

Deletion of the miR172 target site in a TOE-type gene is a strong candidate variant for dominant double-flower trait in Rosaceae

Stefano Gattolin^{1,2}, Marco Cirilli³, Igor Pacheco^{3,4}, Angelo Ciacciulli³, Cássia Da Silva Linge^{3,5}, Jehan-Baptiste Mauroux^{6,7}, Patrick Lambert⁶, Elia Cammarata³, Daniele Bassi³ , Thierry Pascal⁶ and Laura Rossini^{3,*} 

¹PTP Science Park Lodi, Via Einstein, Loc. Cascina Codazza, 26900 Lodi, Italy,

²CNR-IBBA, Via Bassini 15, 20133 Milano, Italy,

³Università degli Studi di Milano – DiSAA, Via Celoria 2, 20133 Milano, Italy,

⁴Instituto de Nutrición y Tecnología de los Alimentos – Universidad de Chile, El Libano 5524, Santiago, Chile,

⁵Department of Plant and Environmental Sciences, Clemson University, 105 Collins St., Clemson, SC, USA,

⁶GAFL, INRA, 84140, Montfavet, France, and

⁷AGRO SELECTIONS FRUITS, La Prade de Mosseillous – CS70001, 66201, Elne, Cedex, France

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*For correspondence (e-mail laura.rossini@unimi.it).

SUMMARY

Double flowers with supernumerary petals have been selected by humans for their attractive appearance and commercial value in several ornamental plants, including *Prunus persica* (peach), a recognized model for Rosaceae genetics and genomics. Despite the relevance of this trait, knowledge of the underlying genes is limited. Of two distinct loci controlling the double-flower phenotype in peach, we focused on the dominant *Di2* locus. High-resolution linkage mapping in five segregating progenies delimited *Di2* to an interval spanning 150 858 bp and 22 genes, including *Prupe.6G242400* encoding an euAP2 transcription factor. Analyzing genomic resequencing data from single- and double-flower accessions, we identified a deletion spanning the binding site for miR172 in *Prupe.6G242400* as a candidate variant for the double-flower trait, and we showed transcript expression for both wild-type and deleted alleles. Consistent with the proposed role in controlling petal number, *Prupe.6G242400* is expressed in buds at critical times for floral development. The indel*Di2* molecular marker designed on this sequence variant co-segregated with the phenotype in 621 progenies, accounting for the dominant inheritance of the *Di2* locus. Further corroborating the results in peach, we identified a distinct but similar mutation in the ortholog of *Prupe.6G242400* in double-flower roses. Phylogenetic analysis showed that these two genes belong to a TARGET OF EAT (TOE)-type clade not represented in Arabidopsis, indicating a divergence of gene functions between AP2-type and TOE-type factors in Arabidopsis and other species. The identification of orthologous candidate genes for the double-flower phenotype in two important Rosaceae species provides valuable information to understand the genetic control of this trait in other major ornamental plants.

Keywords: *Prunus persica* L. Batsch, *Rosa hybrida*, ornamental traits, petal number, linkage mapping, AP2.

INTRODUCTION

Many plant species are grown worldwide as ornamentals for aesthetic characteristics such as flower morphology and size. The *Prunus* genus includes several species traditionally cultivated for ornamental purposes, such as the Japanese apricot (*Prunus mume*), Japanese cherry (*Prunus serrulata*), and dwarf flowering almond (*Prunus glandulosa*). Peach (*Prunus persica* L. Batsch) is a major fruit tree crop and is also appreciated as an ornamental plant, especially in Asian countries such as China, where rich diversity

is present for traits related to tree growth habit and flower type. Various ornamental cultivars are readily available in many countries (Hu *et al.*, 2003, 2005). The peach flower is actinomorphic, and is typically characterized by five sepals, five petals, many spirally arranged stamens and one pistil (Layne and Bassi, 2008), an ancestral single-flower structure shared by several Rosaceae species, including *Rosa* spp. (rose; Potter *et al.*, 2007); however, peach exhibits phenotypic variants, with supernumerary petals that

represent the double-flower trait (Yoshida *et al.*, 2000). Two distinct loci were identified for the control of the double-flower phenotype in peach. The first locus was described as a monogenic recessive trait (*D1/d1*, single flower/double flower) by Lammerts (1945), and is currently assigned to linkage group 2 of the *Prunus* reference map (Dirlewanger *et al.*, 2004). The second locus, harboring a single dominant gene (*Di2/di2*, double flower/single flower), first described by Beckman *et al.* (2012), was recently mapped to chromosome 6, although within a large mapping interval (Pascal *et al.*, 2017). Despite this progress, both the *D1* and *Di2* genes remain unknown.

Flower formation is a pivotal process in angiosperms, as it ensures reproductive success. Flower development requires the formation of four different organ types arranged in concentric whorls: sepals, petals, stamens and carpels. The specification of floral domains was explained through the ABC model described below (Bowman *et al.*, 1991; Coen and Meyerowitz, 1991), initially deduced from loss-of-function mutants in *Arabidopsis* and *Antirrhinum*. According to this model, *Arabidopsis* A-function genes *APETALA 1* (*AP1*) and particularly *APETALA 2* (*AP2*) ensure the development of sepals in the first floral whorl. The activity of B-function genes *APETALA 3* (*AP3*) and *PISTILLATA* (*PI*) overlaps with that of the A-function genes in the second whorl, and this confers petal identity. The C-function gene *AGAMOUS* (*AG*), together with *AP3* and *PI*, promotes stamen identity in the third whorl, whereas *AG* establishes carpel development in the fourth whorl. Further studies revealed additional D-function genes responsible for ovule identity (Pinyopich *et al.*, 2003), and four redundant E-function *SEPALLATA* (*SEP*) genes (Pelaz *et al.*, 2000; Ditta *et al.*, 2004) required for the specification of all floral organ identities, leading to the ABCDE model (Rijkema *et al.*, 2010). Cloning of the ABC genes showed that *AP1*, *AP3*, *PI*, *SEP* and *AG* all encode MADS-box domain proteins (Honma and Goto, 2001). Instead, *AP2* belongs to the euAP2 lineage (a subgroup of the large AP2/ERF Ethylene Responsive Factor superfamily), typically characterized by the presence of two AP2 domains and a target site for microRNA miR172 (Jofuku *et al.*, 1994; Riechmann *et al.*, 2000; Kim *et al.*, 2006). This lineage can be further divided into two types, according to relatedness to *AP2* and *TARGET OF EAT* (*TOE*) genes (Wang *et al.*, 2016). In *Arabidopsis*, the two identified AP2-type genes *AP2* and *TOE3* are involved in the specification of floral organ identity, whereas the TOE-type genes *TOE1*, *TOE2*, *SCHLAFMUTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*) seem to play a role in developmental transitions (Zhu and Helliwell, 2011). miR172-mediated AP2 repression was shown to be essential for the timely termination of floral stem cells and the maintenance of floral meristem size (Zhao *et al.*, 2007). Overexpression of a miR172-resistant version of *AP2* in *Arabidopsis* raised AP2 protein levels and led to an

indeterminate floral meristem producing supernumerary stamens or petals (Chen, 2004). *TOE3* was also shown to be critical for floral patterning, by forming homo- and heterodimers with AP2 in the nucleus (Jung *et al.*, 2014). In contrast to *Arabidopsis*, *ROB1*, *ROB2* and *ROB3* (AP2-type) redundantly repress the B function alone in the first floral whorl of *Petunia*, whereas *BEN* and *BOB* (TOE-type) regulate both B and C functions, suggesting a divergence of euAP2 gene functions between asterids and rosids (Morel *et al.*, 2017).

In order to provide insight into the molecular genetic basis of the dominant double-flower trait, we report the fine mapping of the *Di2* locus in peach and the identification of a candidate causal mutation in the *Prupe.6G242400* gene, encoding a TOE-type member of the euAP2 lineage of transcription factors. We describe the identification of a distinct but similar variant in the rose ortholog of *Prupe.6G242400* that is likely to be involved in the development of supernumerary petals in this genus.

