Peace, a MYB-like transcription factor, regulates petal pigmentation in flowering peach ‘Genpei’ bearing variegated and fully pigmented flowers

Chiyomi Uematsu1*, Hironori Katayama2, Izumi Makino1, Azusa Inagaki1†, Osamu Arakawa3 and Cathie Martin4

1 Botanical Gardens, Graduate School of Science, Osaka City University, Osaka 576-0004, Japan
2 Food Resources Research and Education Center, Kobe University, Hyogo 675-2103, Japan
3 Faculty of Agriculture and Life Science, Hirosaki University, Aomori 036-8561, Japan
4 Department of Metabolic Biology, John Innes Centre, Norwich NR4 7UH, UK

† Present address: Department of Pathology, Graduate School of Medicine, Osaka City University, Osaka 545–8585, Japan
* To whom correspondence should be addressed. E-mail: uematsu@sci.osaka-cu.ac.jp

Received 1 October 2013; Revised 26 November 2013; Accepted 28 November 2013

Abstract

Flowering peach Prunus persica cv. Genpei bears pink and variegated flowers on a single tree. The structural genes involved in anthocyanin biosynthesis were expressed strongly in pink petals but only very weakly or not at all in variegated petals. A cDNA clone encoding a MYB-like gene, isolated from pink petals was strongly expressed only in pink petals. Introduction of this gene, via biolistics gave magenta spots in the white areas of variegated petals, therefore this gene was named as Peace (peach anthocyanin colour enhancement). Differences in Peace expression determine the pattern of flower colouration in flowering peach. The R2R3 DNA-binding domain of Peace is similar to those of other plant MYBs regulating anthocyanin biosynthesis. Key amino acids for tertiary structure and the motif for interaction with bHLH proteins were conserved in Peace. Phylogenetic analysis indicates that Peace is closely related to AtMYB123 (TT2), which regulates proanthocyanidin biosynthesis in Arabidopsis, and to anthocyanin regulators in monocots rather than to regulators in dicots. This is the first report that a TT2-like R2R3 MYB has been shown to regulate anthocyanin biosynthesis.

Key words: Anthocyanin, flowering peach, MYB-like transcription factor, Peace, Prunus persica, variegation.

Introduction

Pigmentation is an important trait for ornamental plants especially flowers. Anthocyanins, one of the major pigments in the flower kingdom, are widely distributed among higher plants. Six basic types of anthocyanidin are found in plants (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin) which provide different colours in flowers of different species depending on the degree of side-chain decoration, (glycosylation and acylation) (Glover and Martin, 2012). Horticulturalists, breeders, and geneticists have been eager to produce unique cultivars with attractive flower colour traits (Forkmann, 1993; Martin and Gerats, 1993; Holton and Cornish, 1995).

There are two categories of genes involved in the biosynthesis of anthocyanins (Martin et al., 1991; Jackson et al., 1992). The first category includes structural genes encoding enzymes catalysing each step of the biosynthetic pathway such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT). A second category includes regulatory genes encoding MYB-like transcription factors (Śablowski et al., 1994; Martin and Paz-Ares, 1997; Kranz et al., 1998; Stracke et al., 2001), basic helix-loop-helix
(bHLH) transcription factors (Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003), and WD repeat proteins (de Vetten et al., 1997; Walker et al., 1999) which regulate the expression of the structural genes co-ordinately (Quattrocchio et al., 1993; Baudry et al., 2004; Zimmermann et al., 2004).

Flowering peach, *Prunus persica* (L.) Batsch. cv. Genpei is one of the ornamental peach cultivars which bears fully pigmented pink flowers and variegated flowers on a single tree. The cultivar name ‘Genpei’ has its origin in Japanese history, therefore it is iconic in Japanese culture with a colour image of red and white. Pink flowers and variegated flowers of Genpei are believed to result from mutation as opposed to a graft chimera. Branches with pink flowers reproducibly bear only pink flowers every year, but branches with variegated flowers occasionally produce fully pigmented flowers. This phenomenon is similar to the unstable variegation caused by insertion and excision of class II transposable elements as observed in snapdragon (Harrison and Carpenter, 1979; Bonas et al., 1984; Martin et al., 1985; Sommer et al., 1985; Upadhyaya et al., 1985; Martin et al., 1988), petunia (Doodeman et al., 1984; Gerats et al., 1990; Snowden and Napoli, 1998; van Houwelingen et al., 1998), morning glory (Epperson and Clegg, 1987; Inagaki et al., 1994; Habu et al., 1998), and carnation (Itoh et al., 2002; Nishizaki et al., 2011). Chaparro et al. (1995) suggested the involvement of an active transposable element as a cause of flower colour variegation in flowering peach cv. Pillar. However, no transposon has yet been detected in Pillar.

Bud mutation has commonly been used as a convenient tool to obtain new cultivars from fruit trees, even though the mechanisms of mutation remain unclear (Imai, 1935; Shamel and Pomeroy, 1936; Whitham and Slobodchikoff, 1981). Flowering peach with variegated flowers could be a good system to reveal the mechanisms of bud mutation in woody species. Generally, fruit trees are genetically heterozygous and have long life cycles, so genetic analysis is problematic. Flowering peach Genpei, has two phenotypes, pink and variegated flowers, within one tree, i.e. two genotypes, wild type and mutant, co-exist within the same tree. These two genotypes probably possess the same genetic background except for the difference in flower colour trait and so are equivalent to isogenic lines.

In some fruit species such as apple, pear, peach, grape, and citrus, many cultivars have been derived from a few cultivars via bud mutation (Shamel and Pomeroy, 1936; Butelli et al., 2012). In Japan, more than two-thirds of peach cultivars are thought to have arisen as chance seedlings or sports by mutation from ‘Hakuto’ or its relatives (Yamamoto et al., 2003a, b). A high frequency of bud mutations in certain cultivars might suggest involvement of a transposable element in their creation. If transposable elements could be found in unstable flowering peach, it might be possible to use them to create new mutations and as tags to isolate the genes responsible for useful traits.

The isolation of the *Peace* gene encoding a Myb transcription factor that regulates petal pigmentation in flowering peach is reported here. *Peace* is expressed strongly only in pink petals and can complement the magenta colour in the white areas of variegated petals when introduced by particle bombardment. Comparison of the deduced amino acid sequence of *Peace* with other Myb transcription factors, suggests that *Peace* plays a role in inducing the anthocyanin biosynthetic pathway in peach petals.

