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Molecular cloning and functional characterization of the flavonoid 3′-hydroxylase gene from *Rubus coreanus* Miquel

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Summary

Rubus coreanus Miquel is a Korean black raspberry used in folk medicine and functional foods. To investigate the biosynthesis pathway of anthocyanin in *R. coreanus* Miquel, the complete coding sequence of flavonoid 3′-hydroxylase (F3′H), designated as RcMF3′H1, was cloned for the first time using the Korean black raspberry transcriptome library. The deduced amino acid sequence of RcMF3′H1 contained the proline-rich "hinge" region, P450 consensus heme-binding domain, and F3′H-specific motifs. Phylogenetic analysis revealed that RcMF3′H1 was clustered into the same subgroup as other plant F3′Hs. In addition, expression analysis by quantitative real-time PCR revealed the involvement of RcMF3′H1 in methyl jasmonate-mediated anthocyanin biosynthesis. Furthermore, the ability of the *RcMF3′H1* gene to complement the *Arabidopsis transparent testa 7-1* mutant suggested that *RcMF3′H1* encodes the functional F3′H enzyme involved in anthocyanin biosynthesis. Taken together, the cloning and molecular characterization of RcMF3′H1 will facilitate a better insight into the anthocyanin biosynthesis pathway in *R. coreanus* Miquel.

Introduction

Flavonoids, widely distributed in the plant kingdom, are ubiquitous secondary metabolites with a C6-C3-C6 general structural backbone (Santos-Buelga and Feliciano, 2017). In plants, flavonoids have several physiological functions including protection against ultraviolet (UV) radiation and phytopathogens, pollen fertility, auxin transport regulation, and pigmentation (Falcone Ferreyra et al., 2012). The interest in these compounds has considerably increased because of their potential applications in nutraceutical, pharmaceutical, medicinal, and cosmetic products (Panche et al., 2016). Although over 9,000 known flavonoid compounds have been identified in various plants (Buer et al., 2010), all flavonoid compounds are derived from the general phenylpropanoid pathway, which converts phenylalanine to 4-coumaroyl-CoA though three different enzymatic reactions (Falcone Ferreyra et al., 2012). Most enzymes in the flavonoid biosynthesis pathway are divided into three classes of enzymes according to their cofactors: oxoglutarate-dependent dioxygenases (flavanone 3-hydroxylase, flavonol synthase, flavone synthase I, leucoanthocyanidin dioxygenase), NADPH-dependent reductases (dihydroflavonol 4-reductase, leucoanthocyanidin reductase, vestitone reductase, and isoflavone reductase), and cytochrome P450 hydroxylases (flavonoid 3′-hydroxylase (F3′H), flavonoid 3′5′-hydroxylase (F3′5′H), flavone synthase II (FSII), etc.) (Winkel-Shirley, 2001).

In the flavonoid biosynthesis pathway, F3′H is an important enzyme for controlling the hydroxylation of naringenin, dihydrokaempferol, kaempferol, and apigenin at the 3′ position in the B-ring to generate eriodictyol, dihydroquercetin, quercetin, and luteolin, respectively (Fig. 1), which are important intermediates for anthocyanin and proanthocyanidin biosynthesis (Xu et al., 2007). In Arabidopsis, transparent testa 7 (*tt7*) mutants (loss-of-function mutation of F3′H) exhibit a yellow seed coat color compared with wild-type seeds with a dark brown seed coat, and there is a lower content level of anthocyanin in the whole plant body of mutant plants compared with wild-type plants (Schoenbohm et al., 2000). In addition, the expression of F3′H has been found to be associated with the accumulation of cyanidin-based anthocyanins in the berry skin of grapevines (Castellarin et al., 2006), suggesting that F3′H is important in determining seed coat pigments and accumulating anthocyanin pigments.