RESULTS

The double-flower phenotype in peach

Peach trees carrying the dominant *Di2* allele develop double flowers with more than five petals, in contrast to the five petals typical of homozygous *di2* wild-type plants. In this study, four parents were used: 'Bounty' (By) and 'Pamirsij 5' (P), characterized by wild-type single flowers, 'NJ Weeping' (W) and 'Weeping Flower Peach' (WFP), exhibiting flowers with supernumerary petals (Figure 1a). On average, W and WFP display flowers with 14.3 and 19.1 petals, respectively (Figure 1b). The F₂ WxBy cross segregated double flowers versus single flowers in a ratio of about 3:1 ($\chi^2 = 0.33\text{--}3.41$), consistent with a monogenic dominant inheritance. Segregation in the F₂ WP² population deviated from the expected pattern ($\chi^2 = 16.10$), showing an excess of single-flower seedlings (Table 1). Segregation distortion for the *Di2* trait has previously been reported (Beckman *et al.*, 2012; Pascal *et al.*, 2017). In double-flower seedlings of the WP² progeny, supernumerary petals varied in a quantitative manner, ranging from 15.8 to 91.0, in parallel with stamen numbers (Appendix S1). A small percentage of seedlings (about 14%) also showed flowers with double the number of sepals (10 versus five).

High-resolution mapping of the *Di2* locus

The *Di2* locus was recently mapped on chromosome 6, in a large genomic region spanning approximately 7 Mbp (Pascal *et al.*, 2017). To fine map the *Di2* locus, the WP² progeny was genotyped with the IPSC 9k SNP array, restricting the mapping interval on chromosome 6 to 194 366 bp, between SNP_IGA_680329 and SNP_IGA_681209 (24 006 441–24 200 807; Figure 2a). The same locus was also identified in the WxBy^A progeny, spanning

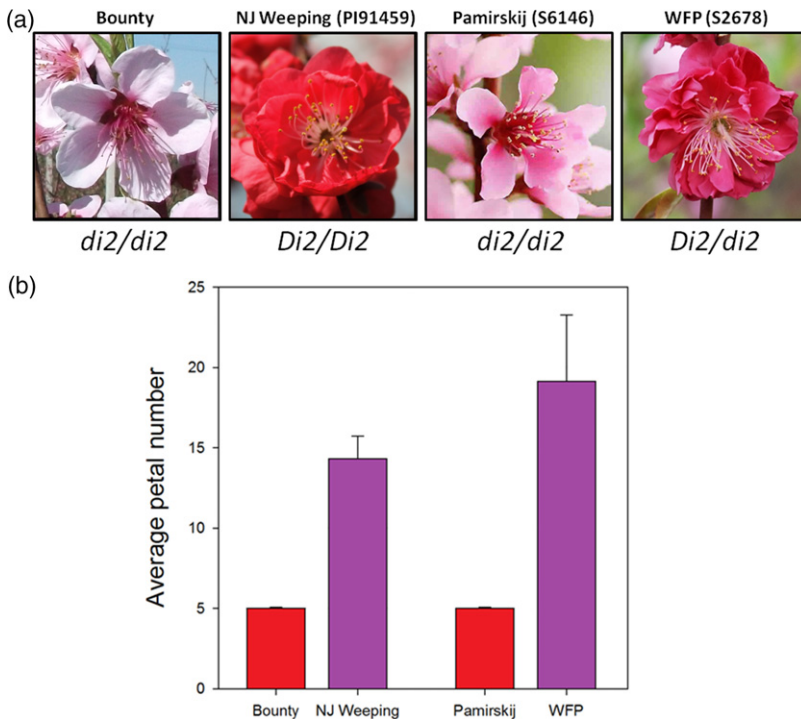


Figure 1. Single and double flower phenotypes in peach. (a) Flower morphology and (b) average petal number of the four peach parental lines used in this study. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1 Segregation of the double-flower trait within the five progenies used in this study

Cross	No. of seedlings	Flower type			χ^2
		Double <i>Di2</i>	Single <i>di2</i>	Expected ratio	
PI91459 (NJ Weeping) × Bounty F ₂ (06004) ^A	123	95	28	3:1	0.33
PI91459 (NJ Weeping) × Bounty F ₂ (06002) ^B	87	60	27	3:1	1.68
PI91459 (NJ Weeping) × Bounty F ₂ (99018) ^C	37	25	12	3:1	1.09
PI91459 (NJ Weeping) × Bounty F ₂ (99019) ^D	61	52	9	3:1	3.41
S2678 (Weeping Flower Peach) × S6146 (Pamirskij) F ₂	313	204	109	3:1	16.10

a region of 496 461 bp, between SNP_IGA_680124 and SNP_IGA_681888 (23 906 028–24 402 489; Figure 2a). Based on the overlap of mapping intervals, we hypothesized that the *Di2* locus was likely to be responsible for the dominant double-flower phenotype in both WxBy and WP² progenies. To further delimit the position of *Di2*, newly developed dCAPS and KASPTM markers were used to genotype the F₂ WxBy^{B,C,D} and the WP² progenies, respectively: recombinant individuals allowed further delimitation of the locus to a 150 858-bp region, ranging between SNP_IGA_680329 and SNP_IGA_680909 (24 006 441–24 157 299; Figure 2b).

Identification of a deletion in an euAP2 family gene as a candidate variant for the *Di2* locus

Based on annotations of peach reference transcripts v2.1, the minimum mapping interval contains 22 gene models, from *Prupe.6G241600* to *Prupe.6G243700* (Table S1). The

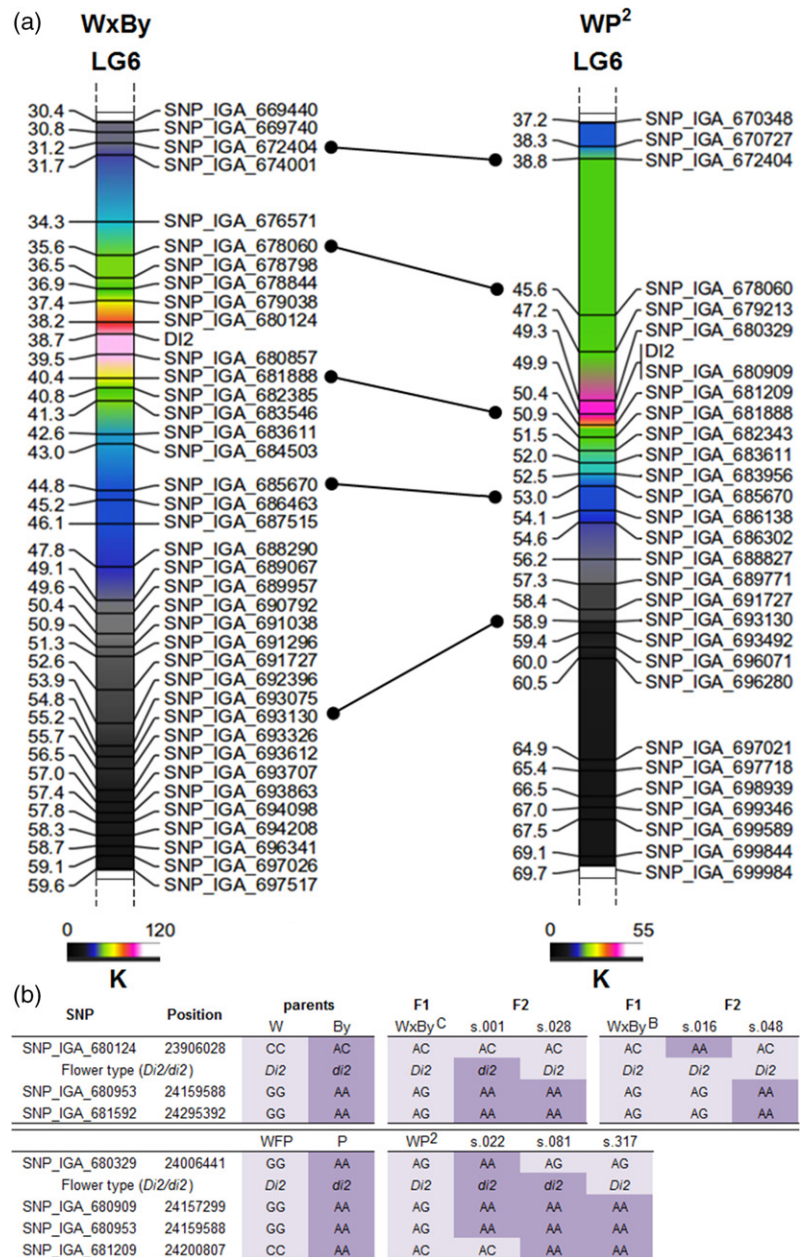
gene inventory includes different transcripts involved in transcriptional regulation (such as a MYB-G-like, a zinc finger and two pentatricopeptide repeat-containing proteins) and disease resistance (four RGA-like proteins). The most promising candidate was clearly represented by *Prupe.6G242400*, showing similarity to the homeotic gene *AP2*, member of the euAP2 subfamily and known to be involved in flower development (Kim *et al.*, 2006).