### Materials and methods

#### Plant material

A weeping type tree of flowering peach (*Prunus persica* cv. Genpei, Plant ID No. 4F0199) bearing double flowers maintained at the Botanical Gardens of Osaka City University, Japan, was analysed in this study (Fig. 1A). Fully pigmented pink flowers and variegated flowers were borne simultaneously within one tree. One branch bearing pink flowers (P2 branch) and another branch bearing variegated flowers (V2) were marked to collect flower material.

#### HPLC assay for pigment analysis

For the pigment analysis using HPLC, flower petals collected from newly opened flowers were frozen in liquid nitrogen and stored at −80 °C until use. Frozen petals from one flower were placed in the pre-cooled mortar with liquid nitrogen and ground thoroughly using a chilled pestle. Extraction buffer containing 300 μl of 15% acetic acid in methanol and 100 μl of 0.1% gentisic acid was added to the ground material and kept on ice for 1 h. The extraction mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered using a Millipore filter of 0.45 μm and then applied for HPLC analysis using NANOSPACE S1-1 (SHISEIDO, Japan) equipped with Photo Diode Array Detector 2017 (SHISEIDO, Japan) and CAPCELL PAK C18 (4.6 mm in diameter × 150 mm in length). Solvent A (1.5% phosphate solution) and solvent B (1.5% phosphate, 20% formic acid, 25% acetonitrile solution) were used with the following gradients: A; 90–70% (0–30 min), 70–65% (30–40 min), 65–55% (40–70 min), 55–0% (70–90 min), and B; 100% after 90 min. The flow rate was 100 μl min−1 and the detection wavelengths were 280, 320, 350, and 530 nm.

In order to identify peaks obtained from HPLC profiles, chlorogenic acid, p-coumaric acid, eriodictyol, naringenin, quercetin dihydrate, quercetin-3-galactoside, catechin, epicatechin, cyanidin chloride, and cyanidin-3-O-glucoside were used as standards for HPLC analysis, each prepared as a 0.1% solution with 15% acetic acid in methanol. For the quantification of identified peaks, 0.1% gentisic acid prepared with 15% acetic acid in methanol was used as an internal standard.

#### DNA extraction and blotting to the membrane

Total DNA was extracted from frozen petals collected separately from pink flowers and variegated flowers just after blooming using a CTAB method (Katayama and Uematsu, 2003). DNA (10 μg) was digested with *BamHI*, *DraI*, *EcoRI* or *HindIII* and fractionated by 0.8% agarose gel electrophoresis then blotted onto the Hybond-N+ (GE Healthcare, UK) nylon membranes.

#### Probe preparation and signal detection

Partial genomic clones of the *CHS*, *CHI*, *F3H*, *DFR*, and *AS* genes were obtained from pink flowers by PCR cloning using degenerate primers as follows:

| Primer | Sequence |
|--------|----------|
| CHS/F2 | 5′-CACCACAYTKGATWSYYTIG-3′ |
| CHS/R1 | 5′-GCWATCCAGAAABDVAGTT-3′ |
| CHI/F1 | 5′-SYYAARTGGARGGYAARC-3′ |
| CHI/R3 | 5′-DGAHBCRTCTTGGARAAGC-3′ |
| F3H/F2 | 5′-CTACAAGCATTCCAGCAACG-3′ |
| F3H/R1 | 5′-CARTCTTGCAWGCTTCTCC-3′ |
| DFR/F8 | 5′-GCTCTGGITTTYATWGGITCATGGC-3′ |
Peace regulates petal pigmentation in flowering peach

DFR/R8 (5'-CGTAGCATSGTGIGARCAAATG-3'),
AS/F2 (5'-CUATCCCIAAAGAGTACATC-3') and
AS/R1 (5'-GAAAACAGCCCATGAAATCC-3').

Degenerate primers contained the following nucleotides; B=T+C+G, D=A+T+G, H=A+T+C, K=T+G, R=A+G, S=C+G,
V=A+C+G, W=A+T, Y=C+T, and I which indicates deoxyinosine.

Primers of Malubi/F (5'-ATGATCGAGGTGGTGCTGAA-3')
and Malubi/R (5'-AGTCCTTGAGGGTGATGTTGG-3') both
designed based on the Malus ubiquitin sequence (GenBank
DT00167) were used to amplify the ubiquitin gene from flowering
peach. Amplified fragments were cloned using a TA cloning kit (Life
Technologies, USA).

Clones of the CHS, CHI, F3H, DFR, ANS, and Ubiquitin
genes were used as homologous probes.

cDNA clones of PAL and UFGT obtained from Antirrhinum majus
(A. majus) were also used as a probes. Hybridization was performed
with ECL non-radioactive DNA labeling and detection kits (GE
Healthcare, UK).

RNA extraction and first strand cDNA synthesis

Two hundred milligrams of petals were collected separately from pink
and variegated flower buds ranging from 6–9 mm in diameter. Petals
removed from flower buds were immediately frozen in liquid nitro-
gen and subjected to total RNA extraction using an RNeasy Plant
Mini kit (Life Technologies, USA). First strand cDNA was prepared
using a cDNA synthesis kit (GE Healthcare, UK) according to the
manufacturer’s instructions. On occasion, an adapter primer having
dT17 (B26) was used (Frohman et al., 1988; Schwinn et al., 2006).
Six micrograms of total RNA was used for one reaction and cDNA was
diluted into 1 ml with sterilized water. 10 μl of first-strand cDNA
solution was used for the RT-PCR and 3' RACE PCR.

Fig. 1. Peach flowers used in this study and pigment analysis. (A) Peach tree ‘Genpei’ bearing fully pink coloured flowers and variegated
flowers within a single tree. (B) HPLC profiles obtained from pink petals and variegated petals, respectively. Absorbance spectrum at the
wave length of 280, 320, 350, and 530 nm are given.

A

B

| Absorbance | Retention Time (min) |
|------------|----------------------|
| pink       | 0                    |
| gentisic acid | 10                   |
| cyanidin-3-glucoside | 20                   |
| quercetin-3-galactoside | 30                   |
| variegated | 0                    |
| gentisic acid | 10                   |
| cyanidin-3-glucoside | 20                   |
| quercetin-3-galactoside | 30                   |
Partial cloning of a peach MYB-like gene by 3’ RACE PCR

The 3’ end of the peach MYB gene was obtained by 3’ RACE amplification using 10 μl of first-strand cDNA solution as a template with the G1709 primer (forward, 5′-AAAGGCTGAGACTTAGGTTGTTGATTATCCTAAGGC-3′) designed for Ros1 amplification in snapdragon (Schwinn et al., 2006) and B25 adapter primer (reverse, 5′-GACTGAGTCGACATCG-3′) (Frohman et al., 1988). PCR amplification was performed using Gene Amp® PCR System 9700 (Life Technologies, USA) with the following thermal conditions; 94 °C for 5 min then a repeat of 30 or 40 cycles of 94 °C for 48 s, 50 °C for 60 s, 72 °C for 3 min and, finally, 72 °C for 10 min for extension. Amplified fragments were cloned using pGEM-T Easy Vector System (Promega, USA).