The Korean black raspberry, the fruit of *Rubus coreanus* Miquel, has traditionally been used as medicine for the treatment of spermatorrhea, enuresis, and asthma in Korea (Lim et al., 2012). In addition, increasing evidence demonstrates that Korean black raspberry extracts have beneficial health effects including antioxidant (Ju et al., 2009), anti-inflammatory (Lim et al., 2012), and anticancer activities (Jeon et al., 2007), suggesting that the bioactivities of Korean black raspberry are possibly linked to the presence of flavonoids such as

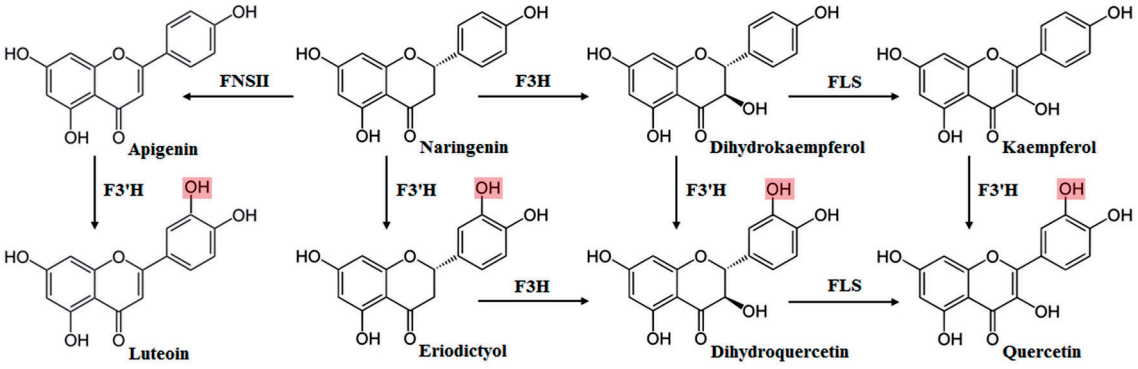

Fig. 1: Schematic diagram of reactions catalyzed by F3′H.

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anthocyanins and catechins (Kim et al., 2011; Yoon et al., 2003). In addition, cinnamate-4-hydroxylase (Baek et al., 2008a) and flavanone-3-hydroxylase (Baek et al., 2008b), which are involved in the flavonoid biosynthesis, were cloned and characterized from Korean black raspberry. Recently, a study has suggested that the increased expression of *R. coreanus* Miquel *F3'H1* (*RcMF3'H1*) may affect the accumulation of cyanidin-based anthocyanins during the ripening process of Korean black raspberry (Hyun et al., 2014); however, further functional characterization of RcMF3′H1 is necessary. Therefore, in this study, we isolated the complete coding sequence of RcMF3′H1 from the Korean black raspberry transcriptome library. To elucidate the RcMF3′H1 biochemical function in plants, we investigated the expression pattern of *RcMF3'H1* in response to stress-related stimuli, and RcMF3′H1 was transformed into the *Arabidopsis transparent testa 7-1* (*tt7-1*) mutant for phenotype-complementation analysis.

Material and methods

Plant materials and stimuli

R. coreanus Miquel plants were cultivated in the Gochang Black Raspberry Research Institute, Republic of Korea. For RNA isolation, dark red fruits were collected from five individual plants. All *Arabidopsis thaliana* wild-type and mutant (*tt7-1*, EMS mutant) plants were in the Landsberg erecta ecotype and grown in environmentally controlled growth chambers with a 16-h light (22 °C)/8-h dark (20 °C) photoperiod.

A comparison of *RcMF3'H1* expression in the presence or absence of stress-related stimuli was performed with the leaf disks of *R. coreanus* Miquel. To avoid non-specific gene expression induced by leaf injury, the leaf discs (5 mm in diameter) were floated on distilled water in 6-well plates. After pre-incubation for 24 h, the leaf disks were treated with 500 mM mannitol, 100 mM NaCl, 50 μM methyl jasmonic acid (MeJA), and 1 mM salicylic acid (SA). Samples were collected at different time points and frozen in liquid nitrogen.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from stress-treated leaf disks using the modified CTAB method (Gambino et al., 2008). In brief, leaf disks were ground with liquid nitrogen and transferred to RNase-free tubes filled with 600 μl extraction buffer (2% cetyltrimethylammonium bromide, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, and 2% β-mercaptoethanol). After incubation at 65 °C for 5 min, an equal volume of chloroform-isoamyl alcohol (24:1) was added, and the mixture was centrifuged (11,000 *g* for 10 min) at room temperature. The supernatant was again mixed with an equal volume of chloroform-isoamyl alcohol and centrifuged (11,000 *g* for 10 min). Then, the supernatant was transferred to a new tube with 1/3 volume of 8 M LiCl and stored at -20 °C overnight. After centrifugation (11,000 *g*) for 20 min at 4 °C, the pellet was washed with 1 ml of 75% EtOH, and the washed pellet was dissolved in 30 μl DEPC-treated water. To avoid genomic DNA contamination, the total RNA was treated with RNase-free DNase I (Promega, USA). The total RNA from dark red fruits was isolated using the RNEasy Plant Mini kit (Qiagen, USA) according to the manufacturer's instructions.