Further insights into genomic variants within the *Di2* locus were obtained by inspecting whole-genome resequencing data of By and W parents. For a more stringent identification of candidate variants, additional resequencing data of 11 single-flower accessions were also analyzed (Appendix S2; Cirilli *et al.*, 2018). According to the dominant inheritance of the *Di2* gene and the segregation pattern in 15 WxBy F₁ individuals (all showing the double-flower phenotype), the causative variant within the target interval should be present and homozygous only in W.

Figure 2. Genetic mapping of the *Di2* double-flower locus.

(a) Location of the *Di2* locus on the linkage group 6 (LG6) map in 'NJ Weeping' (W) × 'Bounty' (By) and 'Weeping Flower Peach' (WFP) × Pamirskij 5 (P) progenies. Marker names and genetic distances (in centimorgans, cM) are listed to the right and left of LG6, respectively. The quantitative trait locus (QTL) is represented as a color gradient corresponding to the respective K scales (reported at bottom).

(b) Recombinants analysis of the *Di2* locus region in WP², WxBy^B and WxBy^C progenies. The genotype of the delimiting markers is indicated in parental lines, F₁ and informative F₂ individuals.



After filtering by these selection criteria, a total of 151 genomic variants were identified in W that were mostly single-nucleotide polymorphisms (SNPs; Appendix S2). About half of them (71 variants) are located in coding regions of the transcripts *Prupe.6G242400*, *Prupe.6G243400* and *Prupe.6G243700*, whereas the rest are almost equally distributed in intergenic or upstream/downstream gene regions. In *Prupe.6G243400* and *Prupe.6G243700*, which code for RGA-like disease-resistance genes, several SNPs were detected causing missense or frameshift mutations in the predicted proteins. The involvement of these two genes in controlling flower morphology seems unlikely,

however, as many of these variants are present in single-flower accessions (Appendix S2). The G/T variant at position 24 074 941 in *Prupe.6G242400* introduces a premature stop codon (S435*) in the 10th exon. Interestingly, this mutation is absent in W, whereas it is homozygous or heterozygous in several single-flower accessions, including By. Through the inspection of W alignment data, a large deletion was found near the C terminus of the same gene (Figure 3a). Sequencing of a 390-bp cDNA fragment obtained by 3'-RACE from the W parent confirmed a 994-bp deletion. The missing genomic portion (24 074 355–24 075 350) covers a region between the ninth intron and

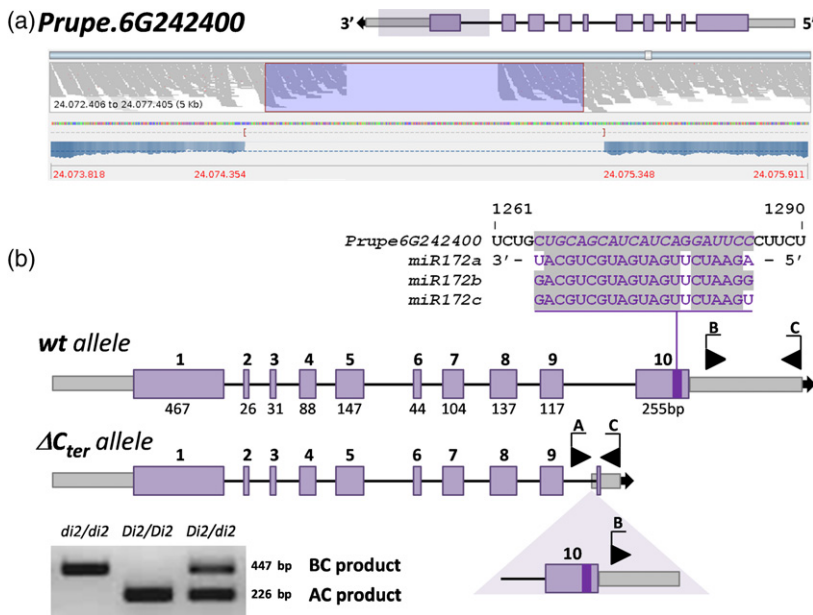


Figure 3. Molecular analysis of sequence variants of the Prupe.6G242400 candidate gene.

(a) Large deletion identified near the C terminus of Prupe.6G242400 (Pp06: 24 074 355–24 075 350) in the W parent.

(b) Prupe.6G242400 and ΔC_{ter} gene models with the position of the A, B and C primers, designed to genotype the two alleles, marked with arrowheads. The putative miR172 binding site within exon 10 is also indicated, along with the sequences of peach ppa-miR172a, b and c (from Zhu *et al.*, 2012). PCR products obtained with the indicated primers in By (*di2/di2*), W (*Di2/Di2*) and F_1 WxB Y^A (*Di2/di2*) are shown in the panel at the bottom left-hand side. [Colour figure can be viewed at wileyonlinelibrary.com].

the 3' untranslated region (3'-UTR) of the annotated gene, ending 474 bp downstream of the stop codon. As a result, the W allele of Prupe.6G242400 (herewith indicated as the ΔC_{ter} allele) lacks the 10th exon and the miR172 binding site within (Figure 3b; Appendix S3). Similar to other (semi)dominant mutations in miRNA binding sites (McConnell *et al.*, 2001; Emery *et al.*, 2003; Juarez *et al.*, 2004; Ochando *et al.*, 2006), the deletion of the miR172 binding site might result in the upregulation of the Prupe.6G242400 gene product, explaining the dominant inheritance of the *Di2* trait.

A co-dominant PCR marker (named indelDi2) developed on the mutated ΔC_{ter} allele was used to genotype the four parents (W, *Di2/Di2*; By, *di2/di2*; WFP, *Di2/di2*; P, *di2/di2*) and the F_2 individuals of all five progenies (621 plants), and showed a complete co-segregation with the phenotype (Appendix S1). This is also supported by the WP^2 progeny, where an excess of single-flower phenotypes is associated with a low frequency of individuals homozygous and heterozygous for the ΔC_{ter} allele. These results provide further evidence for the ΔC_{ter} deletion in Prupe.6G242400 as a strong candidate variant for the *Di2* locus.

Genotyping with the indelDi2 marker also allowed the discrimination of individuals that were homozygous or heterozygous for the mutation, and to test the hypothesis of a dosage effect on petal number (Figure 4). In the WP^2 progeny, supernumerary petals showed a non-normal distribution, with a skewness towards an average of 20–30 petals (Figure 4). Individuals homozygous for the ΔC_{ter} allele (*Di2/Di2*) bear a higher number of petals (with a maximum frequency around 55–60), also accompanied by the presence of 10 sepals (Figure 4) and more than 100

stamens (Appendix S1). The allele-dosage effect may arise from the presence of one or more modulating genes with an additive effect on petal number in the WP^2 progeny only, and (associated with a factor) affecting fertility/viability. In support of this hypothesis, the W parent, although being homozygous for the ΔC_{ter} deletion, shows an average petal number that is lower than 20. Besides the *Di2* locus, no other significant signals emerged from quantitative trait locus (QTL) analysis in the WP^2 progeny using quantitative data for petal number (Figure S1); however, plotting the chi-square value distribution on chromosome 6, segregating markers showed a distortion peak at about 10 Mbp, near SNP_IGA_635084 (Figure S2). A factor located in this genomic region may affect both average petal number in *Di2/Di2* individuals and flower fertility/viability, leading to segregation distortion in neighboring markers and traits.

