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Cloning and Characterization of Three *APETALA1/FRUITFULL*-like Genes in Different Flower Types of *Rosa* × *hybrida* L.

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Abstract To clarify the molecular mechanism of flower development in *Rosa* × *hybrida* L., three different *APETALA1/FRUITFULL* (*AP1/FUL*)-like MADS-box genes were isolated and their expression analyzed in normally developed flowers and in malformed flowers of a stable phenotype. *AP1/FUL*-like genes were designated as *RhAP1-1*, *RhFUL*, and *RhAP1-2*. Alignment of amino acid sequences showed 83% identity between RhAP1-1 and TrAP1 of *Taihangia rupestris* and 82% identity between RhFUL and TrFUL of *T. rupestris*. RhAP1-1 is 97% identical to RhAP1-2 and 58% identical to RhFUL. Expression of *RhAP1-1* and *RhAP1-2* in whorls 1 and 2 of rose flowers exclusively is in accordance with the expression pattern of class A genes in other plant species. In contrast, *RhFUL* showed a unique expression pattern and was expressed only in sepals. The roles of all putative A, B, and C class genes were examined in different flower organs of normally developed flowers and in malformed flowers that are similar to a classic C function mutant from *Arabidopsis* (with petals in whorl 3 and sepals in whorl 4). The expression pattern of the putative class B genes was similar in both normal and malformed flowers. However, the putative class A genes were upregulated and class C genes were downregulated in all flower organs of the mutant. These data suggest that suppression of the class C genes *RhC1* and *RhC2* leads to altered expression of *RhAP1-1*, *RhFUL*, and *RhAP1-2* in whorls 3 and 4 that leads to the mutant flower phenotype.

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Introduction

The genus *Rosa* with several thousand cultivated varieties, marketed as miniature potted roses, garden roses, or cut flowers, is the major floricultural crop marketed globally. The important morphological characteristics of roses are flower color, flower shape, and number of petals. Enhanced petal number within the flower, the so-called double flower, might be caused by a homeotic change of stamens to petals.

The typical angiosperm flower meristem is organized in four distinct concentric whorls representing growth of different organs, namely, sepals, petals, stamens, and carpels. During the last two decades much progress has been made in understanding floral meristem formation and floral organ development. Flower development is known to be a cascade of flower time integrators that sequentially activate floral meristem identity genes and then flower organ identity genes.

Based on studies of flower mutations in *Arabidopsis thaliana* (Bowman and others 1991) and *Antirrhinum majus* (Coen and others 1990; Schwarz-Sommer and others 1990), a theoretical model has been developed that classifies organ identity genes into three different classes, designated as A-, B-, and C-function genes. This model was refined by the addition of D- and E-function genes (Angenent and others 1995; Pelaz and others 2000, 2001) and by the protein-based floral quartet model (Theissen 2001; Theissen and Saedler 2001), which explains flower organ determination by formation of tetrameric transcription factor complexes composed of different subunits with A, B, C, and E function. D genes are required at a later

stage for ovule formation (Angenent and others 1995) and E-gene expression is required in all four whorls (Ditta and others 2004).

Class A-function genes, such as *APETALA1* (*AP1*) from *Arabidopsis* (Mandel and others 1992), lead to formation of sepals in whorl 1. Expression of C-function genes, such as *AGAMOUS* from *Arabidopsis* (Yanofsky and others 1990), are required for formation of carpels. In addition, some genes also function as cadastral genes; C-function genes inhibit expression of A-function genes and they are repressed (among other genes) by A-function genes (*APETALA2*) (summarized in Jack 2004). B-function genes, like *APETALA3* from *Arabidopsis* (Jack and others 1992), are expressed independently of A- and C-function genes in whorls 2 and 3. The combined expression of B- and A-function genes leads to formation of petals in whorl 2 and class B and class C genes function simultaneously, specifying development of stamens in whorl 3. Meristem and organ identity genes code mainly MADS-box transcription factors (Shore and Sharrocks 1995; Theissen and Saedler 1995). All MADS-box proteins contain a highly conserved amino acid motif [of 60 amino acids (aa)] at the N terminus to interact with DNA and proteins like other MADS-box transcription factors (Shore and Sharrocks 1995; Theissen and others 1996). A second, weakly conserved, amino acid region of roughly 70 aa, designated as a K-box, is responsible for protein-protein interactions in type II MADS-box proteins (Ma and others 1991).

MADS-box genes have been cloned from a range of plant species, including petunia (Angenent and others 1993), tomato (Pnueli and others 1991), birch (Elo and others 2001), rose (Kitahara and Matsumoto 2000; Kitahara and others 2001), and apple (Sung and others 1999).