Gene expression confirmed by RT-PCR Southern hybridization

Expression of the structural genes was detected by RT-PCR amplification using the first-strand cDNA solution as a template with primers used for probe preparation and the B25 adapter primer described above. For amplification of MYB-like genes, the G1709 forward primer was used (Schwinn et al., 2006). PCR amplification was performed using the same conditions as used for cloning the peach MYB-like genes. Amplified fragments were fractionated using 1.4% agarose gel electrophoresis then blotted onto a Hybond-N+ membrane. Signals were generated according to the methods described for probe preparation and signal detection.

5’ RACE PCR

The 5’ end of the peach MYB gene was determined by 5’ RACE System (Life Technologies, USA) according to the manufacturer’s instructions using the PG5240 primer (5′-GCTCTTGTGTTCTTCCTGTTGATTATCCTAAGGC-3′) as a GSP1 (Gene-Specific Primer 1) and PG542 primer (5′-GCTCTTGTGTTCTGATTATATTTCCTCTTTGATAT-3′) as a GSP2 (Gene-Specific Primer 2). Amplified product was cloned using the pGEM-T Easy Vector System (Promega, USA) and sequenced.

Preparation of an expression vector harbouring the peach MYB gene

The PMintF primer (5′-TTGCAATTGCCAGAATTTTG-3′) and the PMintR primer (5′-AGGTTATGCTTATGTTGCAATTATT-3′) were designed based on the sequence information from 3’ RACE and 5’ RACE of the peach MYB gene. First-strand cDNA was amplified using this primer set. PCR product was diluted 100-fold and used for GATEWAY cloning (Life Technologies, USA) according to the manufacturer’s instructions. PCR amplification was performed with primers in which 25 bp attB1 and 25 bp attB2 sequences plus four terminal Gs were added to the intermediate F and R primers, respectively. The Entry clone was made by recombination between attB-PCR product of the peach MYB gene and pDONR 207 using the BP reaction. The peach MYB gene (Peace cDNA sequence, GenBank/EMBL/DDBJ accession AB897866) was then transferred to the expression vector of pJAM1500 (a destination vector derived from pIJ160 with a Gateway destination cassette inserted in the Soul site of the polylinker between the double CaMV 35S promoter and the CaMV terminator sequence) using the LR reaction.

Complementation analysis by particle bombardment

Variegated flower buds (just before opening) were bombarded using a particle inflow helium gun (Vain et al., 1993; Schwinn et al., 2006) with genes encoding the transcription factors: Rose1, Rose2, Venos, and Mutabilis from Antirrhinum majus or peach MYB cDNA cloned into the expression vector. About 2 μg of gold particles (size 0.95) were coated with 10 μg of plasmid DNA of each transcription factor and 5 μg of YFP gene DNA prepared in 20 μl by adding 100 μl each of the Xho buffer (30 μl of 5 M NaCl, 5 μl of 2 M TRIS pH 8.0, 965 μl of SDW), 0.1 M spermidine, 25% PEG (mol. wt. 1300–1600), and 2.5 M CaCl₂. This mixture was incubated for a few minutes and then, after pulse centrifugation, the supernatant was removed and the pellet was washed with absolute ethanol. Gold particles were resuspended in 2 μl of absolute ethanol. Horizontally placed tubing was filled with this gold particle suspension using a syringe and then left for a few minutes to settle the gold particles at the bottom of the tubing. A piece of paper was placed at the end of the tubing to drain the ethanol. The tubing was dried completely with nitrogen gas. This dried tubing was then cut and inserted into the cartridge of the gene gun for shooting. Each flower bud was shot three times at 300 psi pressure. After shooting, branches were kept at room temp (about 20 °C). After 24 h, petals were removed from the flowers and placed on glass slides. Magenta spots, generated by complementation, were counted under bright field of a dissection fluorescent microscope (LEICA MZFLIII, Germany) and, in the same field, yellow spots generated by expression of the YFP gene were counted under dark field.

Cloning of the Peace gene using a genomic library

Cloning of the Peace gene (Peace genomic sequence, GenBank/EMBL/DDBJ accession AB897866) from genomic DNA was carried out following the directions for the Stratagene phage cloning system (Stratagene, USA). Total DNA extracted from V2 petals was partially digested with SmaI (TAKARA, Japan). After a partial fill-in reaction, total DNA fragments were ligated with lambda FIX/BamHI and partially fill-in treated DNA (Stratagene, USA), and a packaging reaction was carried out using GigaPack Gold (Stratagene, USA). Recombinant phages were selected by plaque hybridization with Peace cDNA (clone cPP14) as a probe. Plaque hybridization was carried out using an ECL-radioactive DNA labelling and detection kit (GE Healthcare, UK).

Cloning of the PprMYB10 homologue from flowering peach

A partial genomic sequence homologous to the PprMYB10 (Prunus persica MYB10, Lin-Wang et al., 2010) gene was obtained from DNAs of pink petals by PCR cloning followed by sequencing. Three primer sets: A2F (5′-ATGGAGGGTTATGACTTGAGTGAGA-3′) and A2R (5′-TACTTCATCTGGTTACATTATT-3′); B2F (5′-GGTGCAGAGAACGCTGACAAAGCC-3′) and B2R (5′-GACTCCTCAATCGTTGATGTTG-3′); C2F (5′-AAAGACCTATAAGGCAACACCC-3′) and C2R (5′-GTGCTGACTTAAAGGAGAAATATT-3′) were used for amplification. The A2F primer started from the start codon of PprMYB10. Three primer sets were designed to overlap each other and cover almost the entire region of the PprMYB10 gene.

Phylogenetic analysis

Amino acid sequences of 21 MYB transcription factors including Peace were aligned using CLUSTALW [version 2.1]. The phylogenetic tree was generated using NJplot [version 2.3].