The total RNA (1 μg) obtained from leaf disks and fruits was used to synthesize the first-strand cDNA using the QuantiTect® Reverse Transcription kit (Qiagen) in accordance with the manufacturer's recommendations.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on the CFX96™ Real-Time System (BIO-RAD) with SYBR® Green Real-Time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan) according to conditions described previously (HYUN et al., 2014). The expression level of *RcMF3'H1* in different samples was normalized to the constitutive expression level of *GAPDH*, and two technical replicates were performed in each biological replicate. Primer sequences are listed in Tab. 1.

Cloning of *RcMF3'H1* and *Arabidopsis* complementation analysis

RcMF3'H1 was identified in a Korean black raspberry transcriptome library (NCBI Sequence Read Archive, SRX347804). To produce a full-length coding sequence (CDS) of *RcMF3'H1*, its coding region was amplified from fruit cDNA using the primer AscI-RcMF3′H-F and AatII-RcMF3′H-Rev (Tab. 1). After gel extraction using Sol-Gent Gel & PCR Purification System (Solgent Co., Ltd., Korea), the PCR product was ligated into the pCR®-Blunt vector (Thermo Fisher Scientific, USA). The full-length CDS of *RcMF3'H1* was subcloned into the AscI and AatII site of the myc-pBA binary vector under the control of the CaMV *35S* promoter. The *RcMF3'H1* overexpression construct was transformed into *Agrobacterium tumefaciens* strain GV3101, and Agrobacterium-mediated stable transformation of the *Arabidopsis tt7-1* mutant was performed by the floral dip method (Zhang et al., 2006). Transformants were selected by BASTA selection and subjected to immunoblot analysis.

Tab. 1: Primer sequences for RcMF3′H cloning and qReal-time PCR analysis.

Primer	Sequences (5′-3′)
AscI-RcMF3′H-F	GGCGCGCCTATGTTTCTCATAG
AatII-RcMF3′H-Rev	GACGTCTGAGATGATGAAGCA
RcMF3′H-F	AAATCGATGGTGGTGGAGAT
RcMF3′H-Rev	TCGAATCTCTTGTGCAGCTT
GAPDH-F	TGCAATGGAGACGGGAACGG
GAPDH-Rev	TTCCCTGTGACCCAATTCCACT

Sequence alignment and phylogenetic analysis

The BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to obtain the homologs of *RcMF3'H1* and predict the amino acid sequence of *RcMF3'H1*. Multiple-sequence alignments of plant F3′Hs were carried out using ClustalW (http://bioinformatics.ubc.ca/resources/tools/clustalx), and phylogenetic analysis was performed using the Phylogeny.fr server (http://www.phylogeny.fr) with the 'one click' mode. Bootstrap branch support values were estimated in PHYML 3.0 with 100 replicates by default.

Immunoblot analysis

Total protein extraction and immunoblot analysis were performed as described previously (Hyun et al., 2014). In brief, 20 μg of the total protein from each transgenic line was separated on 12% SDS-polyacrylamide gels by electrophoresis and transferred to PVDF membranes (Millipore, USA). After the blocking of nonspecific sites with 1× TBST buffer containing 5% nonfat dried milk, the membrane was hybridized with a primary antibody (mouse anti-c-Myc; GenScript Inc., USA). Then, the membranes were washed three times with TBST buffer and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, USA). The detection of bound antibodies was visualized using a chemiluminescence system according to the manufacturer's instructions.