In the genus *Rosa*, two types of *AGAMOUS*-like genes, designated as *MASAKO C1* and *MASAKO D1* (Kitahara and Matsumoto 2000), were isolated and investigated from wild rose (*Rosa rugosa* Thumb. Ex Murray). Expression analyses of both *AGAMOUS*-like genes, based on the ABC model, verified the predicted expression in the stamen and carpel (Kitahara and Matsumoto 2000). Functions of *MASAKO C1* and *MASAKO D1* were investigated by transgenic approaches in *Arabidopsis thaliana* and *Torenia fournieri*. Overexpression of *MASAKO C1* or *MASAKO D1* resulted in production of flowers with homeotic changes from sepal to carpel and petal to stamen in *Arabidopsis* and sepal-to-carpel transformation in *Torenia*. Based on these results, no functional differences between *MASAKO C1* and *MASAKO D1* were detected (Kitahara and others 2004).

B-, C-, and E-function orthologous genes have been isolated and investigated in wild rose (*Rosa rugosa* Thumb. Ex Murray), and expression patterns were investigated in roses with malformed (phyllody-showing) flower types (Chmelnitsky and others 2003; Matsumoto and Kitahara 2005) from different genetic sources. Transcripts of *RAG*, an *AGAMOUS* ortholog with a homology of 99% to *MASAKO C1* from *Rosa rugosa*, were not detected during early stages of development in *R.* × *hybrida* cv. Motrea with malformed flowers (phyllody-showing) and in *R. chinesis viridiflora* 'green rose' compared to normal flowers (Chmelnitsky and others 2003). In contrast, expression patterns of class B genes *MASAKO BP*, *B3*, and *euB3*, class C genes *MASAKO C1* and *D1*, and class E genes *MASAKO S1* and *S3* showed no differences between flowers of 'green rose' (*R. chinesis viridiflora)* and wild rose (*R. rugosa*) (Kitahara and others 2001; Matsumoto and Kitahara 2005). As described above, B-, C-, and E-function orthologous genes have been isolated and investigated in wild rose (*Rosa rugosa* Thumb. Ex Murray), and expression patterns were investigated in roses with malformed (phyllody-showing) flower types (Chmelnitsky and others 2003; Matsumoto and Kitahara 2005) from different genetic sources. However, no information about A-function genes (like *APETALA1* and *FRUITFULL*-like) in rose is currently available. The group of *FRUITFULL* (*FUL*)-like genes are closely related to *AP1* (*APETALA1*). The FUL protein (AtAGL8) of *Arabidopsis* regulates transcription of genes that are essential for cellular differentiation during fruit and leaf development and it is expressed at high levels in inflorescence meristems (Gu and others 1998).

Here we report the identification and expression analysis of three novel *APETALA1*/*FRUITFULL*-like genes, designated as *RhAP1-1*, *RhAP1-2*, and *RhFUL*. Expression pattern analysis of all A-, B-, and C-function MADS-box genes was undertaken for two different genotypes selected as F1 progeny, a mutant with completely malformed generative organs (similar to a class C gene mutant in other plants) and a genotype with normally developed flowers.

Materials and Methods

Plant Material

Two genotypes varying in floral morphology, one with fertile buds (76/74) and the other with sterile and/or malformed buds (76/72), like a classic class C-function mutant (flower organs: sepals-petals-petals-sepals), were selected from an F1 population of cultivars 'Lavender Kordana' × 'Vanilla Kordana' (W. Kordes' Rosenschulen Co., Germany) (for details see Ahmadi and others 2009). Flowers from genotype 76/72 (developed sepals and petals only) and genotype 76/74 (developed sepals, petals, stamens, and carpels) were collected at the development stages of smaller than 2, 2-6, and 10 mm (Fig. 1). Plants were propagated from cuttings, four cuttings per pot, under the following greenhouse conditions: temperature 22°C/18°C (day/night) and day length extended

to 16 h by SON-T lamps (Osram, 400 W, Philips Co.) supplied 600 μmol m^{-2} s^{-1}.

