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The Horticulture Journal *Preview*
doi: 10.2503/hortj.UTD-212

The Japanese Society for Horticultural Science
http://www.jshs.jp/

Two Independent Spontaneous Mutations Related to Anthocyanin-less Flower Coloration in *Matthiola incana* Cultivars

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Matthiola incana **is a popular winter-flowering plant, and white is considered a valuable flower color for marketed cultivars. In this study, we aimed to identify the genes responsible for white flower coloration in six commercial cultivars of *M. incana* used as cut and potted flowers. The expression levels of chalcone synthase, flavanone 3-hydroxylase, flavonoid 3'-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase (*ANS*), and anthocyanidin 3-*O*-glucosyltransferase in the petals of 'Kiss me White' and 'Pygmy White' were 0%–48% lower than those in the purple flower 'Vintage Lavender', whereas the expression level of basic helix-loop-helix 2 (*bHLH2*) was two-fold higher. Significantly reduced expression levels of *ANS* were also detected in four other white flower cultivars: 'Vintage White', 'Iron White', 'White Wonder No. 2', and 'Quartet White'. All investigated white flower cultivars had a single nucleotide deletion in the first exon of *ANS*, which we designated as *ans-1*. This generates a frameshift mutation and a nonsense codon. In addition to *ans-1*, 'Kiss me White' and 'Pygmy White' have a 481-bp insertion within *bHLH2*. This insertion has features of *hAT*-type transposable elements and was designated as *dTmi1*. All white flower cultivars contain the *ans-1* mutation, whereas 'Kiss me White' and 'Pygmy White' are double mutants containing both *bhlh2*^{dTmi1} and *ans-1*. 'Kiss me Yellow', which accumulates carotenoids, but not anthocyanins, in its petals possesses the *bhlh2*^{dTmi1} allele, but not the *ans-1* allele. Therefore, either *bhlh2*^{dTmi1} alone or *ans-1* alone can lead to a deficiency in anthocyanin production in commercial cultivars of *M. incana*. We also developed co-dominant DNA markers that can distinguish between wild-type and mutant alleles of both *bHLH2* and *ANS*. In combination with other previously developed markers that can distinguish between single- and double-flowered individuals, these markers will be useful for nursery plant management and breeding of commercial *M. incana*.**

Key Words: anthocyanidin synthase, garden stock, PIRA-PCR, transposable element.

Introduction

Matthiola incana (L.) R. Br., commonly known as garden stock or common stock, is a popular winter-flowering plant in Japan. It is used mainly as cut flowers (standard- and spray-types) and as potted flowers. The flowers of *M. incana* cultivars have a wide range of colors, from pure white to purple, pale purple, blue, red, and pale yellow (Dole and Wilkins, 2005). Anthocyanins, particularly pelargonidin and cyanidin derivatives, are responsible for these various *M. incana* flower colors, while delphinidin derivatives have not been detected in its commercial cultivars (Tatsuzawa et al., 2012). A white flower color is a result of anthocyanin deficiency, and white flower cultivars of *M. incana* accumulate kaempferol derivatives in their petals

Received; May 11, 2020. Accepted; August 13, 2020.
First Published Online in J-STAGE on October 24, 2020.
Part of this paper was presented at the Meeting of the Japanese Society for Horticultural Science held in March 2020.
This work was supported by JSPS KAKENHI Grant Number 17H03765.
No conflicts of interest declared.
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(Tatsuzawa et al., 2014).

M. incana has been subjected to advanced genetic studies since the early 20th century (Saunders, 1928). Genes *C* and *R* have long been recognized as essential for the production of anthocyanins in *M. incana* flowers. Thus, a homozygous state in either *c* or *r* results in a white flower phenotype (Crane and Lawrence, 1947; Saunders, 1928). In the absence of anthocyanin, flowers may exhibit three color forms: white, sulfur-white, and cream, with the latter two deriving their yellowish color from the presence of carotenoid (Crane and Lawrence, 1947). These different shades of white are a result of differences in a single gene, and the genotypes of white, sulfur-white, and cream flower colors correspond to *WW*, *Ww*, and *ww*, respectively (Crane and Lawrence, 1947). *M. incana* has been used as a suitable flower to characterize the flavonoid/anthocyanin biosynthetic pathway, resulting in the establishment of various nearly isogenic lines (Seyffert, 1971), from which three white flower mutant lines, designated as *e*, *f*, and *g*, have been isolated (Hemleben et al., 2004). The *f* mutant is deficient in chalcone synthase (CHS) activity (Hemleben et al., 2004; Spribille and Forkmann, 1981). The white flower and glabrous *e* mutant has a single nucleotide change within the *TRANSPARENT TESTA GLABRA1* (*MiTTG1*) gene, leading to an amino acid substitution located in the WD motif (W158R) (Dressel and Hemleben, 2009). In the white flower *g* mutant, anthocyanin biosynthesis can be activated by two *Arabidopsis* basic helix-loop-helix (*bHLH*) genes: *MYC-146* (*ENHANCER OF GLABRA 3* (*EGL3*)) and *GLABRA 3* (*GL3*) (Ramsay et al., 2003). Thus, it has been speculated that the *g* mutant is deficient in an ortholog of the *bHLH* transcription factor for anthocyanin biosynthesis; however, the *bHLH* genes that are responsible for the white flower coloration of *M. incana* have not yet been identified. In addition, it remains to be determined whether other mutations besides *CHS*, *bHLH*, and *TTG1* are present in commercial *M. incana* white flower cultivars.

The structural genes involved in anthocyanin biosynthesis in *M. incana* have been reported previously (Chen et al., 2018; Nuraini et al., 2020; Seitz et al., 2006). More recently, using the *M. incana* cultivar 'Vintage Lavender', we identified the genes *MiMYB1*, *MibHLH2*, and *MiWDR1* (=*MiTTG1*) as components of the anthocyanin biosynthesis-activator of the MYB-bHLH-WDR (MBW) complex (Nuraini et al., 2020). *MiMYB1*, *MibHLH2*, and *MiWDR1* are the orthologs of *Arabidopsis PRODUCTION OF ANTHOCYANIN PIGMENTS 1* (*PAP1*), *EGL3*, and *TTG1* (Nuraini et al., 2020), respectively. The petal-specific expression of *MiMYB1* correlates well with anthocyanin accumulation in petals, and the ectopic expression of *MiMYB1* by the turnip mosaic virus (TuMV) can induce anthocyanin accumulation in leaves. Moreover, we demonstrated that the other bHLH protein, MibHLH1, which is an ortholog of *Arabidopsis* TRANSPARENT TESTA 8 (TT8), may be an enhancer of anthocyanin biosynthesis in *M. incana* petals (Nuraini et al., 2020).