Analysis of anthocyanin accumulation and flavonol staining

Seeds from two independent lines were germinated on MS medium without nitrogen sources (PhytoTechnology Laboratories, USA) as

described by Liu et al. (2010). Anthocyanin accumulation was observed using a stereomicroscope (Olympus, Japan).

For the visualization of flavonols, three-day-old etiolated seedlings were stained for 5 min with staining solution containing 0.25% (w/v) diphenylboric acid 2-aminoethyl ester (DPBA) and 0.02% (v/v) Triton X-100, and the samples were analyzed with an epifluorescence microscope (Olympus, Japan) as described by Stracke et al. (2007).

Results and discussion

Cloning and characterization of the *F3′H* gene from *R. coreanus* Miquel

During the berry ripening process, the differential expression of flavonoid biosynthesis genes including *F3′H* can result in the switch in flavonoid biosynthesis from proanthocyanidins to anthocyanins (Hyun et al., 2014; Jaakola et al., 2002). Among flavonoid biosynthesis genes, *F3′H* is known as a member of the cytochrome P450 superfamily and plays an important role in the production of intermediates for anthocyanin biosynthesis (Winkel-Shirley, 2001). Therefore, F3′H is associated with pigment variation. Since *RcMF3′H1* has been predicted as a dominant gene associated with cyanidin-based anthocyanins in Korean black raspberry, we cloned RcMF3′H1 for functional characterization. *RcMF3′H1* (available from the National Agricultural Biotechnology Information Center (NABIC, http://nabic.rda.go.kr; accession number NU-0111) was found to have an open reading frame of 1551 bp, encoding a protein of 516 amino acids. As shown in Fig. 2, RcMF3′H1 exhibits amino acid sequence identities of 89.5%, 65.9%, and 58.4% with *Fragaria* × *ananassa* F3′H (FaF3′H), *Arabidopsis thaliana* (AtF3′H), and *Oryza sativa* F3′H (OsF3′H), respectively. Multiple sequence alignments of F3′H proteins from these plants revealed that the functional domains are highly conserved. As shown in Fig. 2, the proline-rich "hinge" region may act as a hinge motif necessary for the optimal orientation of the P450 enzyme (Zhou et al., 2016). The asterisk motif ((A/G) GX(D/E)T(T/S)) forms a binding pocket for oxygen molecules required for catalytic activity (Chapple, 1998), and the EXXR motif stabilizes the core structure (Werck-Reichhart and Feyereisen, 2000). The P450 consensus heme-binding domain (FxxGxRxCxG), which is responsible for carbon monoxide-binding ability (Werck-Reichhart and Feyereisen, 2000), was found in RcMF3′H1. In addition, F3′H-specific motifs, VVVAAS (blue box; Boddu et al., 2004) and GGEK (green background box; Brugliera et al., 1999), were also found in RcMF3′H1. However, F3′H-specific VDVKG (yellow background box; Boddu et al., 2004) was present at the V_{429}DIRG$_{433}$ of RcMF3′H1.

Phylogenetic analysis was performed using the amino acid sequence of RcMF3′H1 with other known plant-specific F3′Hs and F3′5′Hs. Based on the phylogenetic tree, F3′H and F3′5′H were separated into two clades (CYP75B and CYP75A, respectively), which were highly supported with 100% bootstrap values. RcMF3′H1 was grouped into the F3′H clade, suggesting that the *RcMF3′H1* gene belongs to the F3′H family (Tab. 2, Fig. 3).

Expression of *RcMF3′H1* in response to stress-related stimuli

In higher plants, anthocyanin accumulation is stimulated by environmental stresses including UV light, pathogen infection, drought, sub- or supra-optimal temperatures, wounding, and nutrient deficiency (Kovinich et al., 2014 and 2015; Olsen et al., 2008; Peng et al., 2008; Zhang et al., 2011). In addition, plant hormones such as cytokinins, gibberellins, brassinosteroids, and MeJA are also regulated by anthocyanin accumulation (Deikman and Hammer, 1995; Loreti et al., 2008; Peng et al., 2011; Shan et al., 2009). Since anthocyanin biosynthesis genes are transcriptionally activated by external stimuli (Lo and Nicholson, 1998), we analyzed the expression patterns of *RcMF3′H1* in response to external stimuli by treatment with mannitol, NaCl, MeJA, and SA. When the leaf disks of *R. coreanus* Miquel were treated with MeJA or mannitol, the expression level of *RcMF3′H1* was increased, whereas NaCl and SA treatments did not induce the transcription of *RcMF3′H1* (Fig. 4). Although *RcMF3′H1* was induced by osmotic stress but not by salt stress (Fig. 4, anthocy-