DNA and RNA Preparation and Reverse Transcription

Total DNA was isolated from 100 mg of leaf tissue using the DNA Plant Mini kit (Macherey–Nagel, Clontech). Total RNA was isolated from 100 mg of ground plant samples from different organs dissected from normally developed and malformed buds with a length of 10 mm (± 1 mm) using the Invisorb® Spin Plant RNA Mini Kit (Invitek) according to the manufacturer's protocols. RNA samples were treated with DNaseI (Fermentas) as follows: 0.5 U DNaseI, reaction buffer (10 mM Tris–HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6), and 20 U RiboLock™ (Fermentas) were added to each 2 μg RNA sample and incubated in a Thermocycler (Biometra) at 37°C for 30 min. To eliminate any DNaseI enzyme residue, 0.1 mM EDTA was added to each RNA sample followed by incubation at 65°C for 10 min. One microgram of RNA from different flower organs was reversely transcribed using M-MLV RT, RNase H^- (Promega). Reverse transcription was carried out using 1 μg oligo-dT_{23} primer, incubating at 70°C for 10 min, and quenching immediately on ice for 5 min. After centrifugation, M-MLV RT buffer (Promega), 0.5 mM dNTPs, 200 U M-MLV RT(H-) enzyme, and 20 U RiboLock were added to the 25 μl RT reaction and incubated at 40°C for an initial 10 min, 50°C for 4 h, and finally 70°C for 5 min.

Cloning of *AP1/FUL*-, *AGAMOUS*-, and *PI*-like Genes in *Rosa* × *hybrida* by PCR

Sequence alignments were made between the known AP1 similar protein sequences of different plant species: AP1-like protein from *Malus* × *domestica* (Acc. No. AAL61543.1), MADS-box protein from *M.* × *domestica* (Acc. No. CAA04321.1), APETALA1 from *Eriobotrya japonica* (Acc. No. AAX14152.1), MADS-box protein AP1a from *Lotus corniculatus* var. *japonicus* (Acc. No. AAX13296.1) from NCBI (http://www.ncbi.nlm.nih.gov). By using homologous regions of these AP1-like proteins, primers AP1_dfor: 5′-TCAGCTKAAGMGSATAGAGA-3′ and AP1_drev: 5′-GTGTCAAGCTGTTGCTCCAA-3′ were constructed. The distance between the position of primer AP1_dfor and that of primer AP1_drev is 128 amino acids, thus an amplicon of 384 bp was expected. Primers were designed using the Primer3 program (Rozen and Skaletsky 2000). PCR was performed using 0.5 ng of cDNA generated from RNA of petals and sepals of the genotype 76/74 (normal flowers) in a final volume of 20 μl containing 0.375 μM of each primer, 0.15 mM of each dNTP, 1 U Hot Start Taq DNA polymerase (Axon), 3 mM $MgCl_2$, and DNA polymerase buffer (Axon). After 10 min of incubation at 94°C, the cDNA was amplified by 40 three-step cycles: 30 s at 94°C, 1 min between 48 and 65°C, and 1.5 min at 72°C using the Primus 96 advanced gradient thermocycler (Peqlab, Isogen Life Science). The cloning of *AGAMOUS* and *PISTILLATA* ortholog genes was completed based on the sequence data from NCBI (http://www.ncbi.nlm.nih.gov). To clone *MASAKO C1* (AB025644), an *AGAMOUS* ortholog from *Rosa rugosa* and *RAG* (U43372), an amplicon of 668 bp was amplified from cDNA of *Rosa* × *hybrida* 'Genotype 76/74' with the primers RrC1_for: 5′-CGTCAAGTCACCTTCTGCAA-3′ and RrC1_rev: 5′-AGTTGCGAGCCTCATGAAAT-3′. Cloning of *MASAKO D1* (AB025643) was done with the primers RrD1_for: 5′-GGAGATCGAGCTGCAAAATC-3′ and RrD1_rev: 5′-TCAGGAAACAG-AAGAGGTGGA-3′. The *PISTILLATA* ortholog gene was cloned in *Rosa* × *hybrida* with primers RrB_for: 5′-ACAGGCAGGTGACCTATTCG-3′ and