M. incana may be either single- or double-flowered, and most cultivars are grown double-flowered individuals because this form is more commercially advantageous (Nakatsuka and Koishi, 2018). However, double-flowered *M. incana* do not produce seeds as they lack reproductive organs. Generally, the eversporting, single-flower trait that is associated strongly with pollen sterility is selected for *M. incana* cultivars, resulting in 50%–60% double-flowered individuals (Dole and Wilkins, 2005). Double-flowered seedlings of eversporting can be selected based on distinct leaf characteristics such as serrated leaves, pale yellow leaves, and round and large cotyledons because the double flower trait is linked strongly to the visible traits of the seedlings (Ecker et al., 1993). The seed testa color in *M. incana* is usually blackish, but that in certain white flower cultivars is yellow (Dole and Wilkins, 2005). Among eversporting lines of some cultivars, the double flower trait is strongly linked to yellow seed color. Therefore, by purchasing only yellow seeds, growers can select mostly double flowered individuals, and these cultivars are commonly known as "all-double" (Fujita, 1994).

White is a commercially important flower color, and several studies have investigated the molecular mechanisms that generate white flowers. In the Japanese morning glory (*Ipomoea nil*), a frameshift mutation in *InMYB1* (anthocyanin biosynthetic regulatory gene) produces white flowers with red stems and colored seeds (Morita et al., 2006). White flower cultivars of the Japanese gentian (*Gentiana triflora*) have either a mutation in the anthocyanidin synthase gene (*ANS*) (Nakatsuka et al., 2005b) or a mutation in the transposable element inserted into *GtMYB3* (anthocyanin biosynthetic regulatory gene) (Nakatsuka et al., 2008). In *Eustoma grandiflorum*, a 94-bp deletion in *ANS* leads to white coloration (Shimizu et al., 2011). In the white flower cultivar *Torenia hybrida* 'Crown White', a retrotransposable element is inserted into a 5' region upstream of the flavanone 3-hydroxylase gene (*F3H*), which suppresses *F3H* promoter activity (Nishihara et al., 2014). White-colored *Lilium speciosum* has two independent mutations (Suzuki et al., 2015). The first is an amino acid substitution in the R2 repeat of *LsMYB*, which leads to reduced transcription of the anthocyanin biosynthetic gene and no tepal pigmentation. The second is a nonsense mutation of the dihydroflavonol 4-reductase gene (*DFR*), which results in the absence of anthocyanin accumulation in tepals and anthers (Suzuki et al., 2015).

Previous studies have demonstrated that the white flower trait is associated with double flowers, pollen sterility, glabrous leaves, and seed color (Dressel and Hemleben, 2009; Hemleben et al., 2004; Saunders,

1928). To identify the genes responsible for the white flower coloration in *M. incana* cultivars, we selected six representative white flower *M. incana* cultivars possessing various genetic characteristics in this study (Table 1). 'Iron White' and 'Quartet White' are typically produced as standard- and spray-type cut flower cultivars in Japan, respectively, and they have hairy leaves. 'White Wonder No. 2' is an all-double-type cut flower cultivar with white flowers and yellow seed color loci that are linked strongly to both loci with regard to double-flower and pollen sterility (Fujita, 1994). 'Pygmy White' is a potted flower cultivar that possesses serrate and glabrous leaves (Fujita, 1994). 'Kiss me White' is a derivative of 'Pygmy White', but does not possess the serrated leaf trait. 'Vintage White' is a potted flower cultivar that has hairy leaves. We hypothesized that the genes responsible for anthocyanin-less phenotypes differ among cultivars with hairy leaves, those lacking hairy leaves, and all-double cultivars. Here, we identified the two independent genes responsible for anthocyanin-less coloration in commercial *M. incana* cultivars.

Materials and Methods

Plant materials

We purchased seeds of 'Vintage Lavender' and 'Vintage White' from M&B flora (Hokuto, Japan), and 'Kiss me White', 'Pygmy White', 'Iron White', 'White Wonder No. 2', 'Quartet White', 'Kiss me Blue', 'Kiss me Rose', 'Kiss me Cherry', 'Kiss me Salmon', and 'Kiss me Yellow' from Takii Seed (Kyoto, Japan). All plants were grown in a greenhouse at Shizuoka University (Shizuoka, Japan) under natural conditions from September 2019 to February 2020. The samples were collected from petals at the S3 and S4 stages of flower development as described previously (Nuraini et al., 2020) and from young leaves. After collection, all samples were frozen immediately using liquid nitrogen and stored at −80°C until use.

Flavonoid analysis

Anthocyanin and flavonol analyses were performed as described previously (Nuraini et al., 2020). For anthocyanins, 200 mg of petal samples at stage 4 of flower development were ground and extracted with 40% methanol and 10% acetic acid for 24 h at 4°C. For flavonols, one gram of petal samples collected from each cultivar was ground using liquid nitrogen and extracted with 5 mL of 80% methanol for 24 h at 4°C. To convert flavonoids to aglycone by hydrolysis, 4.5 mL of 3 M hydrochloric acid was added to 500 µL of extracts, and the mixture was boiled for 1 h. Flavonol aglycones were extracted using ethyl acetate, and the resulting extracts were dried and then resuspended with 500 µL of 80% methanol. The anthocyanin and flavonol aglycone extracts were filtered through a 0.22-µm polytetrafluoroethylene syringe filter (Osaka Chemical, Osaka, Japan). Flavonoid analyses were conducted using the Agilent Infinity 1290 High Performance Liquid Chromatography system (Agilent Technologies, Santa Clara, CA, USA) and Agilent 1200 Diode-Array Detector SL (Agilent Technologies), with the reverse-phase column YMC-Pack Pro C18 RS (4.6 × 150 mm, 5 µm; YMC, Kyoto, Japan). Solvent A [1.5% (v/v) phosphoric acid] and solvent B [1.5% (v/v) phosphoric acid, 20% (v/v) acetic acid and 25% (v/v) acetonitrile] were used for elution at 40°C at a flow rate of 0.8 mL·min^{-1}. Elution was performed with 20%–85% of solvent B for 40 min under gradient elution conditions. Anthocyanins and flavonols were monitored based on their absorbance levels at 520 and 360 nm, respectively. Flavonol concentrations were calculated from a standard curve using authentic standards consisting of kaempferol, quercetin, and myricetin (Extrasynthese, Lyon, France). Five biological replicates were used for flavonoid analyses.

Gene expression analysis of anthocyanin biosynthetic genes

Total RNA was isolated from the petals of each cultivar at the S3 stage of flower development using RNAiso Plus (Takara Bio, Otsu, Japan) and purified using lithium acetate precipitation as described previously (Nuraini et al., 2020). Genomic DNA was eliminated and then cDNA synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio). RT-qPCR was performed for each gene using a Thermal Cycler Dice Real Time System (TP850; Takara Bio) and TB Green Fast qPCR mix (Takara Bio). The reaction mixture (10 µL) comprised 1× Master Mix, 0.2 µM

Table 1. Features of *Matthiola incana* commacal white flower cultivars used in this study.