Results

Pigment responsible for the pink colour in petals

HPLC analysis revealed that cyanidin-3-glucoside was the major pigment in pink peach flowers (Fig. 1B). Pink flowers contained 62.3 μg of cyanidin-3-glucoside per flower. By contrast, variegated flowers contained only 0.6 μg of cyanidin-3-glucoside per flower, so that the level of anthocyanin
in variegated flowers was only 1% of those in pink flowers. Variegated flowers contained higher levels of quercetin-3-galactoside (108.2 μg per flower), which was twice the level of that in pink flowers (56.5 μg). These results suggested that the latter part of the anthocyanin biosynthetic pathway catalysed by DFR, ANS, and UFGT is suppressed in variegated flowers.

**Genomic Southern hybridization**

DNA extracted from pink flowers and variegated ones was digested with restriction enzymes, separated by gel electrophoresis, subjected to Southern hybridization and probed with genomic or cDNA clones encoding genes involved in the anthocyanin biosynthetic pathway (Fig. 2). The only polymorphism observed was in the EcoRI digestion, for the CHS gene. A very weak hybridization signal to a 2.7 kb fragment was seen only in DNA from pink petals, but not in DNA from variegated petals. No other polymorphic patterns were observed for other restriction enzymes such as BamHI, DraI, and HindIII, so it was concluded that it was unlikely that this weak signal was responsible for the difference in flower colour phenotype. Other probes such as PAL, CHI, F3H, DFR, ANS, and UFGT did not show any polymorphisms between pink and variegated petals. No other differences were detected at the DNA level that could explain the different flower colour phenotypes.

**Gene expression in the flower bud**

Expression of structural genes, which encode enzymes in the anthocyanin biosynthetic pathway, was analysed by RT-PCR Southern hybridization using RNAs extracted from young petals of pink and variegated flower buds. All genes were very strongly expressed in pink petals, whereas in variegated petals CHI, F3H, and ANS showed very weak expression compared with pink petals (Fig. 3). PAL, CHS, and DFR gene expression in variegated petals was not detected even after 40 cycles of amplification. This result suggested that the white sectors in variegated flowers involved loss of function of a gene regulating the expression of structural genes of the anthocyanin biosynthetic pathway.

**Complementation of pigment biosynthesis**

To assess whether heterologous transcription factors could regulate the anthocyanin biosynthetic pathway in flowering peach, four genes encoding transcription factors from Antirrhinum majus, i.e. Rosea1, Rosea2, Venosa, and Delila (Schwinn et al., 2006) were bombarded into the white area of variegated petals together with the YFP gene. All introduced genes were cloned into the pJIT60 vector under the control of the double CaMV 35S promoter. Twenty-four hours later, bombarded petals were removed from the flower and observed under the dissection microscope equipped with a fluorescent light source. Magenta spots were observed when an introduced gene for the transcription factor could complement anthocyanin biosynthesis (Table 1). The same area of petal tissue was observed under fluorescent light to count the number of yellow spots generated by expression of the YFP gene. The complementation ratio was calculated from the number of magenta spots divided by the number of yellow spots (Table 1). Rosea1, which encodes one of the MYB transcription factors controlling anthocyanin biosynthesis in A. majus, showed the highest complementation ratio of 25.3%, whereas Rosea2 and Venosa (which also encode MYB transcription factors; Schwinn et al., 2006) and Delila (which encodes a bHLH protein controlling anthocyanin production; Goodrich et al., 1992), showed lower complementation ratios. Therefore, expression of a MYB-like transcription
Magenta spot/yellow spot (%)  

Fig. 8

Fig. 5

Fig. 3. Gene expression in the petals of young flower buds examined by RT-PCR Southern hybridization. P, pink petal; V, variegated petal.

Table 1. Magenta spot ratio observed after particle bombardment

| Transcription factors | Magenta spot/yellow spot (%) |
|-----------------------|------------------------------|
| Negative control      | 0.0                          |
| YFP control           | 0.0                          |
| Rosea1                | 25.3                         |
| Rosea2                | 9.1                          |
| Venosa                | 4.0                          |
| Delta                 | 7.7                          |

factor, functionally homologous to Rosea1, might be disrupted in the white parts of variegated petals of flowering peach, because cyanidin-3-glucoside was not produced there.

Isolation of a MYB-like gene from pink peach flower buds

To confirm the involvement of a MYB-like transcription factor in controlling flower pigmentation, expression of MYB-like genes was examined in pink and variegated petals by RT-PCR Southern hybridization. The G1709 primer, based on the petunia AN2 and maize C1 gene sequences (Schwinn et al., 2006) and the B25 primer for 3′ RACE amplification (Frohman et al., 1988) were used. The Rosea1 gene was used as a probe. cDNA from both pink and variegated petals gave strong signals for a 1.3 kb transcript (Fig. 4A). However, there was a difference in cDNAs from pink and variegated petals for bands of 1.0 kb and 1.1 kb. For both these fragments, pink petals showed stronger expression than variegated petals. Therefore these 1.0 kb and 1.1 kb transcripts could be candidates for genes regulating the difference in anthocyanin biosynthesis in flowering peach.

To obtain genes encoding MYB-like transcription factors regulating the phenotypic difference between pink and variegated petals, 3′ rapid amplification of cDNA ends (RACE) PCR was performed for cDNA from pink petals using the G1709 and B25 primers (Fig. 5). Amplified fragments were cloned into the pGEM-T Easy cloning vector. cDNA clones derived from pink petals had various lengths of insert ranging from 860 bp to 1020 bp, but sequence similarity was very high between these clones. The amino acid sequence of the N-terminal region deduced from the pink clone cPpP14 showed high similarity to other plant MYB transcription factors. Among 21 R2-R3 MYB transcription factors, 33 out of 66 amino acids corresponding to the latter part of R2 and whole R3 repeat were identical (Fig. 8). Furthermore, 50 amino acids in the same region of cPpP14 were identical to TT2, ZmCl1, ZmPl1, and OsC1, whereas 37 were identical to Rosea1, Venosa, An2, VvMYBA1, and PprMYB10. However, the C-terminal region was different from other MYB proteins, which may reflect the specificity of this gene.

When this cPpP14 clone was used as a probe for hybridization to RT-PCR products on Southern blots to confirm the expression of this MYB-like gene in flower buds, the 1.0, 1.1, and 1.3 kb transcripts were detected only in cDNA from pink flower buds (Fig. 4B).