Expression profiles of peach euAP2 lineage genes

Peach euAP2 proteins possess several well-conserved domains (Wang *et al.*, 2016), including the two AP2 domains (AP2-R1 and AP2-R2), except the Prupe.2G220100 that harbors a deletion within AP2-R2. They also contain EAR (LxLxLx or DLNxxP) and nuclear localization signal (NLS) motifs, and a linker domain, whereas the target sequence for miR172 binding also translates into a conserved amino acid sequence (Figure 5).

In order to gain further insight into the potential role of Prupe.6G242400 and other peach euAP2 genes in the control of flower development and morphology, and their possible regulation by miR172, gene expression analysis was carried out on developing peach buds sampled during the period of floral differentiation (from 180 to 240 Julian days,

Figure 4. Distribution frequency and average petal number in $WP^2 F_2$ individuals. Plants are grouped according to heterozygosity ($Di2/di2$) and homozygosity ($Di2/Di2$) for the $\Delta Cter$ allele. Sepal number (five or 10) is represented by horizontal lines. [Colour figure can be viewed at wileyonlinelibrary.com].

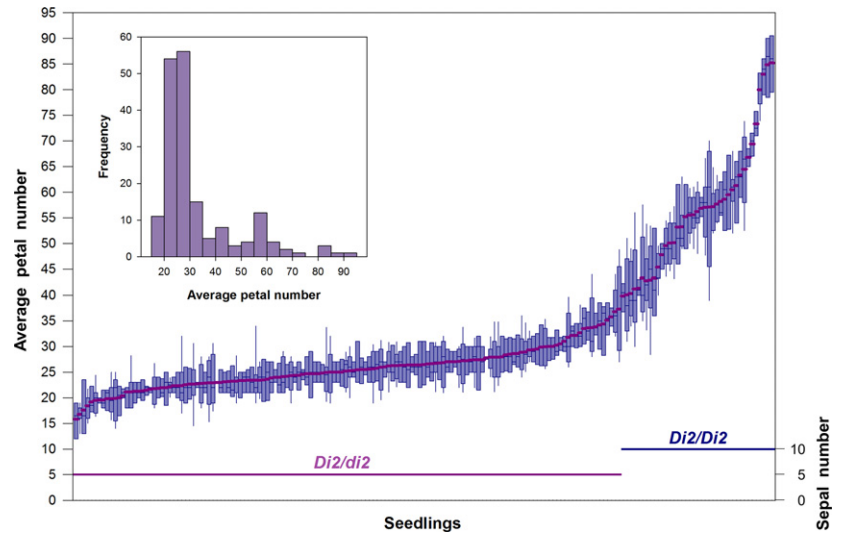
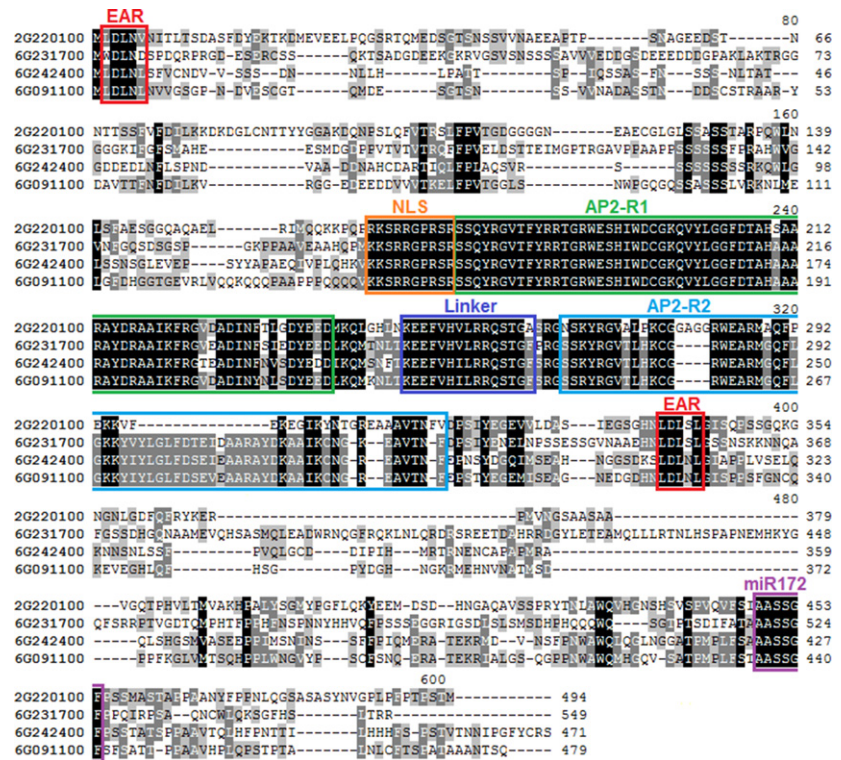


Figure 5. Alignment of deduced amino acid sequences of peach euAP2 proteins. Black and grayscale shading indicate identical amino acid residues and the degree of conservation of amino acid residues, respectively. The two AP2 domains, AP2-R1 and AP2-R2, the EAR motif-like sequences, a putative nuclear localization signal (NLS) motif, a linker domain and the sequence derived from the target site for miR172 binding are also indicated. [Colour figure can be viewed at wileyonlinelibrary.com].



JD) in W (homozygous for the $\Delta Cter$ allele) and By (homozygous for the wt allele). Amplification of both alleles was achieved in all three stages sampled (Figure 6). The expression of *Prupe.6G242400* decreased over time, showing comparable levels in W ($\Delta Cter$) and By (wt), except for higher expression in W at 210 JD. Conversely, miR172 levels tended to increase along the stages sampled, being significantly higher in By. Regarding other euAP2 genes, *Prupe.6G231700* levels strongly increased at 240 JD in W, whereas the expression of *Prupe.6G091100* was higher in

By at 180 JD compared with W, with transcript levels becoming comparable at later stages. Finally, *Prupe.2G220100* transcript was not detected in the tissues analyzed.

Collectively, these results confirm the expression of the $\Delta Cter$ allele and show an increase of miR172 over the course of flower development, which could modulate the activity of *Prupe.6G242400*, as well as that of other euAP2 transcription factors potentially involved in flower morphogenesis.

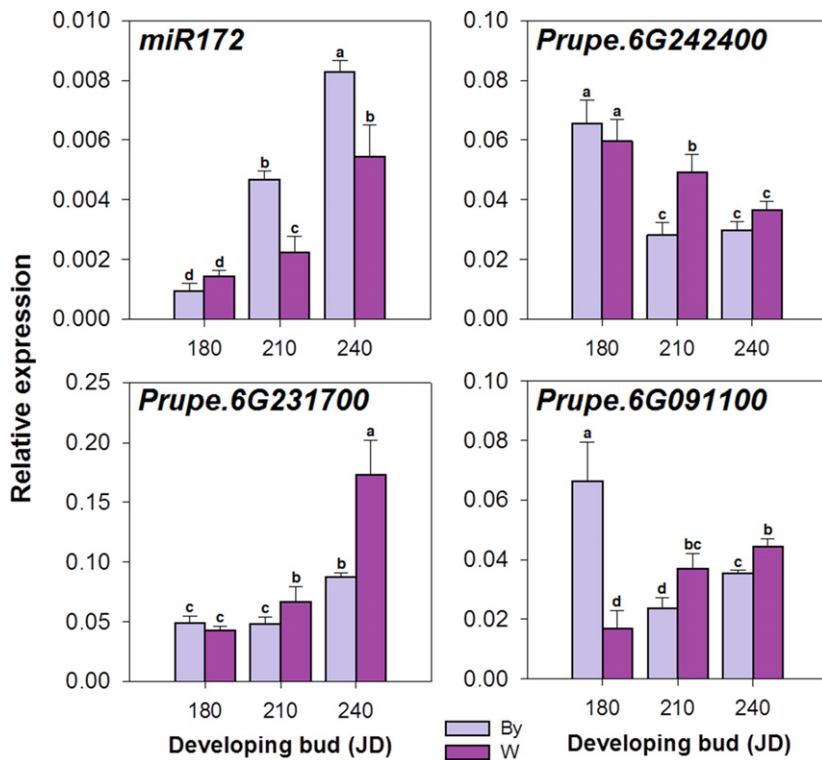


Figure 6. Expression pattern of peach euAP2 genes and miR172 (isoforms a, b and c) in developing buds. RNA was extracted from developing buds collected from on-year shoots at various stages (180, 210 and 240 Julian days) in 'NJ Weeping' (W) and 'Bounty' (By) accessions. The *Actin* gene (Tatsuki *et al.*, 2013) was used as a reference to normalize expression data. Letters indicate significant differences according to one-way ANOVA with Tukey's honest significant difference (HSD) test ($P < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com].