Cultivars	Usage	Flower	Seed	Leaf	Other
Vintage Lavender	potted flower	purple	blackish	hairy	
Vintage White	potted flower	white	yellow	hairy	
Kiss me White	potted flower	white	yellow	glabrous	derived from 'Pygmy White'
Pygmy White	cut flower	white	yellow	glabrous	serrate leaves
Iron White	cut flower	white	yellow	hairy	standard type
White Wonder No. 2	cut flower	white	brown	glabrous	all-double
Quartet White	cut flower	white	yellow	hairy	spray-type

of each primer, and 1 µL of template cDNA. The cycling conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. The primer sets used to quantify *M. incana* anthocyanin biosynthetic genes have been described previously (Nuraini et al., 2020). The specificity of each amplification reaction was checked by a dissociation analysis step added after the cycle reaction. Data were analyzed by second derivative maximum methods using Thermal Cycler Dice Real Time System II version 5.0 software (Takara Bio). The transcript level of each gene was calculated relative to that of the reference gene for actin encoded by *MiACT2* (LC496063) in *M. incana*. RT-qPCR was performed using five biological replicates.

Genomic PCR analysis of anthocyanin biosynthesis-related genes

Genomic DNA was isolated from 10 g of young leaves using the cetyl trimethylammonium bromide method as described previously (Nakatsuka et al., 2005a), with some modifications. In addition, genomic fragments from *CHS*, *DFR*, *ANS*, *3GT*, *MYB1*, *bHLH2*, and *WDR1* in 12 cultivars were amplified. The primer sets used are shown in Table S1. Genomic fragments from *bHLH2* were also amplified using bHLH2-U881 (5'-CGC TTG CTG GAA AGG TTG CTT GTG ATG C-3') and bHLH2-L1405 (5'-ACT CCA ATT CTT GAA CCC GCC TTT GCA GT-3'). The PCR mixture (50 µL) contained 100 ng of genomic DNA, 1× *Ex Taq* buffer, 200 µM dNTPs, 0.4 µM of each primer, and 0.25 units of *Ex Taq* Hot Start (Takara Bio). The reaction conditions were as follows: denaturation at 94°C for 2 min, followed by 35 cycles of 95°C for 20 s, 60°C for 40 s, and 72°C for 2 min, followed by extension at 72°C for 10 min. PCR products were separated on 1.0% or 1.5% agarose gels and then stained with Midori-Green Advance (Nippon Genetics, Tokyo, Japan). The amplified fragments were subcloned into a pGEM-T Easy vector system (Promega, Madison, WI, USA). The sequences of all constructs were confirmed by DNA sequencing (Fasmac, Atsugi, Japan).

To investigate the presence or absence of nucleotide polymorphisms among white flower cultivars and 'Vintage Lavender', we conducted a T7 endonuclease I assay using the genomic PCR fragments described above. Reaction mixtures (19 µL) comprised 2.5 µL of each PCR product, 2 µL of 10× NEBuffer2, and 12 µL of sterile water. PCR was performed under the following conditions: 95°C for 5 min, followed annealing at 95°C–85°C (−2°C/sec) and at 85°C–25°C (−0.1°C/sec). Annealed PCR products were incubated at 37°C for 15 min after the addition of 1 µL of T7 endonuclease I (10 units; New England Biolab Japan Inc., Tokyo, Japan). The reaction products were separated on 1% agarose gels, and then stained with Midori-Green Advance DNA stain.

Development of a PIRA-PCR marker for the ANS mutation

To distinguish between *ANS* and *ans-1* alleles, we used primer-introduced restriction analysis (PIRA)-PCR (Haliassos et al., 1989; Neff et al., 1998). PCR was performed as described above using the following PIRA-PCR primer set designed to distinguish the single nucleotide deletion of *ans-1*: ANS_PIRA-U441, 5'-TTA TCC TCA AGA CAA GAG AGA TCT CTC ACT TTG GCC CAA AAC A**G**C-3' (underlined guanine is substituted for cytosine) and ANS_PIRA-L749, 5'-CAG GCT GAG GAC ATT TTG GGT AAT-3'. Ten microliters of the PCR mixture was added to 10 µL of *Alu* I reaction mixture containing 1× CutSmart buffer and 5 units of *Alu* I (New England BioLab Japan); the mixture was incubated at 37°C for 1 h. The reaction products were separated on 2% agarose gel and then stained as shown above.

Southern blot analysis of bHLH2 and ANS

To determine the number of copies of *bHLH2* and *ANS* in the *M. incana* genome, we performed Southern blot analysis as described previously (Nakatsuka et al., 2005a). Ten micrograms of genomic DNA were digested by *Hin*d III and then separated on a 0.7% agarose gel. The separated DNAs were transferred to a Hybond-N membrane (GE healthcare Life Science, Little Chalfont, England). The open reading frames (ORFs) of *bHLH2* and *ANS* were labeled using the DIG-High Prime DNA Labeling and Detection starter Kit II (Sigma-Aldrich Japan, Tokyo, Japan) and used as probes.

Results

Flavonoid analysis in white flower cultivars

No peaks were detected at 520 nm by HPLC analysis in all six white flower cultivars, which implied that there was no accumulation of anthocyanin pigments in their petals (Figs. 1A and S1). In contrast, post-hydrolysis measurements of flavonol concentrations in the petals of each white flower cultivar showed that flavonol derivatives accumulated in the petals of all six white flower cultivars at levels 2.4- to 3.6-fold higher than that in 'Vintage Lavender', but there was no significant difference among the values (Fig. 1B). These results indicate that the white flower cultivars are deficient in anthocyanin biosynthesis, but not in flavonol biosynthesis.

Comparative expression analysis of anthocyanin biosynthetic genes

To compare the different expression levels of anthocyanin biosynthesis-related genes among white flower cultivars and 'Vintage Lavender', we determined the expressions of these genes using petals at the S3 stage of flower development, when they are expressed strongly (Nuraini et al., 2020).

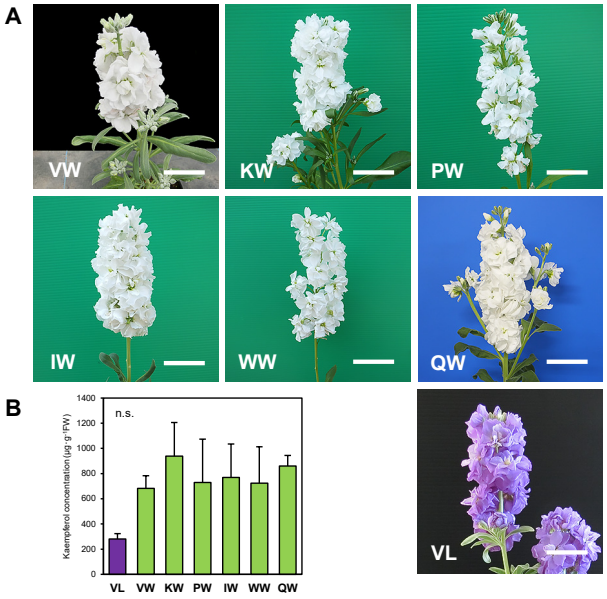

Fig. 1. Phenotypes and flavonol concentrations of white-flowered *M. incana* cultivars. A: VW, 'Vintage White'; KW, 'Kiss me White'; PW, 'Pygmy White'; IW, 'Iron White'; WW, 'White Wonder No. 2'; QW, 'Quartet White'; VL, 'Vintage Lavender' (control). Bar = 5 cm. B: Flavonol accumulation in petals of seven cultivars at the anthesis stage (S4). Flavonoids extracted from each sample were converted to aglycones by hydrolysis and quantified using kaempferol as a standard. Values are expressed as mean ± standard error (n = 5). There was no difference among any values by the Tukey–Kramer method ($P < 0.05$).

