reverse 5'-ATTACACAAACATCACAAGGC-3'). The conditions for PCR amplification were as follows: preheating at 98°C for 5 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 57°C for 5 s, and extension at 68°C for 3 min; and final extension at 72°C for 10 min. A-overhangs were created on the amplified fragments (0.25 U Ex-Taq at 72°C for 30 min) in order to perform TA-cloning. The treated fragments were cloned into *E. coli* HST08 Premium Competent Cells (TaKaRa Bio) with the pGEM-T easy vector

(Promega); plasmid extraction and sequencing analysis were performed as described above.

The *F3'H* gene of purple, red, and white root radishes was analyzed by PCR amplification of gDNA sequences from the root skin and flesh. The PCR was performed in 20 µL reaction mixtures containing 20 ng gDNA, 1X Ex-Taq buffer, 200 µM dNTPs, 0.25 U Ex-Taq (TaKaRa Bio), and 0.2 µM of each primer. Four primers were used: P1 (5'-CTAACCGAGATATGCATGGT-3'), P2 (5'-CTGTCTGAACATGTTTAAAACTCTTC-3'), P3 (5'-GAAGAGGTTGGAACACTCATG-3'), and P4 (5'-GGGCTTGGGTCTGTTTAAAC-3') (Fig. 5). The conditions for the PCR amplifications were as follows: preheating at 94°C for 1 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C (P1 and P2)/57°C (P3 and P4) for 30 s, and extension at 72°C for 5 min; and final extension at 72°C for 10 min. The amplified PCR products were confirmed by electrophoresis on 1% agarose gel (Nippon Genetics Co.) in 0.5X TBE buffer at 100 V for 30 min. Subsequently, the fragments amplified using P1 and P2 primers were cloned using a TOPO-XL PCR cloning kit (Invitrogen, Carlsbad, CA, USA) and *E. coli* HST08 Premium Competent Cells (TaKaRa Bio), and the clones were sequenced as described above. The obtained sequences from 'Chouan-aomaru-koshin' and 'Benikururi 521' were compared with sequence information of purple colored 'Karaine-aka' using Genetyx ver. 11 (Software Development Co.).

Results

HPLC analysis of anthocyanidins

The HPLC analysis of purple, red, and white radishes identified cyanidin in both skin and flesh of the purple radish 'Karaine-aka'. Pelargonidin was detected in both the skin and flesh of the red radish 'Chouan-aomaru-koshin' and 'Benikururi 521'. However, cyanidin derived from the flesh in 'Karaine-aka' and pelargonidin derived from the skin in 'Chouan-aomaru-koshin' was at trace amounts compared with other colored tissues, although the precise anthocyanidin contents in each sample were not checked in this analysis method. However, neither of these anthocyanidins was detected in the white radishes 'Susanoo' or 'Taibyo-soubutori' (Table 1).

Table 1. Anthocyanidin in the skin and flesh of purple, red, and white radishes.

Anthocyanidins	Purple ^z		Red ^z				White ^z			
	'Karaine-aka'		'Chouan-aomaru-koshin'		'Benikururi 521'		'Susanoo'		'Taibyo-soubutori'	
	Skin	Flesh	Skin	Flesh	Skin	Flesh	Skin	Flesh	Skin	Flesh
Cyanidin	+ ^y	+	— ^x	—	—	—	—	—	—	—
Pelargonidin	—	—	+	+	+	+	—	—	—	—

^z Root color.

^y detected.

^x not detected.

Isolation of the *F3'H* homolog from mRNA of the purple root radish 'Karaine-aka'

Two radish *F3'H* sequences were isolated from the purple radish 'Karaine-aka' and termed *RsF3'H-a*

(approximately 1536 bp, DDBJ accession number LC202035), and *RsF3'H-b* (approximately 1536 bp, DDBJ accession number LC202037) (Fig. 3A). The two gene sequences differed at one position only, with