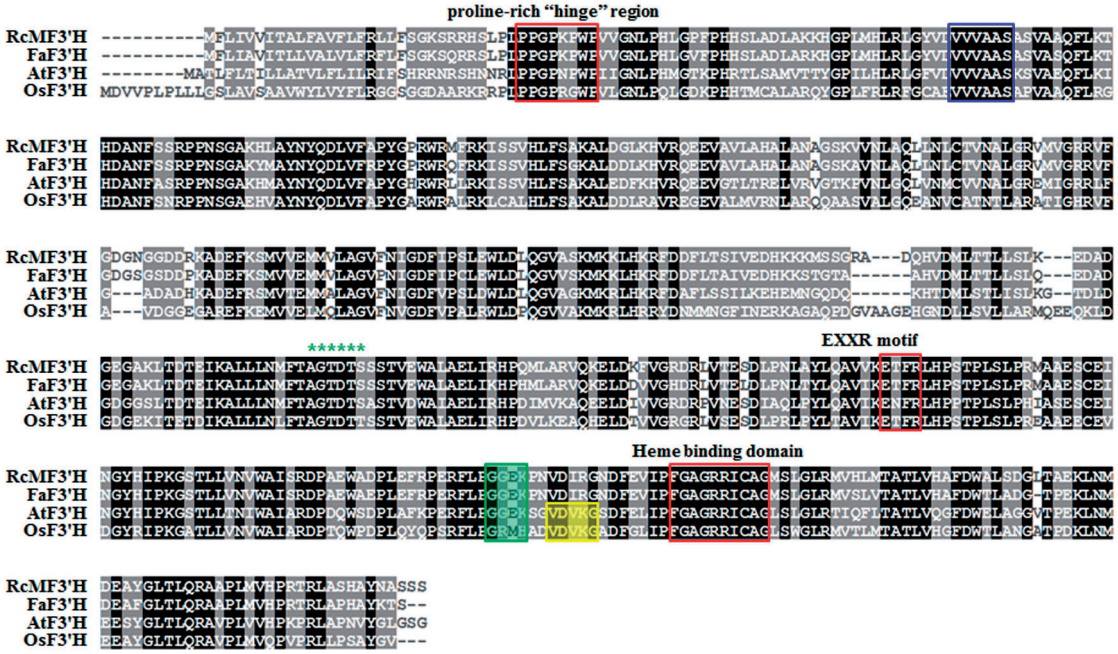

Fig. 2: Multiple sequence alignment of F3′H proteins. Red boxes indicate the proline-rich "hinge" region, EXXR motif, and heme-binding domain. The binding pocket motif for oxygen molecules is labeled with an asterisk. The F3′H-specific motifs "VVVAAS", "GGEK", and "VDVKG" are marked with a blue box, green background box, and yellow background box, respectively. FaF3′H, *Fragaria* × *ananassa* F3′H (BAL63027); AtF3′H, *Arabidopsis thaliana* F3′H (AT5G07990); OsF3′H, *Oryza sativa* F3′H (XP_015613041).