RT-qPCR showed that the expression levels of *CHS*, *F3H*, *F3'H*, *DFR*, *ANS*, *3GT*, and *FLS1* in the petals of 'Kiss me White' and 'Pygmy White' were suppressed by 0%–48% compared with those in 'Vintage Lavender', although there was no significant difference in the expression levels of some genes (Fig. 2). Furthermore, the expression levels of *bHLH2* in both white flower cultivars were two-fold higher than those in 'Vintage Lavender'. The expression levels of *ANS* in 'Vintage White', 'Iron White', 'White Wonder No. 2', and 'Quartet White' were significantly lower than those in 'Vintage Lavender' (Fig. 2). The expression level of *WDR1* in all white flower cultivars was also significantly lower than that in 'Vintage Lavender' (Fig. 2). The expression level of *FLS1* in all white flower cultivars, except 'Vintage White' and 'Iron White', was lower than that in 'Vintage Lavender' (Fig. 2). The expression levels of *CHS*, *CHI*, *F3H*, *DFR*, *3GT*, and *bHLH1* were higher in 'Vintage White' than in 'Vintage Lavender'. The expression level of *F3'H* was lower in all white flower cultivars except 'White Wonder No. 2' (Fig. 2).

Based on expression analysis, we classified the white flower cultivars into two groups. Group 1, comprising 'Kiss me White' and 'Pygmy White', was characterized by suppressed expression of most anthocyanin biosynthetic structural genes. Here, we assumed that mutations in transcriptional factors were responsible for anthocyanin deficiency. In group 2, comprising cultivars 'Vintage White', 'Iron White', 'White Wonder No. 2', and 'Quartet White', the expression levels of most anthocyanin biosynthetic structural and regulatory genes were not suppressed and were often higher compared with those in 'Vintage Lavender'. Therefore, we hypothesized that the anthocyanin deficiency was a result of functional deficiency in certain structural genes in group 2.

Identification of bHLH2 mutation in 'Kiss me White' and 'Pygmy White'

We hypothesized that group 1 white flower cultivars have a mutation in either *MYB1*, *bHLH2*, or *WDR1*, which are components of the MBW complex (Nuraini et al., 2020). The expression level of *bHLH2* in 'Kiss me White' and 'Pygmy White' was increased significantly compared with that in the other four white flower cultivars, and in the latter, it was two times higher than that in 'Vintage Lavender' (Fig. 2). On the other hand, the expression level of *MYB1* in 'Kiss me White' and 'Pygmy White' did not differ significantly from that in other cultivars (Fig. 2). The expression level of *WDR1* in all white flower cultivars was significantly lower than that in 'Vintage Lavender'. Genomic fragments of *MYB1*, *bHLH2*, and *WDR1* were amplified from the genomic DNA of each cultivar. The lengths of the 1.3-kb and 1.0-kb fragments amplified from *MYB1* and *WDR1* were the same in 'Kiss me White' and 'Pygmy White' as in 'Vintage Lavender' and other white flower cultivars (data not shown). In contrast, the 5-kb amplified fragment from the *bHLH2* genome in 'Kiss me White' and 'Pygmy White' was longer than the 4.5-kb fragment in other cultivars (data not shown). To find an insertion in the *bHLH2* genome of both white flower cultivars, PCR was performed using several primer combinations. Approximately 500-bp long fragments were amplified using bHLH2-U881 and bHLH2-L1405 primers in both 'Kiss me White' and 'Pygmy White' compared with 700-bp fragments in other cultivars (Fig. 3A).

The 1.2-kb fragments amplified from *bHLH2* in 'Kiss me White' and 'Pygmy White' were subcloned and sequenced. Sequences from both cultivars had a 481-bp insertion sequence in the 6th exon of *bHLH2* (Fig. 3B). This insertion had an 8-bp target site duplication (TSD) and 13-bp inverted repeats (IRs) at both ends, which are features that correspond to *hAT*-type transposable elements. Here, we designated this as *dTmi1* (Fig. 3C). This insertion does not contain protein-coding sequences; thus, *dTmi1* is likely a non-autonomous transposable element.

Southern blot analysis showed that *bHLH2* exists as a single copy within the *M. incana* genome and that the gene is longer in 'Kiss me White' and 'Pygmy White' due to the *dTmi1* insertion (Figs. S2A and B).