Another MYB-like gene obtained from genomic DNAs of pink petals was almost identical to PprMYB10. The sequence of this gene, 1818 bp in length, was confirmed by connecting overlapped clones. The PprMYB10 gene is 1849 bp in length. The deduced amino acid sequence of this gene started with the same first codon as PprMYB10 and the sequence of this gene, including the 1st and 2nd introns, was identical to PprMYB10. Based on these results the MYB gene from flowering peach was named as FPMYB10. Expression of FPMYB10 in pink and variegated flower buds was investigated by RT-PCR Southern blots using two forward primers, A2F and B2F, in combination with the B25 reverse primer. The 3′ region of FPMYB10, amplified using C2F and C2R primers, was used as a probe. Pink and variegated flower buds showed strong and almost identical levels of expression of the FPMYB10 cDNA amplified using either A2F and B2F primers (Fig. 4C). Therefore at least in petals, differences in expression of the FPMYB10 gene are not the cause of the differences in colour between pink and variegated flowers.

Function of the MYB gene obtained from pink petal cDNA

5′ rapid amplification of cDNA ends (RACE) PCR was performed to obtain the 5′ region of the cPpP14 cDNA clone using the gene-specific primer PGS42 based on the cPpP14 sequence (Fig. 5). The amplified fragment was cloned into the pGEM-T Easy vector and sequenced. The entire coding sequence of the flowering peach MYB gene was reconstructed from the sequence information of the 5′ RACE clone, cPpP33, carrying a 351 bp fragment as an insert and 3′ RACE clone, cPpP14, having a 858 bp insert. The full-length gene was then amplified from cDNA from pink petals with gene-specific primers; namely the PMintF primer designed just upstream of the start codon and the PMintR primer just 3′ to the first stop codon. The amplified product was diluted and used as a template for attB-PCR to add the att-B sequences, required for preparation of an entry vector for the GATEWAY cloning system (Life Technologies, USA). Amplified att-B
product was inserted into the pDONR207 entry vector by BP recombination. The cDNA was transferred by LR recombination to the pJAM1500 vector where expression is driven by the double CaMV 35S promoter. The Peach MYB gene in this expression cassette was bombarded into variegated flower buds together with the YFP gene. Twenty-four hours later, bombarded petals were observed under the dissection microscope. Many magenta spots were observed surrounding aggregated gold particles (Fig. 6). The complementation ratio of 104.1% (Table 2) was much higher than the ratio achieved by bombardment with Rosea1 (Table 1).

It was concluded that this peach MYB gene was controlling the pigmentation of petals of flowering peach. Therefore, this gene was named Peace (peach anthocyanin colour enhancement). In variegated flower petals, Peace expression was not detected by RT-PCR Southern hybridization when probed with clone cPpP14 (Fig. 4B). Consequently, the lack of Peace expression probably resulted in white areas in variegated petals.

Comparison of Peace genomic and cDNA sequences between pink and variegated petals

The gene structure of Peace was confirmed by comparison of cDNA and genomic sequences. The Peace gene was composed of three exons disrupted by two introns, the first intron was 87 bp in length located in the R2 domain at 133 from ATG and the second one was 80 bp in length located in R3 domain at 261 from ATG in cDNA numbering (Fig. 5). These were identical positions to the introns in other plant MYB genes (Martin and Paz-Ares, 1997). Four candidate TATA box sequences were found within 100 bp of the region upstream from the ATG, based on the TFBIND (Tsunoda and Takagi, 1999). One of these candidates, the TATA box situated at 53 bp upstream of the initiating ATG codon, showed the highest score of 0.846 in TFBIND.

To investigate the reasons why the Peace gene was strongly expressed in pink petals but not in variegated petals, a genomic library was constructed from V2 tissue and

Fig. 4. MYB-like gene expression in young petals of variegated (V) and pink (P) flower buds. (A) Amplified using the G1709 primer and probed with the Rosea1 gene of Antirrhinum majus. (B) Amplified using the G1709 primer and probed with clone cPpP14 derived from pink flowers of flowering peach. (C) Amplified using the A2F or B2F primer for PprMYB10 and probed with the 3' region of the PprMYB10 gene. Number of cycles indicates the amplification cycle of PCR performed.
coding and 5′-UTR regions were compared between pink and variegated tissues. The genomic sequence of the coding region and ca. 3 kb of promoter region of the Peace gene was determined by screening the V2 genomic library. Based on this sequence information, sequences of coding and promoter regions of the P2 genome was determined by PCR sequencing. The sequences of coding and promoter regions were completely identical in DNA from pink petals and from variegated petals. No polymorphisms were detected within coding and promoter regions. To confirm whether there was a difference between pink and variegated genomes, genomic Southern hybridization was performed under high stringency conditions using both the full-length cDNA clone of Peace (cPpP3314) and the cPpP14 clone containing just the 3′ region of Peace cDNA as probes. Both pink and variegated genomes carried a single copy of the Peace gene because hybridization patterns showed a single band for each digestion (Fig. 7A). Probes made from the cPpP14 clone were likely to be highly specific for the Peace gene because this part of the cDNA lacked most of the sequences encoding the MYB domain which is highly repeated in plants due to their large number of R2R3 MYB genes. Additional weaker signals were observed when the full-length cDNA clone (cPpP3314) was used as a probe (Fig. 7B). These weaker signals are probably due to hybridization to other genes encoding the conserved R2R3 MYB domain. However, no differences were observed between pink and variegated genomes which could explain the phenotypic differences in flower pigmentation.

To investigate another possible mechanism determining the difference in Peace expression between pink and variegated flowers, sites for DNA methylation were searched in the promoter region. Thirty-five CpG dinucleotides were found to be scattered in the promoter region, but there were no CpG islands in which CpG dinucleotides appeared at high density.

**Table 2.** Magenta spot ratio acquired following introduction of the peach MYB-like gene (Peace) by particle bombardment

| Transcription factors                          | Magenta spot/yellow spot (%) |
|-----------------------------------------------|------------------------------|
| Negative control                              | 0.0                          |
| YFP control                                   | 0.0                          |
| Peach MYB-like gene (Peace)                    | 104.1                        |

**Fig. 5.** Schematic strategy for the cloning of the peach MYB-like gene based on the sequence information of Rosea1 and the peach MYB-like gene structure obtained from cDNA and genomic DNA. Restriction enzyme sites used in Southern blots were indicated by a triangle with B for BamHI, G for BglII, D for DraI, EI for EcoRI, EV for EcoRV, and X for XbaI.