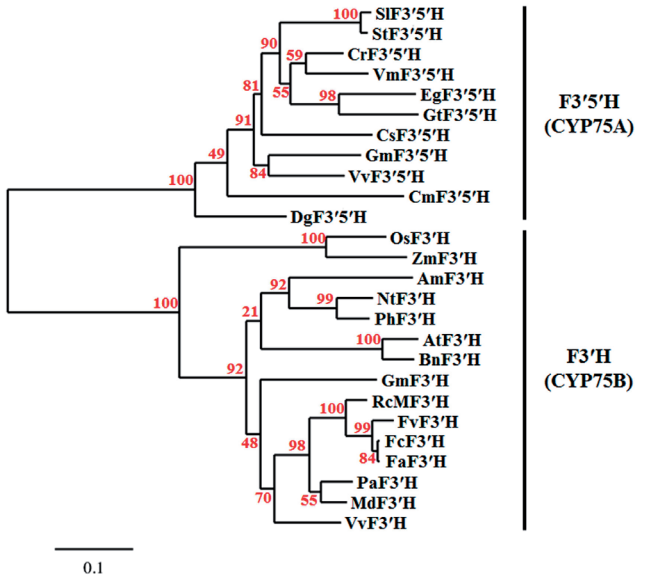

Fig. 3: Phylogenetic analysis of F3′H and F3′5′H.

Tab. 2: List of plant genes used in F3′H and F3′5′H gene phylogenetic analyses.

Gene	Accession Nr.	Species
PaF3′H	ADZ54783	*Prunus avium*
FcF3′H	AEE60888	*Fragaria chiloensis*
FaF3′H	BAL63027	*Fragaria* × *ananassa*
FvF3′H	AEE60887	*Fragaria virginiana*
NtF3′H	AHI58943	*Nicotiana tabacum*
OsF3′H	XP_015613041	*Oryza sativa*
ZmF3′H	AEF33624	*Zea mays*
AtF3′H	AT5G07990	*Arabidopsis thaliana*
AmF3′H	ABB53383	*Antirrhinum majus*
BnF3′H	ABC58723	*Brassica napus*
GmF3′H	ABW69386	*Glycine max*
VvF3′H	ABH06586	*Vitis vinifera*
MdF3′H	ACR14867	*Malus* × *domestica*
PhF3′H	Q9SBQ9	*Petunia* × *hybrida*
CsF3′5′H	ABA40923	*Camellia sinensis*
CmF3′5′H	BAA03440	*Campanula medium*
CrF3′5′H	CAA09850	*Catharanthus roseus*
DgF3′5′H	AAX51796	*Delphinium grandiflorum*
EgF3′5′H	BAA03439	*Eustoma grandiflorum*
GtF3′5′H	BAA12735	*Gentiana triflora*
GmF3′5′H	AAM51564	*Glycine max*
VvF3′5′H	CAI54277	*Vitis vinifera*
SlF3′5′H	ADC80513	*Solanum lycopersicum*
StF3′5′H	AAV85470	*Solanum tuberosum*
VmF3′5′H	BAC97831	*Vinca major*

anin fingerprints were observed to be similar between osmotic (mannitol) and salt (NaCl) stresses (KOVINICH et al., 2014). In addition, three putative RcMF3′H enzymes have been identified in the Korean black raspberry transcriptome library (HYUN et al., 2014), indicating that functional redundancies may exist between *RcMF3′H* genes in response to different stresses.

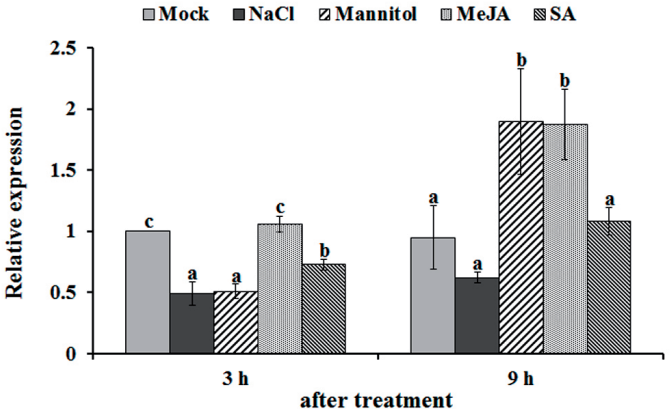

Fig. 4: Expression profile of *RcMF3′H1* genes in response to stress-related stimuli. Transcript levels of *RcMF3′H1* were normalized to the constitutive expression level of *GAPDH* and expressed relative to the values at 0 h. Data represent the mean ± SE of three independent experiments. Different letters correspond to means that are statistically different ($P < 0.05$).