To determine the relationship between the *bhlh2*^{dTmi1}

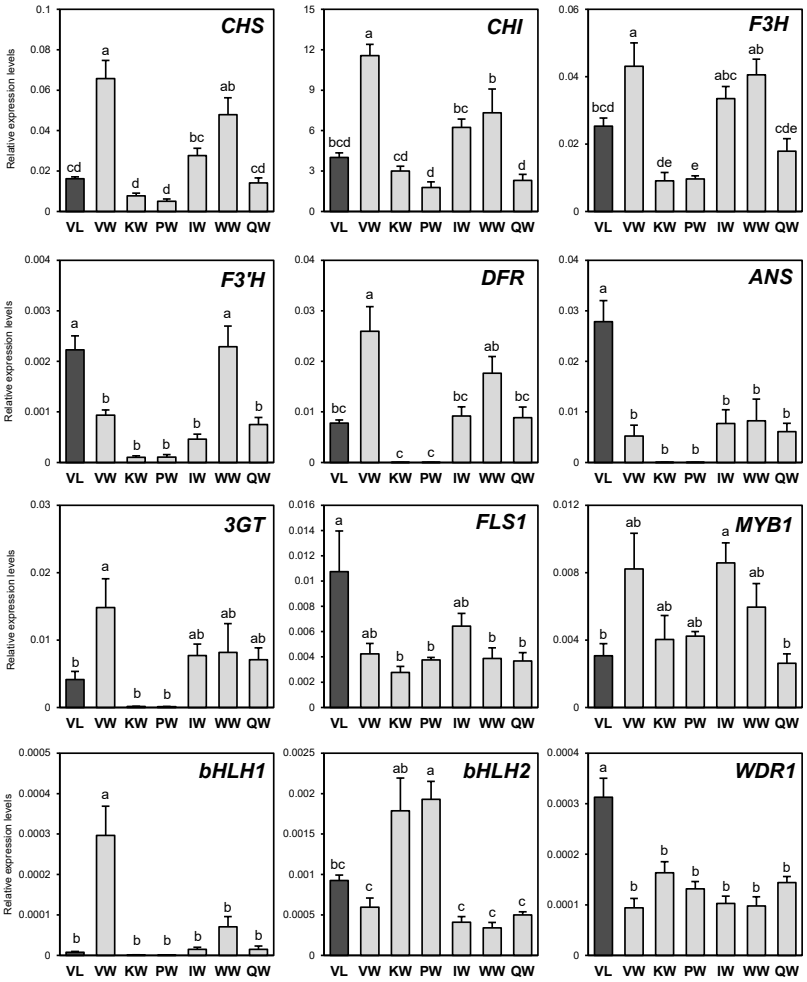

Fig. 2. Relative expression levels of anthocyanin biosynthetic genes in petals of white-flowered cultivars. The expression levels of chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), anthocyanidin 3-*O*-glucosyltransferase (*3GT*), flavonol synthase 1 (*FLS1*), *MYB1*, basic helix-loop-helix 1 (*bHLH1*), *bHLH2*, and WD repeat 1 (*WDR1*) were normalized relative to *ACT2*. Values are expressed as mean ± standard error (n = 5). Different letters indicate significant difference by the Tukey-Kramer method ($P < 0.05$). VL, 'Vintage Lavender'; VW, 'Vintage White'; KW, 'Kiss me White'; PW, 'Pygmy White'; IW, 'Iron White'; WW, 'White Wonder No. 2'; QW, 'Quartet White'.

allele and flower coloration in the 'Kiss me' series of cultivars, we performed genomic PCR for *bHLH2* in various cultivars of the 'Kiss me' series with different flower colors. All cultivars had the glabrous leaf phenotype. 'Kiss me Blue', 'Kiss me Rose', 'Kiss me Salmon', and 'Kiss me Cherry' accumulated anthocyanin pigments in their petals, whereas 'Kiss me Yellow' did not accumulate anthocyanin pigments (Fig. S3). Carotenoid pigment was responsible for the cream flower coloration in 'Kiss me Yellow' (data not shown). Among the six cultivars with different flower colors, a 1.2-kb fragment corresponding to the *bhlh2*^{dTmi1} allele was amplified in 'Kiss me Yellow' and 'Kiss me White'. Hence, the *bhlh2*^{dTmi1} allele correlates well with anthocyanin deficiency in the petals of various cultivars. The enhanced expression level of *bHLH2* and the suppressed expression levels of *CHS*, *F3H*, *DFR*, *ANS*, and *3GT* were also detected in not only 'Kiss me White', but also 'Kiss me Yellow' (Fig. 4).

Identification of the ans-1 mutation in white flower cultivars in group 2

The group 2 cultivars 'Vintage White', 'Iron White', 'White Wonder No. 2', and 'Quartet White' did not contain any mutations in *bHLH2* (Fig. 3A). In these cultivars, we assumed that mutations in either *DFR*, *ANS*, or *3GT* are responsible for anthocyanin deficiency. We amplified fragments approximately 1.5, 1.2, and 1.5 kb in length from genomic regions corresponding to the *DFR*, *ANS*, and *3GT* ORFs, respectively (Figs. S4A, S5A, and S6A). All seven cultivars yielded PCR products of the same fragment length. To investigate whether mismatches of *DFR*, *ANS*, and *3GT* genomic sequences are present between 'Vintage Lavender' and each white flower cultivar, we mixed PCR products and then treated them with T7 endonuclease I (Figs. S4B, S5B, and S6B). PCR products of *ANS* had two partially digested fragments (ca. 500 and 700 bp) in all white flower cultivars, but the fragments were not detected in

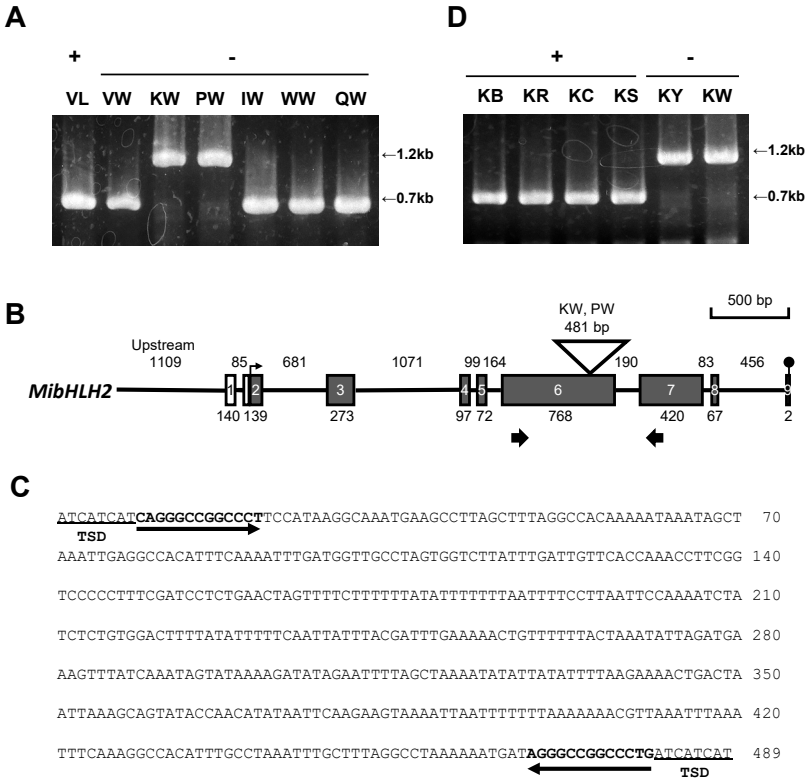

Fig. 3. Genomic PCR analysis of *bHLH2* in white-flowered cultivars. A: Amplified fragments of *bHLH2* using genomic DNAs from seven cultivars including 'Vintage Lavender (VL)', 'Vintage White (VW)', 'Kiss me White (KW)', 'Pygmy White (PW)', 'Iron White (IW)', 'White Wonder No. 2 (WW)', and 'Quartet White (QW)'. '+' represents anthocyanin-accumulating cultivars, whereas '−' represents cultivars that did not accumulate anthocyanin in their petals. Genomic PCR was performed using bHLH2-U881 and bHLH2-L1405 primers. Amplified fragments were separated on 2.0% agarose gels. The 0.7-kb and 1.2-kb fragments correlate to *bHLH2* and *bhlh2*^{dTmi1} alleles, respectively. B: Genome structure of *bHLH2* in 'Vintage Lavender' (accession number LC557498), 'Kiss me White' and 'Pygmy White' (LC557495). Empty and full boxes indicate untranslated regions and CDS within exons, respectively. The numbers below boxes and above lines indicate the number of base pairs of exons and introns, respectively. Arrows indicate the positions of bHLH2-U881 and bHLH2-L1405 primers. The inverted triangle indicates the insertion position of *dTmi1* transposable element. C: Sequences of the *dTmi1* transposable element. TSD is an 8 bp of target site duplication. Arrows indicate 13-bp inverted repeats. *dTmi1* exhibits typical features of *hAT* transposable elements. D: Amplified fragments of *bHLH2* in six cultivars of the 'Kiss me series' including 'Kiss me Blue (KB)', 'Kiss me Rose (KR)', 'Kiss me Cherry (KC)', 'Kiss me Salmon (KS)', 'Kiss me Yellow (KY)', and 'Kiss me White (KW)'.