A mutation disrupting the miR172 target site of the ortholog of *Prupe.6G242400* is present in double-flower roses

Among Rosaceae, the genus *Rosa* is well known for the spectacular variety of flower morphologies and is an interesting model to gain further support for the role of *Prupe.6G242400* and related TOE-type genes in the control of petal number. The flowers of wild roses typically bear five petals, whereas double-flower forms have been selected in most cultivated roses. Similar to peach, a dominant double-flower locus named *Blfo* is known in rose (Spiller *et al.*, 2011). Based on relatedness among Rosaceae genomes, we reasoned that the ortholog of *Prupe.6G242400* might also be involved in controlling this trait in rose. A similarity search was performed on a publicly available rose expressed sequence tag (EST) database assembled from double-flower *Rosa hybrida* 'Samantha' (http://bioinfo.bti.cornell.edu/cgi-bin/rose_454/index.cgi), retrieving two unigenes similar to *Prupe.6G242400*: (i) the first, *RAG04722* (1621 bp), containing the miR172 binding site and encoding a putative TOE-type full-length protein of 461 amino acids with 68% identity and 74.8% similarity to *Prupe.6G242400* (Appendix S4a); and (ii) the second, *RAG04721*, near identical to the first but lacking the miR172 binding site as a result of a divergence in the sequence starting at position 1189 from the putative ATG start codon (Appendix S4b). We hypothesized that these two transcripts represent allelic variants of the rose

ortholog of *Prupe.6G242400*, and confirmed their existence by Sanger sequencing of genomic DNA and cDNA products from flower buds of the double-flower rose 'Meimarmoto' (Figure 7a). This hypothesis was confirmed by mapping the *RAG04721* transcript on the recently released rose genome assembly of the double-flower *Rosa chinensis* 'Old Blush' (Raymond *et al.*, 2018): several retroelement-like features were identified between the annotated gene model *RchiOBHmChr3 g0468481* (chromosome 3: 14 492 546–14 495 427), which encodes the *RAG04721* transcript (5'-UTR plus exon I–exon VIII), and a region annotated as *RchiOBHmChr3 g0468491* aligning to the 3' portion of *RAG04722*, located about 10 kb downstream of *RchiOBHmChr3 g0468481* (chromosome 3: 14 505 776–14 506 765). This is consistent with an insertion within the TOE-like gene encoding transcript *RAG04722*. Based on comparison with cDNA sequences, this insertion is the cause of an alternative splicing event that leads to the production of transcript *RAG04721* encoding a truncated protein and lacking the miR172 target site (Appendix S4). Furthermore, a genetic marker within the *Blfo* locus, *Rh50* (Spiller *et al.*, 2011), maps in proximity (chromosome 3: 13 002 941–13 003 241) to *RchiOBHmChr3 g0468481*, adding evidence for this being the candidate allele for the double-flower phenotype in rose.

Polymerase chain reaction (PCR) primers flanking the insertion junction (Figure 7b) were used to genotype 35 rose accessions, showing a clear correspondence between

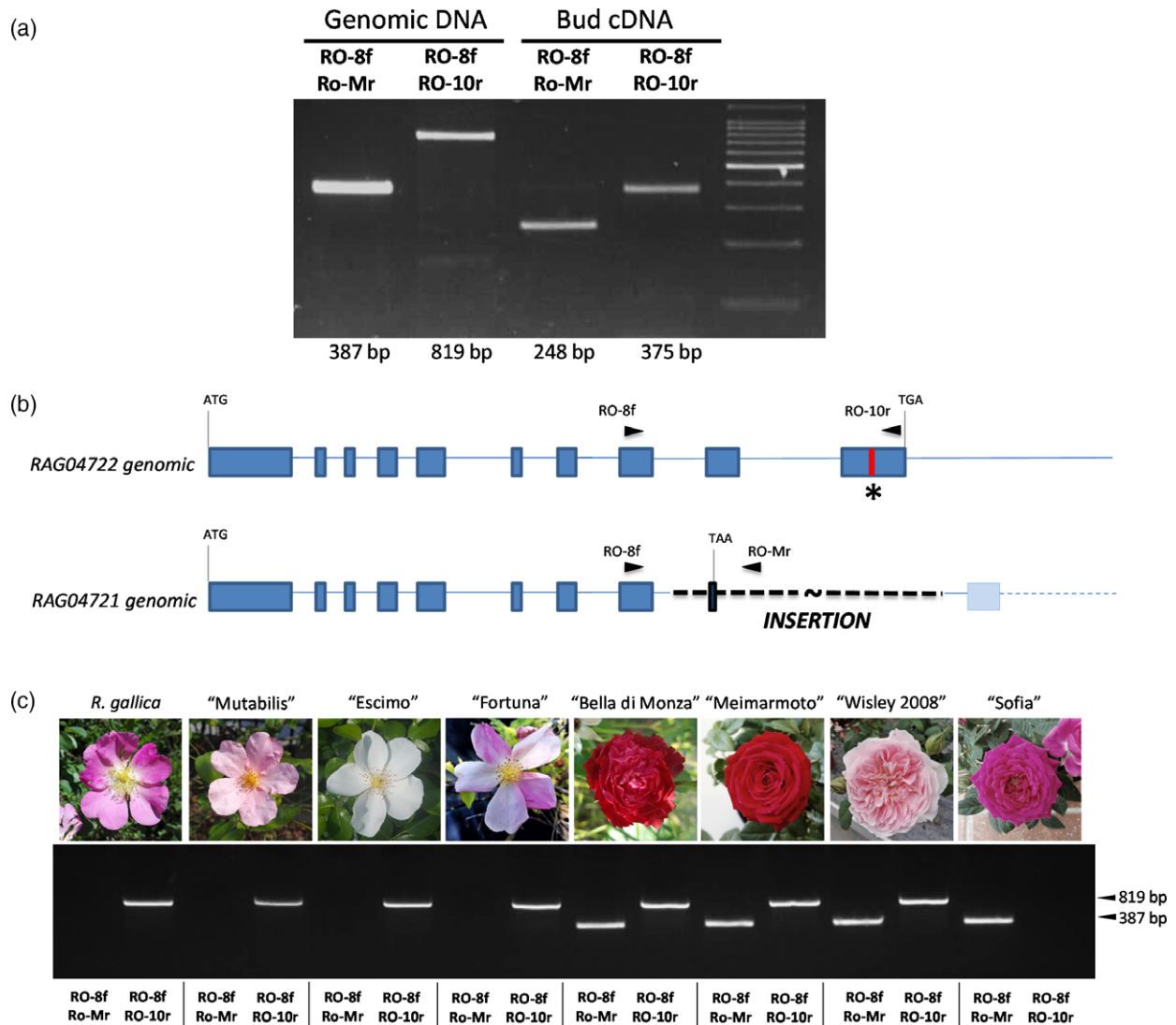


Figure 7. Molecular analysis of the RAG04722 and RAG04721 rose alleles. (a) PCR products obtained with the indicated primers using genomic DNA and flower bud cDNA of rose variety ‘Meimarmoto’ (Red Meilandina® Plus); amplicon sizes are indicated at the bottom. (b) Gene models of wild-type (*RAG04722*) and mutated (*RAG04721*) rose alleles, with the position of the RO-8f, RO-10r and RO-Mr primers designed to genotype them indicated with arrowheads. The putative miR172 binding site within exon 10 is indicated with an asterisk. (c) PCR analysis of four single-flower (left) and four double-flower (right) rose varieties. The primer combinations are specific for the mutated (RO-8f/RO-Mr) or the wild-type allele (RO-8f/RO-10r). [Colour figure can be viewed at wileyonlinelibrary.com].

the double-flower trait and the variant lacking the miR172 site (Appendix S5), similar to what was seen with the $\Delta Cter$ allele in peach. Figure 7(c) shows the genotyping results for a subset of four single-flower and four double-flower roses.