A

	M		
F3'H-a	1	ATGACTAATCTCTCTCCACAACTCTCTCCCTACTTTCATCTCTCTTATTGTC	120
F3'H-b	1	ATGACTAATCTCTCTCCACAACTCTCTCCCTACTTTCATCTCTCTTATTGTC	120
F3'H-a	121	AACCTCCCTCAGATGGGCGCTAAACCCATCAAACTAGCGCCATGGTAACACATACGGCCCTATCTCCACCTACGGCTAGGTTTCGCGCAGCTTGTGGTTGCCGCTCTAAATCC	240
F3'H-b	121	AACCTCCCTCAGATGGGCGCTAAACCCATCAAACTAGCGCCATGGTAACACATACGGCCCTATCTCCACCTACGGCTAGGTTTCGCGCAGCTTGTGGTTGCCGCTCTAAATCC	240
F3'H-a	241	GTGGCGAACAGTTCTTGAAGTTTACAGATGCAATTTTGTAGCGACCACTAACTCCGAGGCAACACATGGCATAACAATTATCAAGATCTTGTCTTTGCCCTTATGACAACGA	360
F3'H-b	241	GTGGCGAACAGTTCTTGAAGTTTACAGATGCAATTTTGTAGCGACCACTAACTCCGAGGCAACACATGGCATAACAATTATCAAGATCTTGTCTTTGCCCTTATGACAACGA	360
F3'H-a	361	TGGAGATGTTGAGGAAGATTAGTCTCTTCAATTATTTTTCAGCTAAAGCTCTGGAAGATTTTAAACATGTTTCGACAGGAAGAGTTTGAACACTATGCGCGAGCTAGCGCTGCAAAC	480
F3'H-b	361	TGGAGATGTTGAGGAAGATTAGTCTCTTCAATTATTTTTCAGCTAAAGCTCTGGAAGATTTTAAACATGTTTCGACAGGAAGAGTTTGAACACTATGCGCGAGCTAGCGCTGCAAAC	480
F3'H-a	481	ACGAACCACTGAACCTAGGCGAGTTGGTGAACATGTGCGTACTCAACGCCCTTGGACGAGAGATGATCGGACGGCGGTGTTTCGCGCGGAGCGGATCACAAGCGGAGGAGTTTCGA	600
F3'H-b	481	ACGAACCACTGAACCTAGGCGAGTTGGTGAACATGTGCGTACTCAACGCCCTTGGACGAGAGATGATCGGACGGCGGTGTTTCGCGCGGAGCGGATCACAAGCGGAGGAGTTTCGA	600
F3'H-a	601	TCAATGGTCACAGAAATGATGGCTCTCGCGGAGTGTTCACATCGGAGATTTCGTGCCCGCACTTGAATGTTTACAGCTTACAAGCGTCTGCTGTTAAATGAAAGCTCTCCACAAGAGG	720
F3'H-b	601	TCAATGGTCACAGAAATGATGGCTCTCGCGGAGTGTTCACATCGGAGATTTCGTGCCCGCACTTGAATGTTTACAGCTTACAAGCGTCTGCTGTTAAATGAAAGCTCTCCACAAGAGG	720
F3'H-a	721	TTCGACGCTTTTCTATCTGCTGATTTTGAAGAGCAGAGATGATGAAGAACGCTCAGGATCAAAAGCACACGAGATGCTTAGCATTAAATCTCGCTTAAGGGGACTGATTTTACGGT	840