Functional characterization of RcMF3′H1 by *Arabidopsis tt7-1* complementation analysis

The yellow or pale-brown mutants of the seeds of Arabidopsis are known as *transparent testa* (*tt*) mutants, which are a group representing 20 loci (*tt1* to *tt16*, *tt18*, *tt19*, *ttg1*, and *ttg2*) (BHARTI and KHURANA, 2003). These mutants are useful for the characterization of flavonoid structural genes. To functionally characterize RcMF3′H1, the *tt7-1* mutant, which has a mutated *F3′H* gene (SCHOENBOHM et al., 2000), was selected. The *Arabidopsis tt7-1* mutant was transformed with myc-tagged RcMF3′H1 under the control of the 35S promoter, and transgene expression was confirmed by immunoblot analysis with the anti-myc tag antibody (Fig. 5A). As shown in Fig. 5B, seeds of the *tt7-1* mutant exhibited a pale-brown phenotype, whereas seeds collected from two independent transgenic lines showed pigmentation characteristic of wild-type Arabidopsis. As a result, T2 seeds,

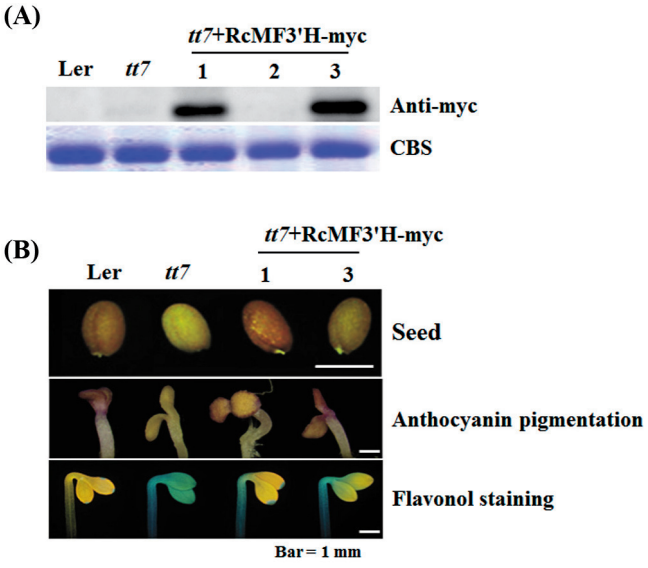

Fig. 5: Complementation analysis of the *RcMF3′H1* gene from the *Arabidopsis tt7-1* mutant. **(A)** Transgenic plants expressing *MYC-RcMF3′H1* were selected by immunoblot analysis using the anti-myc tag antibody. **(B)** Restoration of seed coat color, anthocyanin pigmentation, and flavonol accumulation in transgenic *Arabidopsis tt7-1* mutants expressing *MYC-RcMF3′H1*.

when germinated on MS medium without nitrogen sources, exhibited full complementation of the deficiency in anthocyanin accumulation of *tt7-1* mutant seedlings. The distribution of flavonol in the three-day-old etiolated seedlings of the *tt7-1* mutant, T2 transgenic lines, and wild-type Arabidopsis was visualized with DPBA. The *tt7-1* mutant displayed a greenish fluorescence (kaempferol-specific staining; APPELHAGEN et al., 2014), whereas transgenic plants exhibited an orange fluorescence similar to that of wild-type plants, which indicated the accumulation of flavonols (Fig. 5B). Taken together, our transgenic complementation analysis strongly suggests that *RcMF3′H1* encodes the functional F3′H enzyme.

Conclusions

During the Korean black raspberry repining process, *RcMF3′H1* has been predicted as a dominant gene associated with the accumulation of cyanidin-based anthocyanins (HYUN et al., 2014). Therefore, in this study, *RcMF3′H1* was cloned and characterized in terms of expression and function. The analysis of *RcMF3′H1* gene expression patterns under different stress-related stimuli demonstrated the involvement of RcMF3′H1 in MeJA-mediated anthocyanin biosynthesis. In addition, the complementary activity of RcMF3′H1 indicated that it functions as a F3′H enzyme similar to *tt7*. *RcMF3′H1* is the first reported *F3′H* gene from *R. coreanus* Miquel, and our research will be useful to further understand the anthocyanin biosynthesis pathway in *R. coreanus* Miquel.

Acknowledgments

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