Finally, a phylogenetic analysis was conducted to study the degree of relatedness between the euAP2 protein sequences from different plant species: *Arabidopsis thaliana*, *Cucumis sativus*, *Oryza sativa*, *Prunus persica*, *Petunia axillaris*, *Petunia hybrida*, *Rosa chinensis* and *Vitis vinifera* (Appendix S6). This study revealed four major clades, three

of which included annotated Arabidopsis peptides: (i) TOE2 and SMZ/SNZ; (ii) AP2/TOE3; and (iii) TOE1 (Figure 8). Interestingly, *Prupe.6G242400* belongs to a fourth clade that includes rose *RAG04722*, PhBEN and PhBOB (Morel *et al.*, 2017), but no Arabidopsis orthologs.

DISCUSSION

The elucidation of molecular mechanisms underlying diversity in flower traits is of great importance, both to deepen our knowledge of flower biology and to assist with the development of new varieties for the ornamental

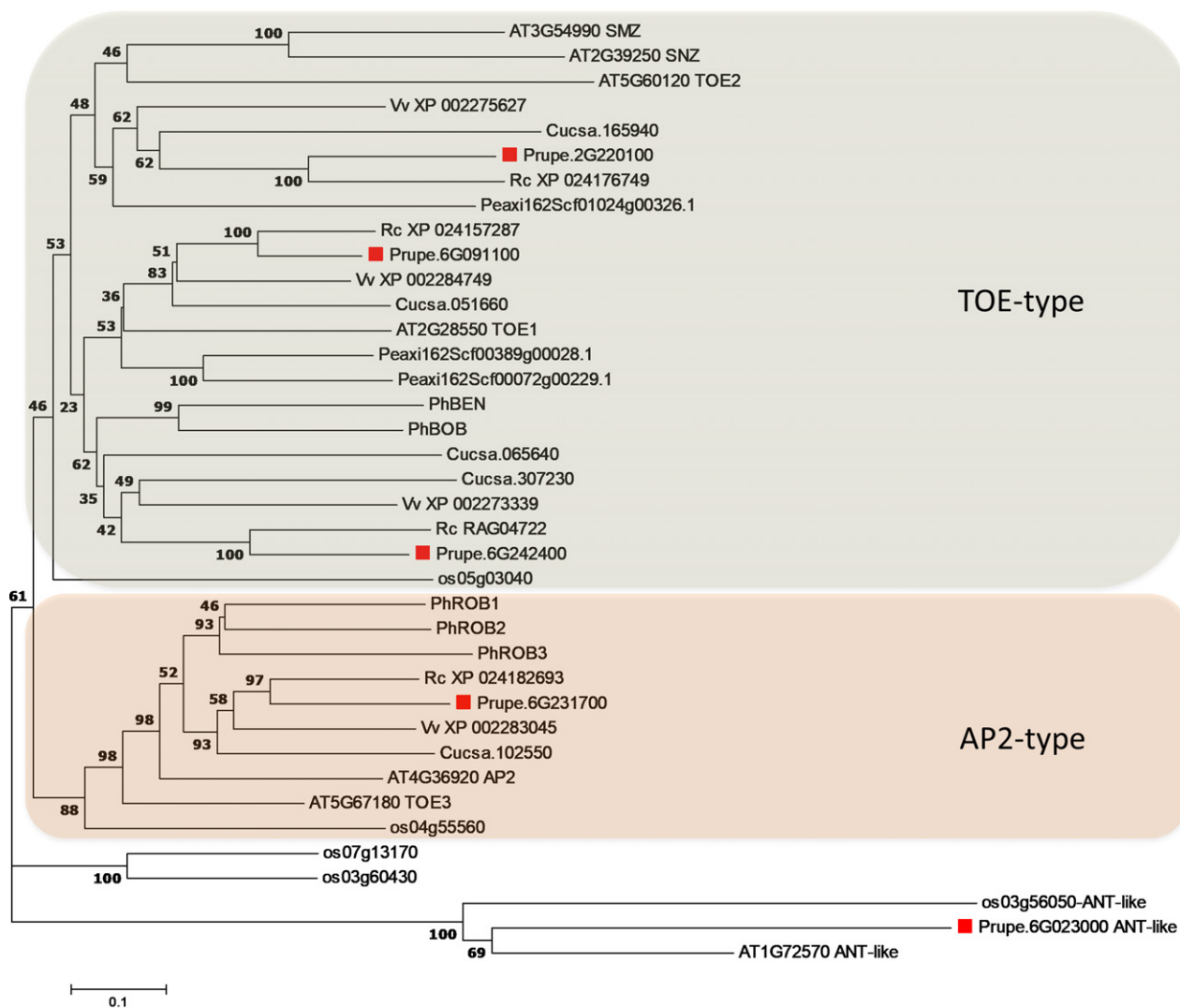


Figure 8. Evolutionary relationships among euAP2 (AP2- and TOE-type) members in peach and other species. Evolutionary relationships were inferred by using the minimum evolution method. The reliability of the phylogenetic tree was estimated by setting 500 bootstrap replicates. Peach sequences are indicated by squares. Peptide sequences are from: *Arabidopsis thaliana* (AT), *Cucumis sativus* (Cucsa), *Oryza sativa* (Os), *Petunia axillaris* (Peaxi), *Petunia hybrida* (Ph), *Prunus persica* (Prupe), *Rosa chinensis* (Rc) and *Vitis vinifera* (Vv). [Colour figure can be viewed at wileyonlinelibrary.com.]

market. Peach is an important fruit tree species and an appreciated ornamental plant for its abundant bloom, as well as a model species for Rosaceae genomics. Wild and cultivated peach germplasm harbors a huge diversity in ornamental traits, particularly those related to tree growth habit (Hollender *et al.*, 2018) and flower appearance. Desirable traits related to flower doubleness have been known for a long time (Lammerts, 1945), and two loci were mapped to chromosomes 2 and 6, but to date the underlying genes remained unknown. Through linkage analyses we narrowed the interval of the dominant *Di2* locus on chromosome 6 from 7 317 354 bp (Pascal *et al.*, 2017) to 150 858 bp. We identified a 994-bp deletion near the C terminus of *Prupe.6G242400* as a prime candidate for *Di2*,

given the critical role played by euAP2 transcription factors in regulating flower development. We then showed that double-flower rose varieties harbor a distinct but similar mutation in the ortholog of *Prupe.6G242400*.

Flower doubleness is not an uncommon trait among eudicots, and recessive factors are known to be responsible for this phenotype in many plants, including *Papaver somniferum*, *Nicotiana glauca* and *Matthiola incana* (Belyaeva, 1995; Zainol *et al.*, 1998; Nakatsuka and Koishi, 2018). Alterations in the expression of C-function genes seem to play a central role in the production of supernumerary petals, because of the negative regulation of AG-like factors on WUSCHEL (WUS), a homeodomain transcription factor that regulates meristem size (Sharma

et al., 2003; Zhao *et al.*, 2007). Indeed, two different regulatory modifications of C-function gene expression were selected during the double-flower domestication of *Camellia japonica* (Sun *et al.*, 2014), and a mutation in the AG homolog in *Prunus lannesiana* leads to the formation of double flowers in this species (Liu *et al.*, 2013). By contrast, the existence of a monogenic dominant locus conferring the double-flower trait has been reported in other species of major ornamental value, including rose, carnation and petunia (Dubois *et al.*, 2010; Yagi *et al.*, 2014; Liu *et al.*, 2016). In rose, the existence of a single locus responsible for the double-flower phenotype, referred to as *Blfo* or *d6*, was confirmed by various authors and mapped to IM-LG3 on the integrated map of diploid roses (Debener and Mattiesch, 1999; Crespel *et al.*, 2002; Spiller *et al.*, 2011). Dubois *et al.* (2010) showed that flower doubleness in rose is a consequence of the downregulation of an ortholog of AGAMOUS (*RhAG*), with an increase in meristem size and the shift of the boundary between A and C function towards the center of the organ, and the consequent conversion of stamens into petals. As *RhAG* does not colocalize with the *Blfo* locus, however, the *Blfo* locus was proposed to specify a dominant master regulator directly or indirectly controlling *RhAG* expression (Spiller *et al.*, 2011).