F3'H-b	721	TTCGACGCTTTTCTATCTGCTGATTTTGAAGAGCAGAGATGATGAAGAACGCTCAGGATCAAAAGCACACGAGATGCTTAGCATTAAATCTCGCTTAAGGGGACTGATTTTACGGT	840
F3'H-a	841	GACGGTGAACACTAACGGATCTAGATCAAGCCCTTGTATTGAACATGTTTACTGCTGGAACGACACGTCAGCAAGTACGGTGGACTGGGCCATAGCTGAACATGATCCGTCACCCG	960
F3'H-b	841	GACGGTGAACACTAACGGATCTAGATCAAGCCCTTGTATTGAACATGTTTACTGCTGGAACGACACGTCAGCAAGTACGGTGGACTGGGCCATAGCTGAACATGATCCGTCACCCG	960
F3'H-a	961	GAGATAATGACAAAAGCCCAAGAGCTTGAATCCGTCGTCGGACGTGAAGGCCCATTAACGAGTCAGACCTCTCTACGCTTCTTACCTTCAGGCGGTATCAAGAGAAATTCAGA	1080
F3'H-b	961	GAGATAATGACAAAAGCCCAAGAGCTTGAATCCGTCGTCGGACGTGAAGGCCCATTAACGAGTCAGACCTCTCTACGCTTCTTACCTTCAGGCGGTATCAAGAGAAATTCAGA	1080
F3'H-a	1081	CTCCATCCCAACACCACTCTCGTTACACACATCGCATCAGAGAGCTGTGAGATCAACGGCTATCATATCCCGAAGGATCAACCCCTTTTACAAACATATGGGCCATAGCCCGTGAC	1200
F3'H-b	1081	CTCCATCCCAACACCACTCTCGTTACACACATCGCATCAGAGAGCTGTGAGATCAACGGCTATCATATCCCGAAGGATCAACCCCTTTTACAAACATATGGGCCATAGCCCGTGAC	1200
F3'H-a	1201	CCGGACCAATGACCGACCCCTTATTGTTTTCGACCCGAGAGATCTTACCCGGTGGTGAAGAACCGCGCTCGATGTGAAGGAAACGACTTCGAGCTTATACCGTTCCGAGCAGGAGG	1320
F3'H-b	1201	CCGGACCAATGACCGACCCCTTATTGTTTTCGACCCGAGAGATCTTACCCGGTGGTGAAGAACCGCGCTCGATGTGAAGGAAACGACTTCGAGCTTATACCGTTCCGAGCAGGAGG	1320
F3'H-a	1321	AGAATCTGCGCGGGCTGAGTTTAGGGTTACGACGATTACGTTTACGCGGACGCTTGTGACGAGATTGAATGGGAACGCGCGGAGGAATACGCGGAGAGCTGAACATGGAG	1440
F3'H-b	1321	AGAATCTGCGCGGGCTGAGTTTAGGGTTACGACGATTACGTTTACGCGGACGCTTGTGACGAGATTGAATGGGAACGCGCGGAGGAATACGCGGAGAGCTGAACATGGAG	1440
F3'H-a	1441	GAGACTTATGGGATCACTCTGCAAAAGAGCGGTTCCTTGGTTGTTTCATCTTAAGCCGAGGTTGGATATGAGTGCTTACGGGCTTGGGTCTGTTTAA	1536
F3'H-b	1441	GAGACTTATGGGATCACTCTGCAAAAGAGCGGTTCCTTGGTTGTTTCATCTTAAGCCGAGGTTGGATATGAGTGCTTACGGGCTTGGGTCTGTTTAA	1536