'Vintage Lavender' (Fig. S5B). Conversely, the digested pattern of *DFR* and *3GT* did not differ between 'Vintage Lavender' and all white flower cultivars (Figs. S4B and S6B). Therefore, *ANS* genomic fragments from white flower cultivars were subsequently subcloned and sequenced.

ANS sequences from white flower cultivars and 'Vintage Lavender' had single-nucleotide polymorphisms, including deletions, only in the white flower cultivars, of a single nucleotide at the 486th position from the start codon of *ANS* (Fig. 5A). This deletion exists in the 1st exon of *ANS* and leads to a frameshift mutation, resulting in a nonsense codon in the 3' position immediately after the deletion. We designated this mutated *ANS* allele in the white flower cultivars as *ans-1*.

Southern blot analysis revealed an approximately 7.0-kb fragment in all cultivars (Fig. S2C). No *Hin*d III-recognizing site was present in the *ANS* genomic region (Fig. S2A). Therefore, these findings suggest that *ANS* exists as a single copy in the *M. incana* genome and that no genome rearrangement has occurred in any of the white flower cultivars.

All white flower cultivars belonging to group 2 exhibited significantly suppressed levels of *WDR1* (Fig. 2). However, we were unable to find any mutation within each respective *WDR1* gene sequence (data not shown).

Development of a marker to distinguish between ANS and ans-1 alleles

A single nucleotide deletion in the 1st exon of *ANS* induced a frameshift mutation, resulting in the *ans-1* allele (Fig. 5A). The *ans-1* allele is indistinguishable from the *ANS* allele based on their respective amplified fragment lengths, unlike the situation between *bHLH2* and *bhlh2*^{dTmi1}. We thus employed PIRA-PCR (Haliassos et al., 1989; Neff et al., 1998) to generate a specific marker for each *ANS* allele. We designed a PIRA-PCR marker to distinguish between the *ANS* and the *ans-1* alleles, and the appropriate mismatch was

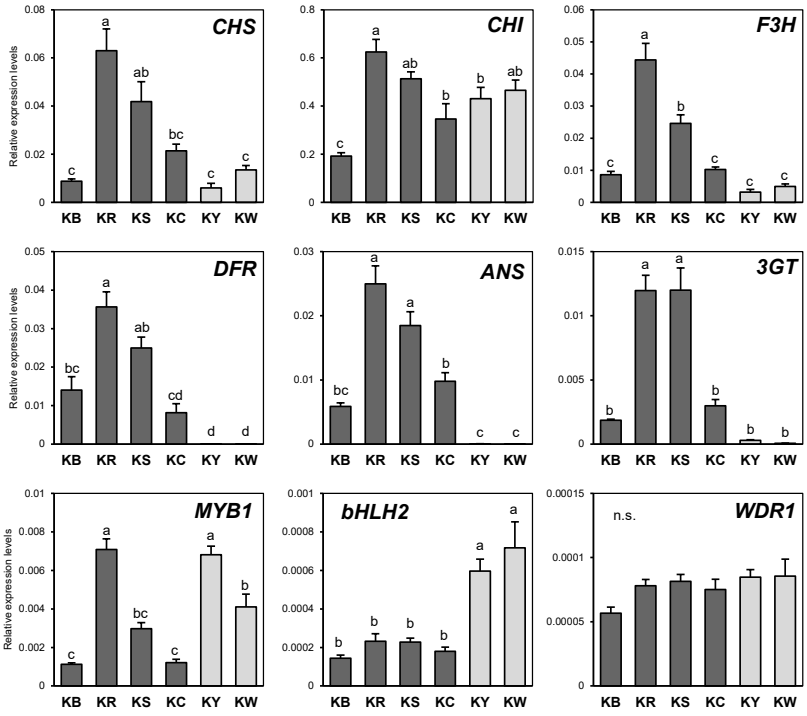

Fig. 4. Relative expression levels of anthocyanin biosynthetic genes in petals of the 'Kiss me series'. The expression levels of chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), anthocyanidin 3-*O*-glucosyltransferase (*3GT*), *MYB1*, basic helix-loop-helix 2 (*bHLH2*), and WD repeat 1 (*WDR1*) were normalized relative to *ACT2*. Values are expressed as mean ± standard error (n = 5). Different letters indicate significant difference by the Tukey–Kramer method ($P < 0.05$). n.s. indicates no significant difference among all cultivars. KB, 'Kiss me Blue'; KR, 'Kiss me Rose'; KC, 'Kiss me Cherry'; KS, 'Kiss me Salmon'; KY, 'Kiss me Yellow'; KW, 'Kiss me White'.

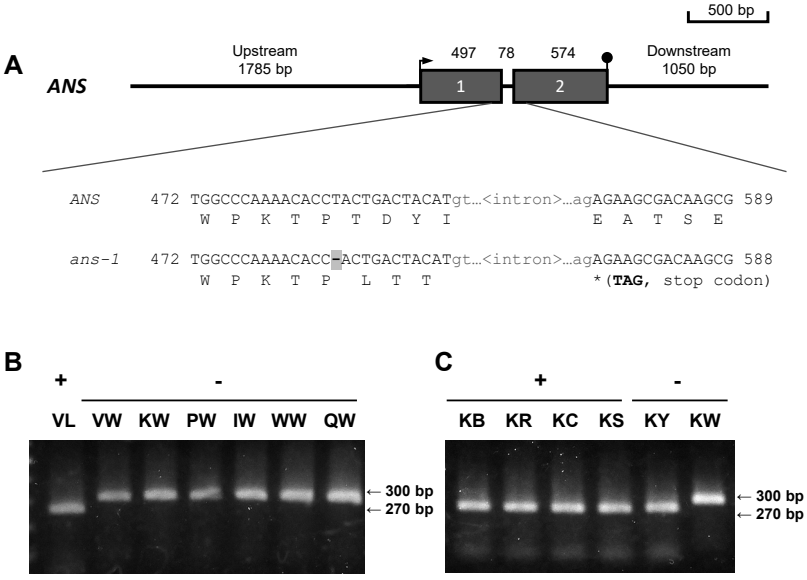

Fig. 5. Genomic sequences of *ANS* in white-flowered cultivars. A: Genomic structure of *ANS* in colored- (accession number LC557496) and white-flower cultivars (LC557497). A single nucleotide at the 486th position from the initiation codon of the *ANS* gene is deleted in white cultivars. '−' indicates the deletion of *ans-1*, resulting in frameshift and nonsense mutations. Full boxes indicate untranslated regions and CDS within exons. The numbers above boxes and lines indicate the number of base pairs of exons and introns, respectively. Upper and lower case exhibit exon and intron sequences, respectively. B: PIRA-PCR analysis for *ANS* alleles in seven cultivars including 'Vintage Lavender (VL)', 'Vintage White (VW)', 'Kiss me White (KW)', 'Pygmy White (PW)', 'Iron White (IW)', 'White Wonder No. 2 (WW)', and 'Quartet White (QW)'. '+' represents anthocyanin-accumulating cultivars, whereas '−' represents cultivars that do not accumulate anthocyanin in their petals. Genomic PCR was performed using ANS-PIRA-U441 and ANS-PIRA-L749 primers, and the amplified fragments were digested with *Alu* I. Afterwards, fragments were separated on 2.0% agarose gels. The 270-bp and 300-bp fragments correlate to the *ANS* and *ans-1* alleles, respectively. C: PIRA-PCR analysis for *ANS* alleles in six cultivars of the 'Kiss me series' including 'Kiss me Blue (KB)', 'Kiss me Rose (KR)', 'Kiss me Cherry (KC)', 'Kiss me Salmon (KS)', 'Kiss me Yellow (KY)', and 'Kiss me White (KW)'.