The *Prupe.6G242400* allele identified in peach ($\Delta Cter$) gives origin to a truncated transcript lacking the 10th exon, and the miR172 binding site within (Appendix S3), but still encoding all the conserved functional domains described for euAP2 transcription factors: two EAR motifs, two AP2 binding domains and an NLS. This mutation may therefore result in a fully functional euAP2 transcription factor that is not post-transcriptionally regulated by miR172, which in turn may affect floral meristem size (Wurschum *et al.*, 2006; Zhao *et al.*, 2007). Previous studies in Arabidopsis found that expression of miR172-resistant versions of AP2-type genes under their native promoters resulted in enlarged floral meristems and the production of supernumerary stamens, whereas overexpression through a constitutive promoter also led to supernumerary petals and an indeterminate floral meristem (Chen, 2004; Zhao *et al.*, 2007; Jung *et al.*, 2014). Phylogenetic analysis revealed that *Prupe.6G242400* belongs to a TOE-type clade, and clusters with similar sequences from various dicotyledonous species, although none of the Arabidopsis euAP2 peptides appears to fall into this group. In particular, the genome of petunia was recently reported to encode two gene products clustering with *Prupe.6G242400*, named BEN and BOB (Morel *et al.*, 2017). Interestingly, a *ben/bob* double-knockout line was characterized by a strong reduction in petal and stamen number, and these genes are required for the proper development of whorls 2 and 3, redundantly regulating both B and C functions. In petunia, the three AP2-type ROB genes did not antagonize the C

function in the perianth, suggesting functional divergence between AP2-type and TOE-type factors in Arabidopsis and petunia (Morel *et al.*, 2017). Likewise, our data support the importance of a TOE-type gene, rather than an AP2-type gene, in determining flower doubleness in Rosaceae, as further supported by the identification of a mutation in the *R. x hybrida* ortholog to *Prupe.6G242400*, which results in a transcript *RAG04721* lacking the miR172 target site, similar to the peach $\Delta Cter$ transcript. This variant originates from an insertion event also annotated in the recently released *R. chinensis* genome (Raymond *et al.*, 2018).

Quantitative PCR analysis showed that expression levels of miR172 in peach buds increased with the progression of flower development in both W and By plants. The higher levels of miR172 observed in By could be explained with an enrichment of fourth-whorl tissues in By flower bud samples, compared with W buds, in which tissue from whorls 2 and 3 are more abundant. The expression patterns of miR172 and *Prupe.6G242400* transcript across bud development in By and W appear to be inversely related; however, wt and $\Delta Cter$ transcripts were detected at comparable levels in By and W, respectively, with significantly higher levels of $\Delta Cter$ at 210 JD. Previous studies in Arabidopsis suggest that miR172-mediated regulation of AP2 mainly occurs through the modulation of protein translation rather than post-transcriptional mRNA degradation (Aukerman and Sakai, 2003; Chen, 2004; Schwab *et al.*, 2005). Conversely, in *N. benthamiana* transgenic plants overexpressing Arabidopsis AP2, mRNA levels are controlled by endogenous miR172 via transcript cleavage (Mlotshwa *et al.*, 2006). In peach, the *Prupe.6G242400* transcript cleavage by miR172 has been confirmed both experimentally (Gao *et al.*, 2012) and by degradome sequencing (Luo *et al.*, 2013). Future work aimed at assessing *Prupe.6G242400* peptide accumulation may help to determine the effect of the $\Delta Cter$ mutation on the final protein levels. Finally, *Prupe.6G091100* and *Prupe.6G231700* were also found to be expressed in peach buds, and higher levels of expression of *Prupe.6G231700* were detected in W at later stages of flower differentiation. This could also be a consequence of petal tissue enrichment in W buds, as the petunia AP2-type factors ROB1, ROB2 and ROB3 were found to be required for the normal development of petals (Morel *et al.*, 2017).

A PCR marker developed for the $\Delta Cter$ mutation, *indelDi2*, co-segregated with the double-flower phenotype in all five progenies and allowed one to differentiate heterozygous from homozygous plants. In agreement with the dominant inheritance of the *Di2* locus, plants carrying one copy of $\Delta Cter$ exhibit the double-flower phenotype. Genotyping analysis revealed a complex allele-dosage effect on average petal number in homozygous $\Delta Cter$ (*Di2/Di2*) individuals of the WP² progeny. Additional modulating factor(s) could be at work in determining petal number in these individuals, in agreement with what has been

described in rose, where a major QTL associated with flower traits was mapped in proximity of *Blfo* (Hibrand-Saint Oyant *et al.*, 2008; Roman *et al.*, 2015). Environmental factors are likely to play an important role as well, and it was recently reported that in double-flower *R. chinensis*, RcAP2 (an AP2 type, identical to Rc_XP_024182693 in Figure 8) is involved in the conversion of stamens into petals in response to temperature (Han *et al.*, 2018). The interaction between different euAP2 and environmental factors is likely to be very complex, and it would be of great interest to direct future studies on the dissection of these mechanisms in peach as a model for other ornamentals.

CONCLUSIONS

We used peach as a model to dissect the genetic determinism of the dominant double-flower phenotype, a trait of major importance in ornamental plants. We mapped the dominant *Di2* locus with high resolution on peach chromosome 6 and identified a strong candidate causal mutation in the TOE-type gene *Prupe.6G242400*. As a consequence of this mutation, the encoded transcription factor may escape miR172-mediated repression, in turn resulting in an increased number of petals (and stamens). A similar mutation in the orthologous gene in rose is likely to cause the double-flower phenotype in this plant. Our work provides important insights into the process of flower formation in Rosaceae, and paves the way for the investigation of similar mutations in other important ornamental species.

EXPERIMENTAL PROCEDURES

Plant material and phenotyping

Five peach F₂ progenies were used in this work (Table 1). One progeny, already used for the initial mapping of the *Di2* locus, included 313 individuals derived from the cross S2678 ('Weeping Flower Peach') × S6146 ('Pamirskij 5') (WP²), and is located in Avignon Domaine Expérimental des Garrigues (INRA, France; Pascal *et al.*, 2017). Four progenies derived from the self-pollination of four F₁ seedlings from the cross 'PI91459' ('NJ Weeping', carrying the double-flower trait) × Bounty: 123 (WxBy^A) and 87 individuals (WxBy^B) located in the experimental field of CRPV (Centro Ricerche Produzioni Vegetali, Cesena, Italy), and 37 (WxBy^C) and 61 (WxBy^D) individuals located in the experimental field of the University of Milan, Azienda Didattico Sperimentale F. Dotti (Lodi, Italy). The double-flower trait was scored as a qualitative phenotype for mapping and co-segregation analyses on all five segregating progenies. For the WP² progeny, quantitative data about sepal, petal and stamen numbers were also collected. Rose plant material was collected from Roseto 'Niso Fumagalli' (Monza, Italy, <http://www.airosa.it>) or obtained from local garden centers (Appendix S5).