B

AtF3'H	1	MTNLELTILLATVFLILRIFSHRRNRSHNNRI	PPGPNPWF	IIGNLPHMGT	KPHRTLSAMVTTYGPILHLRLGFE	VVVAASKSVABQFL	90
BrF3'H	1	MTNLELTILLATVFLILRIFSHRRNRSHNNRI	PPGPNPWF	IIGNLPHMGT	KPHRTLSAMVTTYGPILHLRLGFE	VVVAASKSVABQFL	86
RsF3'H-a	1	MTNLELTILLATVFLILRIFSHRRNRSHNNRI	PPGPNPWF	IIGNLPHMGT	KPHRTLSAMVTTYGPILHLRLGFE	VVVAASKSVABQFL	86
RsF3'H-b	1	MTNLELTILLATVFLILRIFSHRRNRSHNNRI	PPGPNPWF	IIGNLPHMGT	KPHRTLSAMVTTYGPILHLRLGFE	VVVAASKSVABQFL	86
AtF3'H	91	KIHDANFASRPNSGAKHMAINYQDLVFAPYQHRWMLRKISSVHL	FSAKALED	FKHVRQEEVGT	IRELVRVGEKFPVNLGQLVNMVNVN		180
BrF3'H	87	KVHDANFASRPNSGAKHMAINYQDLVFAPYQHRWMLRKISSVHL	FSAKALED	FKHVRQEEVGT	IRELVRVGEKFPVNLGQLVNMVNVN		176
RsF3'H-a	87	KVHDANFASRPNSGAKHMAINYQDLVFAPYQHRWMLRKISSVHL	FSAKALED	FKHVRQEEVGT	IRELVRVGEKFPVNLGQLVNMVNVN		176
RsF3'H-b	87	KVHDANFASRPNSGAKHMAINYQDLVFAPYQHRWMLRKISSVHL	FSAKALED	FKHVRQEEVGT	IRELVRVGEKFPVNLGQLVNMVNVN		176
AtF3'H	181	ALGREGIMGRRLFGADADHKAEEFRSMVTEMMALAGVFNIGDFVPSLD	DLDLQGVAGKMKRLHKKRFDALFSSILKEHE	--MNGQDQKHDTM			268
BrF3'H	177	ALGREGIMGRRLFGADADHKAEEFRSMVTEMMALAGVFNIGDFVPSLD	DLDLQGVAGKMKRLHKKRFDALFSSILKEHE	--MNGQDQKHDTM			266
RsF3'H-a	177	ALGREGIMGRRLFGADADHKAEEFRSMVTEMMALAGVFNIGDFVPSLD	DLDLQGVAGKMKRLHKKRFDALFSSILKEHE	--MNGQDQKHDTM			266
RsF3'H-b	177	ALGREGIMGRRLFGADADHKAEEFRSMVTEMMALAGVFNIGDFVPSLD	DLDLQGVAGKMKRLHKKRFDALFSSILKEHE	--MNGQDQKHDTM			266
AtF3'H	269	LSTLISLKGTDLDGGGSLTDTEIKALLNMFT	AGTDTST	ASTVDWAIAELIRHPEIMTKAQ	QELDSVVGGRCPINESDLSQLPYLQAVIK		358
BrF3'H	267	LSTLISLKGTDLDGGGSLTDTEIKALLNMFT	AGTDTST	ASTVDWAIAELIRHPEIMTKAQ	QELDSVVGGRCPINESDLSQLPYLQAVIK		356
RsF3'H-a	267	LSTLISLKGTDLDGGGSLTDTEIKALLNMFT	AGTDTST	ASTVDWAIAELIRHPEIMTKAQ	QELDSVVGGRCPINESDLSQLPYLQAVIK		356
RsF3'H-b	267	LSTLISLKGTDLDGGGSLTDTEIKALLNMFT	AGTDTST	ASTVDWAIAELIRHPEIMTKAQ	QELDSVVGGRCPINESDLSQLPYLQAVIK		356
AtF3'H	359	ENFRSHPTPLSLPHIASCEINGYHIPKGSTLLTNIWAIA	RDPDQWSDPLIFRPERFLPGGEKSGVDVKGSDFELIE	FGAGRRICAG			448
BrF3'H	357	ENFRSHPTPLSLPHIASCEINGYHIPKGSTLLTNIWAIA	RDPDQWSDPLIFRPERFLPGGEKSGVDVKGSDFELIE	FGAGRRICAG			446
RsF3'H-a	357	ENFRSHPTPLSLPHIASCEINGYHIPKGSTLLTNIWAIA	RDPDQWSDPLIFRPERFLPGGEKSGVDVKGSDFELIE	FGAGRRICAG			446
RsF3'H-b	357	ENFRSHPTPLSLPHIASCEINGYHIPKGSTLLTNIWAIA	RDPDQWSDPLIFRPERFLPGGEKSGVDVKGSDFELIE	FGAGRRICAG			446
AtF3'H	449	SLGLRTIQELTATLVGGEFELAGGV	TPEKLNMEESTYGLTLQRAVPLVVHKKPR	LDMSAYGLGSA			513
BrF3'H	447	SLGLRTIQELTATLVGGEFELAGGV	TPEKLNMEESTYGLTLQRAVPLVVHKKPR	LDMSAYGLGSA			511
RsF3'H-a	447	SLGLRTIQELTATLVGGEFELAGGV	TPEKLNMEESTYGLTLQRAVPLVVHKKPR	LDMSAYGLGSA			511
RsF3'H-b	447	SLGLRTIQELTATLVGGEFELAGGV	TPEKLNMEESTYGLTLQRAVPLVVHKKPR	LDMSAYGLGSA			511