introduced into the ANS_PIRA-U441 primer. Although the ANS_PIRA-U441 and ANS_PIRA-U749 primers amplified 300-bp fragments in all cultivars, the recognition sequence of the restriction enzyme *Alu* I (AG|CT) was present only in the *ANS* allele. After digestion with *Alu* I, a 270-bp fragment was generated only in 'Vintage Lavender', but not in all the white flower cultivars (Fig. 5B). Similarly, the *ANS* gene of six cultivars belonging to the 'Kiss me' series was genotyped using PIRA-PCR. The 270-bp *Alu* I fragment, which indicated the *ANS* allele, was detected in 'Kiss me Blue', 'Kiss me Rose', 'Kiss me Cherry', 'Kiss me Salmon', and 'Kiss me Yellow', but not in 'Kiss me White' (Fig. 5C). Therefore, this PIRA-PCR marker can distinguish between *ANS* and *ans-1* alleles. These results indicate that the loss of anthocyanin in 'Kiss me Yellow' is due solely to the mutation in *bhlh2*^{dTmi1}, which differs from 'Kiss me White', in which a *bhlh2*^{dTmi1} and *ans-1* double mutation occurs (Figs. 3B and 5C).

Discussion

We previously investigated the expression profiles of anthocyanin biosynthetic genes in the purple flower cultivar 'Vintage Lavender' (Nuraini et al., 2020). In this cultivar, the regulatory complex, which is composed of MYB1, bHLH2, and WDR1, activates the transcription of anthocyanin biosynthetic structural genes, resulting in the accumulation of anthocyanin in the petals of *M. incana* (Nuraini et al., 2020). In the present study, we aimed to identify the genes responsible for the white-flower coloration in commercial *M. incana* cultivars.

None of the white flower cultivars accumulated anthocyanin in their petals (Fig. S1), although their flavonol levels tended to be higher than those of 'Vintage Lavender' (Fig. 1B). Based on flavonoid analysis, we hypothesized that white flower cultivars have a mutation in any of three anthocyanin biosynthetic structural genes, namely *DFR*, *ANS*, and *3GT*, or three regulatory genes, namely *MYB1*, *bHLH2*, and *WDR1*. Previous studies have reported that single nucleotide substitutions of *CHS* and *WDR1* were responsible for the white flower color phenotype in *f* and *e* mutants, respectively (Dressel and Hemleben, 2009; Hemleben et al., 2004). After confirming the genomic sequences of both *CHS* and *WDR1* in the six white flower cultivars, we did not find any base substitutions in their genomic sequences (data not shown).

In 'Kiss me White' and 'Pygmy White', the expression levels of *CHS*, *F3H*, *F3'H*, *DFR*, *ANS*, and *3GT* were suppressed to less than half of those in anthocyanin-accumulated cultivars (Fig. 2). We speculate that in both potted cultivars, white flower coloration arises by the same mutation. 'Kiss me White' is a cultivar derived from 'Pygmy White' (Plant variety protection, <http://www.hinshu2.maff.go.jp/index.html>). In our study, group 1 was assumed to have a mutation in one of three regulatory genes: *MYB1*, *bHLH2*, and *WDR1*. Among them, the expression level of *bHLH2* in 'Kiss me White' and 'Pygmy White' increased two-fold compared with that in 'Vintage Lavender' (Fig. 2). The expression level of *MYB1* in these two cultivars did not significantly differ from that in 'Vintage Lavender', and there were no mutations in the *MYB1* genomic sequence. The expression level of *WDR1* in both white flower potted cultivars was suppressed significantly compared with that in 'Vintage Lavender', but they did not have any mutations.

Genomic PCR analysis showed that a fragment amplified from *bHLH2* in group 1 was approximately 500 bp longer than that in the other cultivars (Fig. 3A). Sequence analysis revealed a 481-bp insert in the 6th exon of *bHLH2* in group 1 cultivars (Fig. 3B). This insertion exhibits features of *hAT*-type transposable elements (Kunze and Well, 2002), including 8-bp of TSD and 13-bp of IR at both ends. In addition, these transposable elements are assumed to be non-autonomous because no proteins are encoded within their sequences (Fig. 3C). This is the first report identifying a transposable element in *M. incana*, and we designate this transposable element *dTmi1*.

In many ornamental flowers, transposable elements contribute to white flower formation. For example, in the Japanese gentian 'Polarno White', *GtMYB1*, one of the anthocyanin regulatory genes, is mutated via insertions of either a *hAT*-type transposable element (*dTgt1*) or an uncharacterized sequence (Nakatsuka et al., 2008). In the torenia cultivar 'Crown White', an LTR-type retrotransposable element *TORE1* is inserted into the 5' region upstream of *TfF3H* (Nishihara et al., 2014). The bHLH domain of *bHLH2*, located in the 6th and 7th exons (Fig. 3B), is predicted to bind DNA. Regions outside the bHLH domain are also crucial for stabilizing dimerization and DNA-binding specificity (Feller et al., 2011). In group 1 white flower *M. incana* cultivars, transcripts from the *bhlh2*^{dTmi1} allele did not contain the bHLH domain due to a nonsense mutation within the 481-bp insert (Fig. 3B, C). However, we could not confirm why the expression level of *bHLH2* was enhanced in both white flower cultivars. In dahlia, the spontaneous yellow ray floret variety 'Michael J Yellow (MJY)' accumulates flavone and butein, but not anthocyanin; moreover, *DvCHS1*, *DvF3H*, *DvDFR*, *DvANS*, and *DvIVS* are suppressed (Ohno et al., 2011). *DvIVS* encodes the bHLH transcription factor for anthocyanin biosynthesis, and in 'MJY', a 5.4-kb CACTA transposable element, *Tdv1*, is inserted into *DvIVS*. In pale aleurone pigmented maize, the *r-mi* mutation is caused by a 396-bp *Ds* transposable element in the bHLH domain of *R* (Liu et al., 1998). Thus, mutations in the *bHLH* transcription factor lead to the non-accumulation of anthocyanin in several plants. Furthermore, the downregulation of *CHS*, *F3'H*, *DFR*, *ANS*, and *3GT* in *bhlh2*^{dTmi1} mutant cultivars (Figs. 2 and 4)

implies that the activation of these genes is controlled by the MYB1-bHLH2-WDR1 complex.