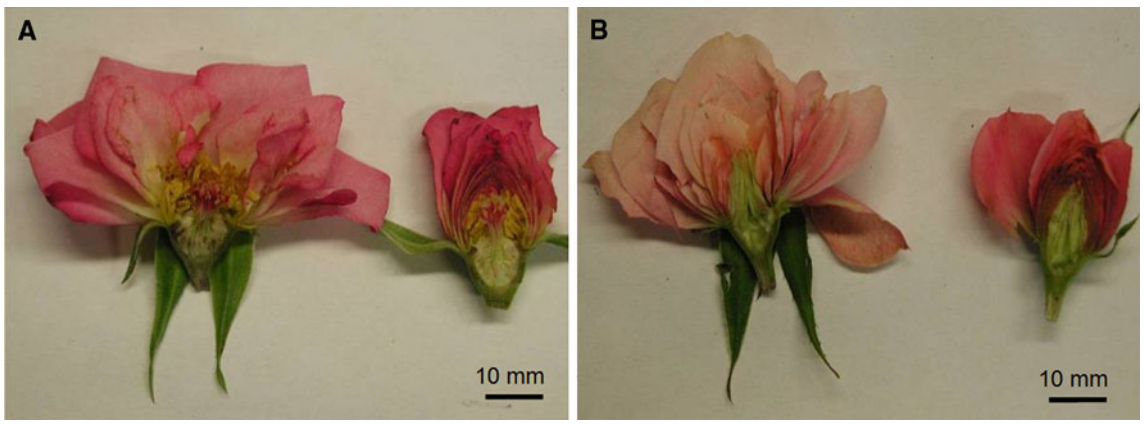

Fig. 1 **a** Two different developmental stages of fertile flower harvested from rose genotype 76/74. **b** Two developmental stages of malformed, sterile flower from rose genotype 76/72. Scale bar = size standard, 10 mm

RrB_rev: 5′-AAGCAAGACGGA-GATCATGG-3′ constructed from *MASAKO B3* (AB055966) from *R. rugosa*. All amplicons obtained were separated by flatbed electrophoresis using 1% agarose gels in TAE buffer and sizes were estimated by comparison to a 100-bp ladder (Fermentas). PCR and RT-PCR amplicons were cloned using a TOPO TA Cloning Kit (Invitrogen). Alignments between *R. rugosa* and *R. hybrida* for *MASAKO C1* and *MASAKO D1* partial sequences were 99.8% homologous (data not shown). The primers designed for the *MASAKO C1* homolog amplicon were designated as *RhC1*, and those for *MASAKO D* and *MASAKO B3 RhC2* and *RhB*, respectively (Table 1).

Cloning, Sequencing, and Phylogenetic Analysis

Plasmids were recovered using the NucleoSpin® Plasmid Kit (Macherey–Nagel, Clontech) and sequencing was accomplished by Eurofins MWG Operon (Ebersberg, Germany). Isolated sequences were analyzed using the ClustalW program [European Bioinformatics Institute (EMBL; Higgins 1994)] and the BLASTN program [National Center for Biotechnology Information (NCBI; Altschul and others 1997)]. Specific primers were designed using the Primer3 program (Rozen and Skaletsky 2000).

Unique *AP1*- and *FUL*-like sequences were identified by BLAST searches (Altschul and others 1997) and were included in the analysis. Phylogenetic analysis and construction of phylogenetic trees were based on amino acid alignment using MEGA 4 (http://www.megasoftware.net; Tamura and others 2007) with implemented method "Neighbor-Joining"; bootstrap confidence values from 1000 replicates and distances were calculated with Poisson corrections for multiple substitutions.

RACE (Rapid Amplification of cDNA Ends)

To isolate the full-length cDNA of interest, the 5′/3′ RACE Kit (Roche) was used according to the supplier's instructions. The first cDNA strand was synthesized using an oligo-dT anchor primer for reverse transcription, as previously described. To amplify the 3′ end of cDNA of *RhAP1-1* and *RhAP1-2*, nested PCR was completed using the anchor primer together with the specific primer 5′-GATCTCTGTCTTGTGCGATGC-3′ in the first PCR and the specific primer 5′-GGAGAAGATTTGGATTC-ATT-3′ in the second PCR. To amplify the 3′ end of the cDNA of *RhFUL*, the first PCR was completed using the anchor primer and the specific primer 5′-GGGATCCTG-GAGCGATATGA -3′, and the second PCR was completed using the anchor primer and the specific primer 5′-CA-AGCTTACGGCAAGGATTGA-3′.

To amplify the 5′ cDNA of *RhAP1-1* and *RhAP1-2*, the first cDNA strand was synthesized using RhAP1_1 primer 1 (5′-GATTCCAAATCAGGTTCGAC-3′) in reverse transcription, as previously described. Nested PCR was completed using the oligo-dT anchor primer and the RhAP1_1 primer (5′-TGCGTACGAGTACCGCTCAT-3′), and the second PCR was completed with the anchor primer and specific primer 5′-CAAAGCAACCTGAGCATCG-3′. To amplify the 5′ cDNA of the *RhFUL*, the first cDNA strand reverse transcription was completed with the specific

Table 1 Gene-specific primer pairs used for qRT-PCR and for southern probe amplification