Fig. 3. Isolation of *RsF3'H* genes from 'Karaine-aka'. A: Comparison of *RsF3'H-a* and *RsF3'H-b* mRNA sequences from the start codon to stop codon. The symbol M and asterisk indicate the start and stop codon, respectively. A black box indicates different sequences in *RsF3'H-a* and *RsF3'H-b* sequences, respectively. Close triangles indicate insertion of retrotransposon and its long terminal region, and open triangles indicate insertion of a poly (T)₂₂ sequence. Horizontal and vertical arrows indicate the primer region for expression analysis and intron regions, respectively. B: Multiple sequence alignment of *Arabidopsis* F3'H (NP_196416), *Brassica rapa* F3'H (ABY89687), and the deduced amino acid sequences for *RsF3'H-a* and *-b*. Black line boxes are P450 motif PPGPNPWF, AGTDTST, FGAGRRICAG, and E-R-R triad residues, and black dashed-line boxes are the F3'H-specific motifs VVVAAS, GGEK, and VDVKG, respectively.

guanine in *RsF3'H-a* and cytosine in *RsF3'H-b*. A comparison of the predicted amino acids from these two sequences with published F3'H amino acid sequences showed that both *RsF3'H-a* and *RsF3'H-b* showed 97% identity with *Brassica rapa* F3'H (ABY89687) and 90% identity with *Arabidopsis* F3'H (AAF73253). The deduced amino acid sequences of the P450 motif P₃₀PGPNWP₃₇, A₃₀₀GTDT₃₀₅, F₄₃₆GAGRRICAG₄₄₅, and E₃₅₇-R₃₆₀-R₃₉₉ triad residues, and F3'H-specific motifs V₇₃VVAAS₇₈, G₄₁₈GEK₄₂₁, and V₄₂₄DVKG₄₂₈ encoded by *RsF3'H-a* and *RsF3'H-b* were homologous to those in F3'H (Xu et al., 2007) (Fig. 3B).

Analysis of expression of anthocyanin biosynthesis genes

Expressions of *RsCHS*, *RsF3'H*, *RsDFR*, and *RsANS* were analyzed using a semi qRT-PCR assay (Fig. 3). This analysis showed that these genes had the highest expression in the skin of 'Karaime-aka', the flesh of 'Chouan-aomaru-koshin', and both the skin and flesh of 'Benikururi 521'. However, expression of *RsDFR* and *RsANS* was not found in white radishes. *RsF3'H* was mainly expressed in the skin of 'Karaime-aka', whereas *RsF3'H* expression was not found in red radishes, and was lower in white radishes (Fig. 4A). An RT-qPCR assay to confirm the patterns of *RsF3'H* expression in 5 radishes showed that the gene was more highly expressed in 'Karaime-aka' than in other radishes (Fig. 4B).

Analysis of *RsF3'H* genes from red radish gDNA

The *RsF3'H* gene was sequenced by isolating *RsF3'H-a* and *RsF3'H-b* from 'Karaime-aka' gDNA. *RsF3'H-a* and *-b* were approximately 3039 bp and 3017 bp in length, respectively (DDBJ accession numbers LC202034 and LC202036, respectively). Analysis of the structure of the gDNA sequences indicated that three introns were present and that the first intron differed in length between the two genes (Fig. 5A).