**Fig. 6.** Complementation of pigment synthesis confirmed by particle bombardment into the white area of variegated petals with the Peace gene. (A) Yellow spots were generated by the YFP gene. (B) Magenta spots were generated by the Peace gene. Both observed in the same field of vision.
Peace regulates petal pigmentation in flowering peach

Therefore, it seems unlikely that differential DNA methylation controls Peace gene expression in flowering peach.

Relationship between Peace and other plant MYB transcription factors

The amino acid sequence deduced from the full-length Peace cDNA revealed the protein to contain well-conserved R2 and R3 MYB domains. Peace aligned well with amino acid sequences of 20 MYB proteins belonging to subgroups 4, 5, 6, 7, and 15, chosen for their functions in regulating flavonoid biosynthesis or by close phylogenetic relationships to regulators of flavonoid metabolism from other plants (Fig. 8) (Stracke et al., 2001; Bailey et al., 2008; Dubos et al., 2010). Among 22 MYB transcription factors, 45 deduced amino acids out of 104 in R2 and R3 repeats were identical. The region of highest conservation was the latter part of the R2 domain and middle and C-terminal part of the R3 domain. Five tryptophans, considered to be important to forming the two repeats of the helix-helix-turn-helix structure in the R2 and R3 domains, were completely conserved in Peace. Furthermore, Peace has the conserved amino acid signature motif [DE]Lx3[RK]x3Lx3Rx6 which is important for interaction with bHLH proteins from sub-group IIIf (Heim et al., 2003; Zimmermann et al., 2004; Lin-Wang et al., 2010). These results suggest Peace can act to regulate anthocyanin biosynthesis in petals of flowering peach in combination with bHLH proteins. However, neither the [A/S/G]N[D/A/N]V motif (Lin-Wang et al., 2010) nor the KPXFR[S/T]F motif, both known to be conserved in R2R3MYB proteins promoting anthocyanin biosynthesis, were present in Peace. The C-terminal region of Peace was quite divergent compared with those of other plant MYB proteins.

To compare the amino acid sequences of Peace, phylogenetic analysis was undertaken for it and 20 additional MYB transcription factors (Fig. 9). The phylogenetic tree indicated that Peace was most closely related to AtMYB123 (TT2) of Arabidopsis which regulates procyanidin biosynthesis (Nesi et al., 2001; Baudry et al., 2004). Monocotyledonous MYB proteins, C1 and Pl of Zea mays and OsC1 of Oryza sativa, involved in the anthocyanin biosynthesis (Paz-Ares et al., 1987; Cone et al., 1993) were also closely related to Peace. However, PpMYB10 of Prunus persica (peach) was not closely related to Peace. It was clustered together with MdMYB10, VvMYBA1, and VvMYBA2 (Kobayashi et al., 2004).

Discussion

The Peace gene encoding an R2R3 MYB-like transcription factor that is expressed in pink petals of flowering peach, Prunus persica cv. Genpei which bears pink flowers and variegated flowers on a single tree has been identified. This gene is strongly expressed in fully pigmented pink petals but very weakly expressed in variegated petals. Introduction of the Peace gene into the white areas of variegated petals by means of particle bombardment complemented pigment biosynthesis and magenta spots appeared. It was, therefore, concluded that the Peace gene is likely to be responsible for colouration in the petals of flowering peach.
All structural genes involved in the anthocyanin biosynthetic pathway were strongly expressed in young petals of pink flower buds, whereas CHI, F3H, and ANS genes showed only weak expression and no expression of PAL, CHS, and DFR genes were observed in variegated flower buds. These results agreed well with the result of pigment analysis which showed that pink petals contained >100-fold more cyanidin-3-glucoside than variegated petals. It seems likely that a change affecting expression of all the anthocyanin biosynthetic genes has occurred in a gene encoding a transcription factor.

Fig. 8. Alignment of the deduced amino acid sequences of 21 R2-R3 MYB transcription factors belonging to subgroup 4, 5, 6, 7, and 15. Peace is marked using pink. Conserved tryptophans are marked using green. The amino acid signature motifs of [DE][Lx2][RK]x3[Lx6]Lx3R, important for interaction with bHLH proteins, are indicated by a yellow square, the [A/S/G]N[D/A/N]V motif and the KPXPR[S/T]F motif, known to be conserved in anthocyanin regulators, are indicated by a red square and a blue square, respectively. The conserved amino acid sequence [DNEI] is shown by a black square. The horizontal line indicates the amino acid sequence of the R2-R3 region derived from the cPpP14 clone.
Peace regulates petal pigmentation in flowering peach. In this context, the expression pattern of Peace, which is strongly expressed in pink petals but not expressed in variegated petals, supports the view that Peace regulates the anthocyanin biosynthetic pathway and that it is variation in its expression that gives variegated petal pigmentation in flowering peach petals.

When Peace was compared with 20 other MYB transcription factors from plants, high similarity was seen between the 104 amino acids of the R2R3 domain, including the identity in some key amino acid positions which are important for interaction with bHLH proteins. This conservation also suggests that Peace encodes a transcription factor controlling anthocyanin biosynthesis in the petals of flowering peach, although a gene encoding a bHLH protein partner has not yet been identified.

Peace does not have the [A/S/G][N][D/A/N]V motif, which appears near the end of the R3 repeat and can be an identifier for anthocyanin regulators (Lin-Wang et al., 2010). However, at the same position, Peace shares the amino acid sequence DNEI with monocotyledonous anthocyanin regulators; ZmC1, ZmPl, and OsC1, and with AtMYB5, AtMYB32, and AtMYB123 (TT2). This suggests that Peace is functionally

Fig. 9. Phylogenetic tree of R2R3 MYB transcription factors showing the relationship between Peace and another 20 accessions belonging to subgroups 4, 5, 6, 7, and 15; the same as shown in Fig. 8.
more similar to AtMYB123 and AtMYB5 which are known
to regulate proanthocyanidin production partially redundant-
ly and to monocotyledonous anthocyanin regulators such as ZmC1, ZmPI or OsC1 (Gonzalez et al., 2009; Dubos et al., 2010). VvMYB5a, the protein from grapevine most similar to AtMYB5 has been shown to activate anthocyanin biosynthesis when expressed ectopically in tobacco (Deluc et al., 2006).

Phylogenetic analysis showed that Peace clustered with
AtMYB123 and anthocyanin regulators of monocotyledo-
 nous plants, such as ZmC1, ZmPI, and OsC1. Stracke et al. (2001) showed a correlation between functional conserva-
tion and sequence similarity in MYB transcription factors, for example, AtMYB123 and ZmC1 both regulate pigmentation.