Primer name	Acc. No.	Primer pair	Sequence (5′-3′)	RT-PCR (base pairs)
RhAP1_1/2	FJ970026	Forward	TGCTCAGGTTGCTTTGATTG	472
		Reverse	ATGGTTTTGCTGCTGTTGCT	
RhAP1_1	FJ970026	Forward	CAACAGCTTGACAATTCTCTTA	335
		Reverse	CATTGAGTATATAATACCTTAGCATCA	
RhFUL	FJ970028	Forward	TCATCCTCCTTTCCCCTTTC	446
		Reverse	GGACCAGTTTCCCTGTGATT	
RhAP1_2	FJ970027	Forward	TTAAGGAGAAGGAGAAGAATGT	338
		Reverse	TCAGGTTCACATTATAGCAAAA	
RhC_1	AB025644	Forward	TCTGTGCTCTGTGATGCTGA	438
		Reverse	TATGCTTTGCTGGTGCCTCT	
RhC_2	AB025643	Forward	AAATGACAGGGCACAACAGC	259
		Reverse	ACCAGGCTGCCTTCTAGCAT	
RhB	AB055966	Forward	GAGCTCACGGTTCTGTGTGA	332
		Reverse	TTGAGCACGTGGTACTTTCG	
Rhßactin	AB239794	Forward	TGCTCCCGCTATGTATGTTG	398
		Reverse	GGACTTCTGGGCATCTGAAA	

primer 5′-ACTTTGAAGCTCCCTCAAGC-3′. Nested PCR was completed using an oligo-dT anchor primer and the RhFUL primer 5′-GTCTAAATCCTCTCCCGTGT-3′, and the second PCR was completed with the anchor primer and the specific primer 2 (5′-TTCAATCCTTGCCGTAA GC-3′). To confirm the highly similar sequences of *RhAP1-1* and *RhAP1-2*, the entire cDNA sequences were amplified using the forward primer 5′ CACTGCCTTTTGCAGTTTT G-3′ and the reverse primer 5′ TTTTCGTCAAGTCAT-CAAGTTCA-3′. To exclude potentially cloning chimeric *RhAP1* sequences, PCR was repeated with primers designed for the different 3′ cDNA ends. Specific amplification of the *RhAP1-1* cDNA was completed with the forward primer 5′-CACTGCCTTTTGCAGTTTTG-3′ and the reverse primer 5′-CATTGAGTATATAATACCTTAG CATCA-3′. Specific amplification of the *RhAP1-2* cDNA was completed with the forward primer 5′ TGGCTAATTT ATAAGCTACAAGAAGAA-3′ and the reverse primer 5′-TCAGGTTCACATTATAGCAAAA-3′. Flatbed electrophoresis was used for separation and cloning of amplicons. RT-PCR amplicons were cloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced.

RT-PCR and qRT-PCR

To evaluate the expression pattern of the B-function genes, RT-PCR was carried out in a 20-µl reaction mixture containing 10 ng cDNA template from different flower organs, 150 µM each dNTP, 0.25 µM primer RhBfor, 0.25 µM RhBrev (Table 1), 0.5 U Taq DNA polymerase (Invitek), 10 mM Tris–HCl, 50 mM KCl, and 2 mM MgCl. PCR amplification was conducted in the thermocycler (Biometra) under the following conditions: 30 s at 94°C for initial denaturation, followed by 30 cycles consisting of 30 s at 94°C for denaturation, 1 min at 65°C for annealing, 1 min at 72°C for polymerase extension, and a final extension step of 72°C for 10 min.

Gene expression patterns from leaves, roots, floral buds (length<2, 2-6, and 10 mm), and flower organs of normal and malformed flowers (length 10 mm) were determined by qRT-PCR with at least three independent biological replicates. The qRT-PCR was performed using a Rotor-Gene 3000 (Corbett Life Science, Qiagen) with three technical replications per run and at least two replications of each run.

The reaction mixture was made up to a volume of 20 µl containing 2 ng cDNA template (reverse transcription as described previously), 150 µM each dNTP, 0.25 µM from each primer (gene-specific primers are given in Table 1), 1 U Hot Start Taq DNA polymerase (Axon), 3 mM $MgCl_2$, and DNA polymerase buffer (Axon) and SYBR® Green by dilution 1:40,000 of stock solution (Roche). After 5 min of incubation at 94°C, the cDNA was amplified by 45 three-step cycles: 10 s at 94°C, 30 s at 62°C, and 30 s at 72°C. To normalize all samples, expression levels of β-actin primer (see Table 1) were assayed in each sample in parallel with the genes of interest. Constant expression of the internal control β-actin was tested and confirmed across developmental stages and flower organs. PCR conditions were optimized for high amplification efficiency ($\geq$ 95%) for all primer pairs used. Plasmids containing the respective target sequence as insert were diluted to generate templates from 10^6 to 10^3 copies and used for standard curves for estimation of copy number and quality of each cDNA sample. In addition, a mixture composed of cDNAs from different flower organs was used for standard curves for estimation of primer efficiency as recommended by Livak and Schmittgen (2001).