In addition to mutations in *bHLH2*, all investigated white flower cultivars had a single nucleotide deletion in the 1st exon of *ANS* (Fig. 5). This deletion results in a nonsense codon via a frameshift mutation; therefore, the *ans-1* allele functions nonenzymatically (Fig. 5A). White flower coloration in lisianthus also involves an *ANS* mutation, specifically a 94-bp deletion in exon 2 (Shimizu et al., 2011). Japanese gentian lacks the *ANS* locus, which is responsible for its white coloration (Nakatsuka et al., 2005b). Therefore, white flower coloration due to a deficiency in *ANS* may be a conserved trait in many ornamental plants. In the present study, we identified two mutated genes, *bhlh2*^{dTmi1} and *ans-1*, that were independently responsible for white flower coloration in commercial *M. incana* cultivars. In Japanese gentian and *L. speciosum*, two distinct pairs of spontaneous mutations are involved in white flower development, i.e., *GtMYB1* and *ANS* (Nakatsuka et al., 2005b, 2008) and *LsMYB12* and *DFR* (Suzuki et al., 2015), respectively. In three white flower mutant lines of *M. incana* established by Seyffert (1971), *e*, *f*, and *g* alleles were thought to be *WDR1*, *CHS*, and *bHLH* mutants, respectively (Dressel and Hemleben, 2009; Hemleben et al., 2004; Ramsay et al., 2003). Among the aforementioned mutants, the *g* mutant was complementary to anthocyanin accumulation when two *Arabidopsis bHLH* genes (*EGL3* and *GL3*) were introduced transiently into its petals (Ramsay et al., 2003). *bHLH2* is an ortholog of *Arabidopsis EGL3* and *GL3* (Nuraini et al., 2020); therefore, the *g* mutant may correspond with the *bhlh2*^{dTmi1} mutant identified in this study. On the other hand, Saunders (1928) also defined the *R* and *C* genetic loci as being responsible for anthocyanin accumulation in *M. incana*. Both *R* and *C* have been also known to regulate the leaf trichome phenotype (Saunders, 1928). Trichome formation on leaf surfaces is also regulated by the MBW complex in *Arabidopsis* (Ramsay and Glover, 2005). Therefore, we assumed that *R* and *C* may correspond with *bHLH2* and *WDR1*. Furthermore, we posited that *ans-1* is a new locus related to white flower coloration in *M. incana*.

'Kiss me White' and 'Pygmy White' are *bhlh2*^{dTmi1} and *ans-1* double mutants (Figs. 3 and 5). This raises the question of whether these two cultivars need both mutations or a single mutation for white flower coloration. In a collection of isogenic *M. incana* lines, three white flower lines (*e*, *f*, and *g* mutants) were identified, with the *e* (*WDR1*) and *g* (*bHLH2*) mutants exhibiting the glabrous phenotype (Seyffert, 1971). The 'Kiss me' and 'Pygmy' series are potted cultivars that exhibit the glabrous leaf phenotype (Table 1). Therefore, we speculated that *bhlh2*^{dTmi1} contributes to the glabrous leaf phenotype in these cultivars irrespective of anthocyanin production. We additionally investigated the presence of mutant *bHLH2* and *ANS* alleles in other cultivars of the 'Kiss me' series that produce anthocyanin or carotenoid pigments and have glabrous leaves (Figs. 3D and 5C). Among cultivars of the 'Kiss me' series, *bhlh2*^{dTmi1} alleles were detected in two anthocyanin-less cultivars, 'Kiss me Yellow' and 'Kiss me White' (Fig. 3D). On the other hand, the *ans-1* allele was detected in only 'Kiss me White' (Fig. 5C). The expression profiles of anthocyanin structure and regulatory genes in 'Kiss me Yellow' were similar to those in 'Kiss me White' (Figs. 2 and 4). Although all cultivars of the 'Kiss me' series have glabrous leaf phenotypes, this phenotype is not correlated with the presence of *bhlh2*^{dTmi1} (Fig. 3D). Moreover, the petals of 'Kiss me Yellow' accumulated carotenoid, but not anthocyanin, pigments. This is correlated with a single mutation designated as *bhlh2*^{dTmi1} (Figs. 3D and S3). Therefore, we suggest that either *bhlh2*^{dTmi1} or *ans-1* alleles are enough to induce deficient anthocyanin production in *M. incana* flowers.

'White Wonder No. 2' is an all-double type cultivar and is utilized as a cut flower. This cultivar exhibits a strong linkage between the double flower and white flower loci (Fujita, 1994). Therefore, we hypothesized that the gene or allele responsible for the white flower coloration is different from that in other white-flowered cultivars. Our results revealed that the white flower coloration in 'White Wonder No. 2' is associated with the *ans-1* mutation (Fig. 5B). However, Southern blot analysis did not detect any genome rearrangement within the 'White Wonder No. 2' *ANS* locus (Fig. S2). Further investigation into the generation of mostly double flowers in cultivars such as 'White Wonder No. 2' is necessary to understand this unique phenomenon.

DNA markers for anthocyanin biosynthesis-related genes have been developed for several plants (Matsubara et al., 2006; Nakajima et al., 2005; Nakatsuka et al., 2012; Nishihara et al., 2015; Shimizu et al., 2011). In the present study, we utilized the bHLH-U881 and bHLH2-L1405 primer set to generate PCR products of distinct lengths that are able to distinguish between the *bHLH2* and *bhlh2*^{dTmi1} alleles (Figs. 3A and D). The *ans-1* allele is the result of a single nucleotide deletion, and it is difficult to distinguish between *ans-1* and *ANS* based solely on PCR. To distinguish between *ans-1* and *ANS*, we employed PIRA-PCR (also known as derived CAPS, dCAPS), a method that does not require specialized and expensive equipment (Haliassos et al., 1989; Nakatsuka et al., 2012; Neff et al., 1998). *Alu* I digestion of amplified fragments from *ans-1* and *ANS* resulted in the generation of a fragment that was 30-bp shorter in the latter (Fig. 5B, C). The DNA markers we developed for differentiating between *bHLH2*/*bhlh2*^{dTmi1} and *ANS*/*ans-1* alleles were then applied to genotypes in other cultivars, including the 'Kiss me' series. As a result, we identified the genotype of 'Kiss me Yellow' as *bhlh2*^{dTmi1} (Figs. 3D and 5C). Southern blot analysis revealed that *bHLH2* and

ANS exist as single copies in the *M. incana* genome (Fig. S2). Therefore, we consider the results of genotyping using our DNA markers to be accurate.

We successfully identified two distinct spontaneous mutated alleles, *bhlh2*^{dTmi1} and *ans-1*, that are of importance and are involved in anthocyanin-less flower coloration in *M. incana* commercial cultivars. In addition, we also developed DNA markers that can be used to distinguish between the genotypes of both alleles of *ANS* and *bHLH2*. Along with other DNA markers that can distinguish between single- and double-flower individuals (Nakatsuka and Koishi, 2018), these DNA markers will be helpful in future nursery plant management and further breeding of commercial *M. incana*.

Acknowledgements

We thank Ms. Nobue Nakamura from Shizuoka University for technical support.

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