Therefore it seems possible that Peace has retained the capacity to regulate anthocyanin biosynthesis by a similar manner as the regulators of anthocyanin biosynthesis from monocots or it has arisen de novo from a proanthocyanidin regulatory ancestor. It may be that the regulatory activity of Peace reflects the ability of other regulators of proantho-
cyanidin biosynthesis from dicots to regulate anthocyanidin biosynthesis, a function not previously recognized because of their concomitant activation of ANR and proanthocya-
nidin production. Recently, Ravaglia et al. (2013) suggested that MYB10 and MYBPA1 of nectarine (Prunus persica) exclusively regulate anthocyanin biosynthesis and proantho-
cyanidin biosynthesis, respectively, in the fruit skin and flesh. However, genes similar to Peace may also contribute to regulat-
ing anthocyanin biosynthesis and proanthocyanidin bio-
synthesis in peach.

The mechanisms regulating the difference in the expression of the Peace gene between pink and variegated petals remains unclear: FPMYB10, a PrMYB10 homologue, was a possible candidate to control the expression in flower colour or the differ-
ence in Peace gene expression. However, both in pink and
variegated flower buds, FPMYB10 is expressed at same level (Fig. 4C) and therefore it was difficult to consider this gene to be the cause of the difference in flower colour or Peace expression between pink and variegated flowers.

Another possible mechanism regulating the difference in Peace expression, could involve miRNA or siRNA. These are known to regulate expression of transcription factors including MYB proteins (Rhoades et al., 2002; Allen et al., 2007). Furthermore SERRATE in Arabidopsis could affect miRNA function to regulate gene expression (Lobbes et al., 2006). Recently, miR156-targeted SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) gene was revealed negatively regulating anthocyanin biosynthesis in Arabidopsis by dis-
rupting the MYB-bHLH-WD40 complex (Gou et al., 2011). In order to understand the mechanism regulating the differ-
ence in Peace expression, further investigations are required.

Both pink and variegated genomes possess a single copy of the Peace gene, according to the results of genomic Southern hybridization. Sequence analysis, in addition to genomic Southern blots, suggests the Peace gene to be identical in pink and variegated flowers, refuting previous suggestions that variegation results from insertion of a transposable ele-
ment (Chaparro et al., 1995). It is possible that the variegated

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (KAKENHI Grant number 07660011), by the Sumitomo Foundation for Grants for Basic Science Research Projects (Grant number 030678), and by The Daiwa Anglo-Japanese Foundation (Daiwa Foundation Small Grants to CU). Special thanks are due to co-author IM, who died suddenly from a brain tumour in 2011 at the age of 32. This report stands as a memorial to her sincere work and warm personality.

References

Allen RS, Li J, Staehle MI, Dubroue A, Gubler F, Milliar AA. 2007. Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis miR159 family. Proceedings of the National Academy of Sciences, USA 104, 16371–16376.

Bailey PC, Dicks J, Wang TL, Martin C. 2008. IT3F: a web-based tool for functional analysis of transcription factors in plants. Phytochemistry 69, 2417–2425.

Bailey PC, Martin C, Toledo-Ortiz G, Quail PH, Huq E, Heim MA, Jakoby M, Werber M, Weisshaar B. 2003. Update on the basic helix-loop-helix transcription factor gene family in Arabidopsis thaliana. The Plant Cell 15, 2497–2502.

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L. 2004. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana. The Plant Journal. 39, 366–380.

Bonas U, Sommer H, Harrison BJ, Saedler H. 1984. The transposable element Tam1 of Antirrhinum majus is 17 kb long. Molecular and General Genetics 194, 138–143.

Butelli E, Licciardello C, Zhang Y, Liu J, Mackay S, Bailey P, Reforgiato-Recupero G, Martin C. 2012. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. The Plant Cell 24, 1242–1255.
Chaparro JX, Werner DJ, Whetten RW, O’Malley DM. 1995. Characterization of an unstable anthocyanin phenotype and estimation of somatic mutation rates in Peach. Journal of Heredity 86, 186–193.

Cone KC, Coccione SM, Burr FA, Burr B. 1993. Maize anthocyanin regulatory gene pl is a duplicate of c1 that functions in the plant. The Plant Cell 5, 1795–1805.

de Vetten N, Quattrrocchio F, Mol J, Koes R. 1997. The an11 locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. Genes and Development 11, 1422–1434.

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Merillon JM, Hamdi S. 2006. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiology 140, 499–511.

Doodeman M, Boersma EA, Koomen W, Bianchi F. 1984. Genetic analysis of instability in Petunia hybrida. Theoretical and Applied Genetics 67, 345–355.

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. 2010. MYB transcription factors in Arabidopsis. Trends in Plant Science 15, 573–581.

Epperson BK, Clegg MT. 1987. Instability at a flower color locus in the morning glory. Journal of Heredity 78, 346–352.

Forkmann G. 1993. Genetics of flavonoids. In: Harborne JB, ed. The flavonoids: advances in research since 1986. London: Chapman & Hall, 537–564.

Frohman MA, Dush MK, Martin GR. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proceedings of the National Academy of Sciences, USA 85, 8998–9002.

Gerats AG, Huits H, Vrijlandt E, Maraña C, Souer E, Beld M. 1990. Molecular characterization of a nonautonomous transposable element (dTPh1) of petunia. The Plant Cell 2, 1121–1128.

Glover BJ, Martin C. 2012. Anthocyanins. Current Biology 22, R147–R150.

Gonzalez A, Mendenhall J, Huo Y, Lloyd A. 2009. TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation. Developmental Biology 325, 412–421.

Goodrich J, Carpenter R, Coen ES. 1992. A common gene regulates pigmentation pattern in diverse plant species. Cell 5, 955–964.

Gou JY, Filippes FF, Liu CJ, Weigel D, Wang JW. 2011. Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. The Plant Cell 23, 1512–1522.

Habu Y, Hisatomi Y, lida S. 1998. Molecular characterization of the mutable flaked allele for flower variegation in the common morning glory. The Plant Journal 16, 371–376.

Harrison BJ, Carpenter R. 1979. Resurgence of genetic instability in Antirrhinum majus. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 63, 47–66.

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC. 2003. The basic helix–loop–helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Molecular Biology and Evolution 20, 735–747.

Holton TA, Cornish EC. 1995. Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell 7, 1071–1083.