To verify single-product amplification, melting curves were analyzed immediately after finishing PCR by heating the reaction 1°C/s from 72 to 95°C under permanent fluorescence detection. Analysis of data was completed using Rotor-Gene software version 6.1.81. The relative quantification of transcript abundance of target genes in individual plant samples was determined by the $2^{-\Delta\Delta Ct}$ method that enabled fold-change values for various genes relative to a calibrator to be calculated for each replicate of each sample (Livak and Schmittgen 2001).

Southern Hybridization

Genomic DNA was isolated from tetraploid *R.* × *hybrida* L. genotype (76/74) with normal flowers. For genomic Southern blots, 10 µg genomic DNA from each sample was digested with *Eco*RI restriction enzyme overnight and then run together with the DNA Molecular Weight Marker III (Dig-, 0.56-21.2 kb; Roche, 1 µg) on a 0.8% agarose in 1 TAE gel. DNA was blotted onto a positively charged nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) using a vacuum blotter (Bio-Rad). The probes (genomic amplicons containing no *Eco*RI sites) for *RhAp1-1/2* (472 bp) and *RhFUL* (446 bp) (Table 1) were labeled with DIGdNTP (Roche) using plasmid containing the gene of interest.

Membrane hybridization, post-hybridization washing, and detection were done under high stringency conditions performed as described in Sriskandarajah and others (2007).

Results

Selection of the Plant Material

One genotype (76/72) with a change of stamens to petals and carpel to sepals was identified among 232 F1 progeny generated from a cross between two commercial miniature rose varieties (previous investigations by Ahmadi and

others 2009). In the present study, this genotype (76/72) (Fig. 1b) was compared with another genotype (76/74) with fertile flower organs originated from the same F1 progeny (Fig. 1a). Several plants with intermediate forms between normally developed and malformed flowers were detected among the progeny. However, flower morphology of almost all these phenotypes was strongly influenced by environmental conditions, including temperature, light, and day length (data not shown).

Cloning and Sequence Analysis of the Three Different *APETALA1*-like Genes

RT-PCR with degenerate primer and cDNA generated from buds as a template resulted in one amplicon with the expected size of 380 bp. After cloning and sequence analysis, two different *AP1*-like cDNAs were detected: cDNA1 of 382 bp and 87% similarity to *TrAP1* of *Taihangia rupestris*, designated as putative *RhAP1-1* (*Rosa* × *hybrida APETALA1*-like 1) and cDNA2 of 385 bp and 84% similarity to *TrFUL* of *T. rupestris* designated as putative *RhFUL* (*Rosa* × *hybrida FRUITFULL*-like).

To clone the whole mRNA sequence of *RhAP1-1* and *RhFUL*, RACE amplification was carried out. The full-length cDNA sequence of *RhAP1-1* (Acc. No. FJ970026) is 1086 bp and encoding a 247-aa polypeptide, and the full-length cDNA sequence of *RhFUL* (Acc. No. FJ970028) is 1323 bp and encoding a 257-aa polypeptide. However, two 3′ cDNA ends of *RhAP1-1* with different sequences that lead to the cloning of a second *AP1*-like cDNA were found. Additional amplification with primers designed for the different 3′ cDNA ends confirmed the existence of two *AP1*-like cDNAs in the transcriptome. This additional AP1 homolog with a full-length sequence of 1155 bp encoding a 247-aa polypeptide was designated as putative *RhAP1-2* (*Rosa* × *hybrida APETALA1*-like 2) (Acc. No. FJ970027).

Sequence Analysis and Characterization of the *APETALA1*-like Genes *RhAP1-1*, *RhFUL*, and *RhAP1-2*

All three cloned *AP1*-like genes encoded conserved MADS-box domains of 58 aa and K-box domains of 70 aa (Fig. 2). RhAP1-1 and RhAP1-2 were 97.2% identical, whereas RhAP1-1 and RhFUL were only 58.4% identical.