The reduced expression of *RsF3'H* in the red radish was investigated by investigating the genomic gene structures. PCR amplification using primers P3 and P4 generated a band of approximately 1 kb in all radishes. The primers P1 and P2 produced a band of approximately 500 bp (I) in 'Karaime-aka', 'Susano', and 'Taibyo-soubutori', two bands of approximately 5 kb (II) and 500 bp (IV) in 'Chouan-aomaru-koshin', and two bands of approximately 5 kb (II) and 800 bp (III) in 'Benikururi 521' (Fig. 5B). Sequencing of the two PCR products from 'Chouan-aomaru-koshin', showed that bands II and IV were 5871 bp (DDBJ accession number LC205688) and 590 bp (DDBJ accession number LC205690) in length, respectively, and those from 'Benikururi 521' were 5871 bp (II) and 889 bp (III, DDBJ accession number LC205689). Comparison of these sequences showed that *RsF3'H* from 'Chouan-aomaru-koshin' had two insertion sequences (5322 bp

Fig. 4. Expression analysis of the *F3'H* gene in the purple root radish 'Karaime-aka' (KA), red root radishes 'Chouan-aomaru-koshin' (CAK) and 'Benikururi 521' (BK), and white root radishes 'Susano' (SU) and 'Taibyo-soubutori' (TS). A: Expression analysis using semi qRT-PCR in root skin (S) and flesh (F). Radish *ACTIN* was used as an internal control. B: Expression analysis of RT-qPCR in skin and flesh. Radish 26S was used as an internal control. The data represent the mean and standard errors obtained from three technical replicates. Different letters indicate significant differences at $P < 0.01$ (Tukey's multiple comparison tests).

and 27 bp) in the first exon, and that *RsF3'H* from 'Benikururi 521' had two insertion sequences (5322 bp and 340 bp) in the first exon. The 5322 bp insertion sequence identified a target site duplication (TSD; ACCAC) and a long terminal repeat (LTR; 340 bp); moreover, the inserted sequences identified a *gypsy-Ty3* retrotransposon that showed 52% identity with *Beta vulgaris* (AFK13856) in a BLASTx search of GenBank. The 340 bp insertion sequence also identified a TSD (ACCAC) and the inserted sequences showed the LTR of a retrotransposon. The 27 bp insertion sequence identified a TSD (GAAGATTTTAAACATGTT) and a poly(T)₂₂ sequence, but the inserted sequences showed no significant homologies in BLASTx and BLASTn searches of GenBank (Fig. 5C).

Discussion

This study was initiated to elucidate the basis of the red root character in the radish. Mutant type pelargonidin pigments accumulated in plants which accumulated cyanidin pigments in the wild type (Beale, 1941). Analyses of anthocyanidin contents and gene expres-

Fig. 5. Genomic structures of *RsF3'H* genes in radishes. A: Genomic structure of *RsF3'H* genes from 'Karaine-aka'. Closed boxes and E1–E4 indicate exons and first exon–fourth exon, respectively, and lines among exons indicate introns. Numbers of closed boxes (up) and lines (down) indicate length of exons and introns, respectively. Horizontal arrows indicate the primer region and direction for PCR analysis, respectively. B: Amplified PCR products of two primer sets (P1+P2; amplify region between 5' upstream region and first exon and P3+P4; amplify region between second exon and 3' UTR). M: 1 kb DNA Ladder (Nippon Genetics). 'Karaine-aka' (KA), 'Chouan-aomaru-koshin' (CAK), and 'Benikururi 521' (BK). 'Susanoo' (SU) and 'Taibyosoubutori' (TS). NC: Negative control. C: Genomic structure of the first exon of *RsF3'H* genes from five cultivars, I: 'Karaine-aka', 'Susanoo', and 'Taibyosoubutori', II and IV: 'Chouan-aomaru-koshin', II and III: 'Benikururi 521'. LTR: long terminal repeats, gypsy-Ty3: a retrotransposon of gypsy-Ty3, Poly (T): Poly (T)₂₂ sequence.

sion were conducted to test the hypotheses that the red root character was caused by accumulation of pelargonidin-based anthocyanin and not of cyanidin-based anthocyanin, and that accumulation of the former was associated with regulation of *RsF3'H* gene expression.

In anthocyanin analyses, Tatsuzawa et al. (2008, 2010) reported that red and purple radishes contain pelargonidin- and cyanidin-based anthocyanins, and Kato et al. (2013) reported that the aglycones of purple and red radishes contain cyanidin and pelargonidin, respectively. Similarly, in the present study, cyanidin and pelargonidin were detected in purple and red radishes, respectively (Table 1), and pelargonidin-based anthocyanin was accumulated in the roots of 'Chouan-

aomaru-koshin' and 'Benikururi 521'.