Imai Y. 1995. The mechanism of bud variation. The American Naturalist 69, 587–595.

Inagaki Y, Hisatomi Y, Suzuki T, Kasahara K, lida S. 1994. Isolation of a Suppressor- mutator/Enhancer-like transposable element, Tpn1, from Japanese morning glory bearing variegated flowers. The Plant Cell 6, 375–383.

Itoh Y, Higeta D, Suzuki A, Yoshida H, Ozeki Y. 2002. Excision of transposable elements from the chalcone isomerase and dihydroflavonol 4-reductase genes may contribute to the variegation of the yellow-flowered carnation (Dianthus caryophyllus). Plant and Cell Physiology 43, 578–585.

Jackson D, Roberts K, Martin C. 1992. Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of Antirrhinum majus. The Plant Journal 2, 425–434.

Katayama H, Uematsu C. 2003. Comparative analysis of chloroplast DNA in Pyrus species: physical map and gene localization. Theoretical and Applied Genetics 106, 303–310.

Kobayashi S, Goto-Yamamoto N, Hirochika H. 2004. Retrotransposon-induced mutations in grape skin color. Science 304, 982.

Kranz HD, Denekamp M, Greco R, et al. 1998. Towards functional characterisation of the members of the R2R3-MYB gene family from Arabidopsis thaliana. The Plant Journal 16, 263–276.

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairnetam S, McGhie T, Espley R, Hellrens R, Allan A. 2010. An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biology 10, 50.

Lobbes D, Rallapalli G, Schmidt DD, Martin C, Clarke J. 2006. SERRATE: a new player on the plant microRNA scene. EMBO Reports 7, 052–058.

Martin C, Carpenter R, Sommer H, Saelder H, Coen ES. 1985. Molecular analysis of instability in flower pigmentation of Antirrhinum majus, following isolation of the pallida locus by transposon tagging. EMBO Journal 4, 1625–1630.

Martin C, Gerats T. 1993. The control of flower coloration. In: Jordan BR, ed. The molecular biology of flowering. Wallingford: CAB International, 219–255.

Martin C, Mackay S, Carpenter R. 1988. Large-scale chromosomal restructuring is induced by the transposable element tam3 at the nivea locus of Antirrhinum majus. Genetics 119, 171–184.

Martin C, Paz-Ares J. 1997. MYB transcription factors in plants. Trends in Genetics 13, 67–73.

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E. 1991. Control of anthocyanin biosynthesis in flowers of Antirrhinum majus. The Plant Journal 1, 37–49.

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L. 2001. The arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. The Plant Cell 13, 2099–2114.

Nishizaki Y, Matsuba Y, Okamoto E, Okamura M, Ozeki Y, Sasaki N. 2011. Structure of the acyl-glucose-dependent anthocyanin 5-O-glucosyltransferase gene in carnations and its
disruption by transposable elements in some varieties. *Molecular Genetics and Genomics* **286**, 383–394.

**Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H.** 1987. The regulatory c1 locus of Zea mays encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. *EMBO Journal* **6**, 3553–3558.

**Pelsy F.** 2010. Molecular and cellular mechanisms of diversity within grapevine varieties. *Heredity* **104**, 331–340.

**Quattrocchio F, Wing JF, Leppen H, Mol J, Koes RE.** 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. *The Plant Cell* **5**, 1497–1512.

**Ravaglia D, Espley RV, Henry-Kirk RA, Andreotti C, Ziosi V, Hellens RP, Costa G, Allan AC.** 2013. Transcriptional regulation of flavonoid biosynthesis in nectarine (*Prunus persica*) by a set of R2R3 MYB transcription factors. *BMC Plant Biology* **13**, 68.

**Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP.** 2002. Prediction of plant microRNA targets. *Cell* **110**, 513–520.

**Sabolowski RW, Moyano E, Culianez-Macia FA, Schuch W, Martin C, Bevan M.** 1994. A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. *EMBO Journal* **13**, 128–137.

**Schwinn K, Venail J, Shang Y, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davies K, Martin C.** 2006. A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *The Plant Cell* **18**, 831–851.

**Shamel AD, Pomeroy CS.** 1936. Bud mutations in horticultural crops. *Journal of Heredity* **27**, 487–494.

**Snowden KC, Napoli CA.** 1998. *Psl*: a novel *Spm*-like transposable element from *Petunia hybrida*. *The Plant Journal* **14**, 43–54.

**Sommer H, Carpenter R, Harrison BJ, Saedler H.** 1985. The transposable element Tam3 of *Antirrhinum majus* generates a novel type of sequence alterations upon excision. *Molecular and General Genetics* **199**, 225–231.

**Stracke R, Werber M, Weisshaar B.** 2001. The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**, 447–456.

**Toledo-Ortiz G, Huq E, Quail PH.** 2003. The *Arabidopsis* basic-helix-loop-helix transcription factor family. *The Plant Cell* **15**, 1749–1770.

**Tsunoda T, Takagi T.** 1999. Estimating transcription factor bindability on DNA. *Bioinformatics* **15**, 622–630.

**Upadhyaya KC, Sommer H, Krebbers E, Saedler H.** 1985. The paramutagenic line niv-44 has a 5kb insert, Tam 2, in the chalcone synthase gene of *Antirrhinum majus*. *Molecular and General Genetics* **199**, 201–207.

**Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer J.** 1993. Development of the particle inflow gun. *Plant Cell, Tissue and Organ Culture* **33**, 237–246.

**van Houwelingen A, Souer E, Spelt K, Kloos D, Mol J, Koes R.** 1998. Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida*. *The Plant Journal* **13**, 39–50.

**Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC.** 1999. The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *The Plant Cell* **11**, 1337–1350.

**Whitham TG, Slodochikoff CN.** 1981. Evolution by individuals, plant–herbivore interactions, and mosaic of genetic variability: The adaptive significance of somatic mutations in plants. *Oecologia* **49**, 287–292.

**Yamamoto T, Mochida K, Hayashi T.** 2003a. Shanhai Suimitsuto, one of the origins of Japanese peach cultivars. *Journal of the Japanese Society for Horticultural Science* **72**, 116–121.

**Yamamoto T, Mochida K, Imai T, Haji T, Yaegaki H, Yamaguchi M, Matsuta N, Ogiwara I, Hayashi T.** 2003b. Parentage analysis in Japanese peaches using SSR markers. *Breeding Science* **53**, 35–40.

**Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF.** 2004. Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. *The Plant Journal* **40**, 22–34.