Alignment of the aa sequences of RhAP1-1 and RhAP1-2 by BLAST and ClustalW indicated 83% identity to TrAP1 of *Taihangia rupestris* (Acc. No. ABB59991) and AP1 from *Malus* × *domestica* (Acc. No. AAL61543). BLAST of the aa sequence of RhFUL showed 82% identity to TrFUL of *Taihangia rupestris* (Acc. No. ABB59990) and 60% to MdMADS-12 of *Malus domestica* (Acc. No. AJ320187) (Fig. 2). Both species, *Taihangia rupestris* and *Rosa* × *hybrida*, originate from the same supertribus Rosodae, which may explain the sequence similarity between these *AP1/FUL*-like genes. The relationship between proteins encoded by the three novel *APETALA1*-like genes from *Rosa* × *hybrida* and other AP1-like proteins from other species were determined using a phylogenetic tree based on the "Neighbor-Joining" method using MEGA 4 (Fig. 3).

According to the phylogenetic tree, two main groups of AP1-like proteins were detected. RhAP1-1 and RhAP1-2 were grouped closely with the TrAP1 protein of *T. rupestris* and with several AP1-like proteins from different members of the Rosaceae family (*P. persica*, *M.* × *domestica*, *P. pyrifolia*, *E. japonica*), and with lower similarity to AP1-like protein from *Arabidopsis*, *L. corniculatus*, and *A. majus* (SQUA) (Fig. 3). All of these proteins have a C-terminal conserved euAP1 motif (Vandenbussche and others 2003) (Fig. 2). Within a second clade, several other members of AP1-like proteins of *Prunus* and *Malus* were grouped with AGL8 (FUL) from *Arabidopsis*. RhFUL clustered in a third clade with several AP1-like proteins (Fig. 3). This group can be divided in two subgroups. One subgroup clustered together with RhFUL and the two AP1 homologs of *T. rupestris* (TrFUL), *M.* × *domestica* (MdMADS-12), and *B. pendula* (BpMADS4). AP1 homologs of the species *H. macrophylla* (HmAPETALA1), *P.* × *hybrida* (PhMADS-FBP29), and *A. majus* (AmDEF28) cluster within a second subgroup (Fig. 3). All of these proteins have conserved C-terminal sequences typical of most FUL homologs (Fig. 2) (Litt and Irish 2003).

Evaluation of Three *RhAP1* Homologs in the Rose Genome

By use of a probe derived from *RhAP1-1*, a hybridization pattern with two signals after digestion with *Eco*RI was detected (Fig. 4), which confirmed the presence of two highly homologous genes *RhAP1-1* and *RhAP1-2* in the rose genome. Hybridization with a probe from *RhFUL* resulted in one signal, verifying the existence of only one of these types in the rose genome (Fig. 4).

Expression of the Genes *RhAP1-1*, *RhFUL*, *RhAP1-2*, *RhB*, *RhC-1*, and *RhC-2*

The mRNA of *RhAP1-1* and *RhAP1-2* was not detectable in vegetative tissues (leaves and roots; data not shown); however, transcripts of *RhFUL* were detected in leaves (but not in roots, Fig. 5).

In buds at stage 2-6 mm, expression of *RhFUL* was higher in malformed than in normal buds (Fig. 6c). However, no differences between malformed and normal buds in the early (<2 mm) or late (10 mm) bud stages