Genotyping and mapping

The F₂ progenies WxBy^A and WP² (95 individuals) were genotyped using the 9K International Peach SNP Consortium (IPSC) SNP array v1 (Verde *et al.*, 2012), as previously described (Mauroux *et al.*, 2013, 2017; Da Silva Linge *et al.*, 2015). SNP marker

positions were recalibrated based on Peach Reference Genome v2.0 (Verde *et al.*, 2017). Genetic maps were built using JOINMAP 4.0 (Van Ooijen, 2006). QTL analyses on WP² were carried out using the software MAPQTL 6.0 (Van Ooijen, 2009). For the mapping of the *Di2* locus, a non-parametric Kruskal–Wallis (KW) rank sum test was used. The association was considered as significant for a KW test *P* value above the 0.005 threshold. The segregation pattern of the double-flower trait was also included as a dominant marker (*Di2* and *di2* for double and single flowers, respectively). For fine mapping, SNPs flanking the *Di2* locus (identified from the WxBy genetic map) were converted into dCAPs (derived cleaved amplified polymorphic sequences) or KASP[™] (competitive allele-specific PCR) markers to genotype the progenies WxBy^B, WxBy^C, WxBy^D and an additional 218 individuals from WP². Primers for dCAPs and KASP[™] are listed in Table S2. Peach and rose genomic DNA was extracted from young leaf tissue using the DNeasy 96 Plant Kit (Qiagen, <https://www.qiagen.com>), and 10 ng of genomic DNA was used in PCR reactions using GoTaq (Promega, <https://www.promega.com>) in a total volume of 10 µl. Additional primers IndelDi2_A, IndelDi2_B and IndelDi2_C were designed to genotype the mutated region in peach with the co-dominant IndelDi2 marker, whereas primers RO-8f, Ro-Mr and Ro-10r were designed to discriminate between the two variants in rose (Table S2).

Whole-genome sequencing

The sequencing of 'PI91459' ('NJ Weeping') and 'Bounty' parents was performed at the Genomics Platform of Parco Tecnologico Padano (Lodi, Italy) following the protocol described by Cirilli *et al.* (2017). Functional annotation of the variants was performed using SNEFFECT 2.0 (Reumers *et al.*, 2006). Sequences for predicted peach gene models were retrieved from the Phytozome 12.0 database (Goodstein *et al.*, 2012).

3'-RACE, RT-PCR and qPCR expression analysis

Peach buds were collected from on-year developing shoots of W and By parental lines at 180, 210 and 240 JD, during the period of floral differentiation (Andreini and Bartolini, 2008). 'Meimarmoto' (Red Meilandina[®] Plus) rose buds were collected from a plant in full bloom during the month of May. Total RNA was extracted using a Quick-RNA Miniprep Kit (Zymo, <https://www.zymoresearch.eu>), following the manufacturer's protocol, with the addition of 2% polyvinyl pyrrolidone (PVP) and 4% beta-mercaptoethanol (Sigma-Aldrich, <https://www.sigmaaldrich.com>), just before use. First-strand cDNA was obtained with GoScript Reverse Transcriptase (Promega), using either a standard oligo dT primer or B36 containing an adaptor sequence (modified from Frohman *et al.*, 1988). The reactions were diluted 1:10, and 1 µl was used as a template for 3'-RACE or RT-PCR using GoTaq (Promega). RACE analysis was carried out in two steps. First, a pre-amplification step using primers RACE2for and B35 with a program consisting of one cycle (1 min at 95°C), 15 cycles (30 sec at 94°C; 30 sec at 55°C; 45 sec at 72°C) and one cycle (1 min at 95°C) in a 25-µl final volume. The reaction was subsequently diluted 1:10 with water. Second, nested PCR was performed using 1 µl of the diluted reaction and primers Exon9for and B35 in a 25-µl reaction using GoTaq. The program consisted of one cycle (1 min at 95°C), 32 cycles (30 sec at 94°C; 30 sec at 55°C; 30 sec at 72°C) and one cycle (1 min at 95°C). For qPCR and miR172 quantification, total RNA was extracted from bud tissues using a miRNeasy kit (Qiagen) and cDNA synthesized by a miScript II kit (Qiagen) using HiFlex buffer and modified oligo-dT primers with a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of both mature miRNA and mRNAs. Primers for ppa-miR172 were designed to amplify the

three isoforms ppa-miR172a, b, c according to the method described by Zhu *et al.* (2012). Specific primers for peach euAP2 genes and *Actin* (*Prupe.6G163400* used as reference, according to Tatsuki *et al.*, 2013) are listed in Table S2.

Molecular phylogenetic analysis by maximum likelihood method

Members of the euAP2 lineage were identified by tBLASTn searches against the current peach reference genome assembly v2.0 (Verde *et al.*, 2017), and publicly available databases for other plant species. Petunia sequences were as described by Morel *et al.*, (2017). Annotated gene models were used for full-length protein prediction. Phylogenetic relationships were estimated in MEGA 7 (Kumar *et al.*, 2016). Peptide sequences (Appendix S6) were aligned by MUSCLE with default settings. Evolutionary relationships among euAP2 members were inferred by using the minimum evolution method and the close-neighbor-interchange algorithm. Initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining algorithm. The reliability of the phylogenetic tree was estimated by setting 500 bootstrap replicates.

Plant material availability

Plant materials used in this study are available upon reasonable request. Authors responsible for the distribution of plant materials are: DB for 'PI91459' ('NJ Weeping'), Bounty and their progeny; and TP for S2678 ('Weeping Flower Peach'), S6146 ('Pamirskij 5') and their progeny.

ACCESSION NUMBERS

Whole-genome resequencing data for 'PI91459' ('NJ Weeping') and Bounty peach accessions are available under SRA BioProject ID: PRJNA479850, biosamples SAMN09606844, SAMN09606845.

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CONFLICT OF INTEREST

A patent application partially related to this research has been submitted by PTP with SG and LR as inventors.

AUTHOR CONTRIBUTIONS

SG conducted the genotyping, identified the candidate variants, developed and validated the associated molecular markers, conceived and performed the experiments on rose and drafted the manuscript. MC performed the analyses of gene expression and of genome-wide resequencing data, and drafted the manuscript. IP, CDSL, EC and TP collected phenotypic data. AC, CDSL, IP and JBM performed linkage mapping. PL performed QTL analysis. DB and TP selected the genetic materials for the study. LR and TP conceived the peach study and critically revised the manuscript for important intellectual content. TP coordinated analyses on the WP² progeny and LR coordinated the whole study. All authors read and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Quantitative trait loci (QTL) analysis for petal number in the WP² progeny, using interval mapping (IM). The limit of detection (LOD) threshold is indicated by the horizontal line.

Figure S2. Chi-square value distribution on chromosome 6 in the WP² cross, showing a peak of distortion at about 10 Mbp, near SNP_IGA_635084.

Table S1. List of gene models in the minimum mapping interval on chromosome 6 (24 006 441–24 157 299), ranging between SNP_IGA_680329 and SNP_IGA_680909.

Table S2. List of all primers used in this study.

Appendix S1. Genotypic and phenotypic data of the five progenies: WP² and WxB^{A,B,C,D}.

Appendix S2. List of variants annotations and effects (annotated by the SNPEff tool) from whole-genome sequencing assembly of 'NJ Weeping' and 'Bounty' parents and 11 single-flower accessions within the fine-mapped interval on chromosome 6.

Appendix S3. (a) Alignment between the genomic regions of reference *Prupe.6G242400* (Pp06:24074192..24077922, reverse complement) and Δ Cter alleles. Predicted CDS are shaded. (b) Alignment between the predicted peptide sequences. Functional domains are underlined.

Appendix S4. (a) Alignment between *RAG04722* and *Prupe.6G242400* predicted peptide sequences and their miR172 binding sites. (b) Alignment between *RAG04722* and *RAG04721* unigenes. (c) Alignment and translation of putative *RAG04722* and *RAG04721* CDS sequences. (d) Alignment between the predicted peptide sequences. Functional domains are underlined.

Appendix S5. PCR analysis of 15 single-flower and 20 double-flower rose accessions. The primer combinations indicated are specific for the mutated (RO-8f/RO-Mr) or the wild-type (RO-8f/RO-10r) allele: '+' indicates the presence of the PCR band.

Appendix S6. List of peptide sequences used for phylogenetic analysis.

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