In addition to the anthocyanidin analysis, when we performed expression analysis of genes related to anthocyanin synthesis, *RsF3'H* expression differed between purple and red radishes (Fig. 4). Tsuda et al. (2004) reported that in red petunias, the downregulation of the endogenous *F3'H* gene and over-expression of a rose-derived *DFR* gene (producing cyanidin) yielded an orange flower containing pelargonidin. Similarly, in *Osteospermum*, *F3'H* suppression by RNAi and the introduction of gerbera *DFR* resulted in pelargonidin accumulation (Seitz et al., 2007). Nakatsuka et al. (2007) also reported that gerbera *DFR* overexpression and *F3'H* and *FLS* suppression in tobacco produced red flowers. In the present study, we found a lower level of *RsF3'H* expression in the flesh of 'Chouan-aomaru-koshin' and both the skin and flesh of 'Benikururi 521' than that of the 'Karaine-aka' root skin (Fig. 4B), suggesting that the *RsF3'H* function could be related to cyanidin-based anthocyanin synthesis.

Our gene sequencing analysis identified a retrotransposon, gypsy-Ty3, in the first exon of the *F3'H* homolog of both 'Chouan-aomaru-koshin' and 'Benikururi 521' (Figs. 5B and C). Moreover, poly(T)₂₂ and a retrotransposon LTR sequence were found in the same exon in 'Chouan-aomaru-koshin' and 'Benikururi 521', respectively (Fig. 5C). In *Ipomoea purpurea*, a pink flower is caused by a mutation (a large insertion in the third exon) of the *IpF3'H* gene (Zufall and Rausher, 2003). In *Dianthus caryophyllus*, a change in flower color from purple to deep pink was attributed to an active *hAT* type transposable element, *Tdic101*, which was inserted into the second intron of *F3'H* (Momose et al., 2013). From this, a retrotransposon inserted in the *RsF3'H* gene indicated that *F3'H* gene expression is decreased in red root radishes, and that the pelargonidin-based anthocyanin accumulation of red color in radishes is caused by the mutant *RsF3'H* gene because the red root color is recessive to the purple root color in anthocyanin accumulation in radishes (Hoshi et al., 1963).

The mutant *RsF3'H* gene and anthocyanidin were not found in roots of white radishes (Fig. 5B; Table 1). In a genetic analysis of radishes (*R. sativus* L. var. *sativus*) called 'Hatsuka Daikon' in Japan, white radishes were recessive to colored radishes (such as purple and red colors) and the white color was controlled by a single gene because the ratio between colored and white radishes was 3:1 in the F₂ population (Tatebe, 1938). Park et al. (2011) showed that the genes *RsDFR* and *RsANS* of the anthocyanin biosynthesis pathway were expressed more highly in the red tissues of red Chinese radishes, and were not expressed in white radishes or white tissues of red Chinese radishes. In the present study, we confirmed the expression patterns of these two genes (Fig. 4A). Anthocyanin biosynthesis is regulated by a transcription factor in a wide range of plant species (Koes et al., 2005). In radishes, overexpression

of the R2R3-MYB transcription factor gene, *RsMYB1*, actively and positively regulates anthocyanin biosynthesis and promotes anthocyanin production by triggering the expression of endogenous basic helix-loop-helix (*bHLH*) genes as potential binding partners for *RsMYB1* (Lim et al., 2016). It is possible that non-anthocyanin accumulation of white radishes was caused by mutations of *DFR*, *ANS*, or *MYB*. Thus, more experiments are needed to clarify the reason for non-anthocyanin accumulation.

In conclusion, we showed that two late genes that are active in the anthocyanin biosynthesis pathway were expressed in purple and red radishes, and that the red root character of radishes is caused by a lack of F3'H function, which is a retrotransposon insertion in the mutant *RsF3'H* gene. In a future study, we will analyze the mode of inheritance of the mutant *RsF3'H*. Moreover, to enable selection of the red character for efficient radish breeding, we will develop a gene-based marker using the *RsF3'H* mutant allele.

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