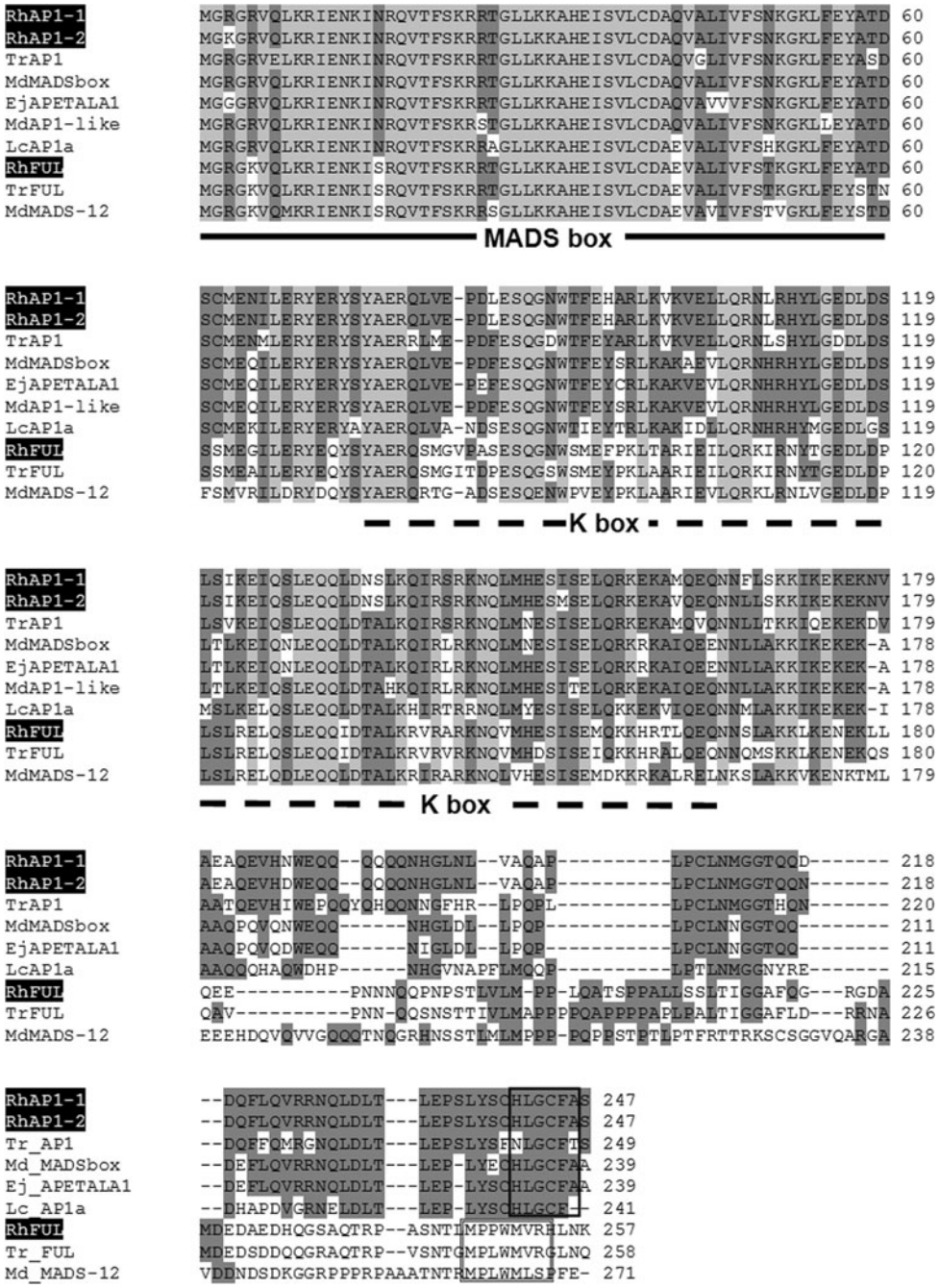

Fig. 2 Comparison of AP1 homologous proteins RhAP1-1, RhFUL, and RhAP1-2 from *Rosa hybrida* with AP1 homologs of other species: TrFUL, *Taihangia rupestris* (ABB59991); TrAP1, *Taihangia rupest*ris (ABB59990); MdMADS-12, *Malus x domestica* (AJ320187); MdMADS-box protein, *Malus x domestica* (CAA04321); EjAPETALA1, *Eriobotrya japonica* (Acc. No. AAX14152); LcAP1a, *Lotus corniculatus* var. *japonicus* (AAX13296); solid line: MADS box; dashed line: K box; bold-lined box: AP1 conserved motive; double-lined box: FUL conserved motive (Litt and Irish 2003; Vandenbussche and others 2003)

were detectable (Fig. 6c). Surprisingly, the expression of *RhAP1-1* and *RhAP1-2* was always higher in normal buds compared to malformed buds in all investigated developmental stages (Fig. 6a, b).

The highly similar cDNA of *RhAP1-1* and *RhAP1-2* showed similar expression patterns in the sepals and petals of normally developed flowers (Fig. 7a, b), indicating a common origin. This expression pattern is similar to that for *AP1* from *Arabidopsis* (Mandel and others 1992). Expression of *RhAP1-1* and *RhAP1-2* was detected in all investigated flower organs (sepals, petals, inner petals, and inner sepals) of the malformed flowers (Fig. 7a, b).

RhFUL was expressed only in sepals of normally developed flowers and in both the sepals of whorls 1 and 4 of the malformed flowers (Fig. 7c). Besides this sepal specificity, expression of *RhFUL* was detectable only in petals of whorl 3 in malformed flowers (Fig. 7c).

The B-function gene *RhB* showed expression in petals and anthers of both normal and malformed flowers (Fig. 8). Expression analysis of the gene *MASAKO B3* of *Rosa*

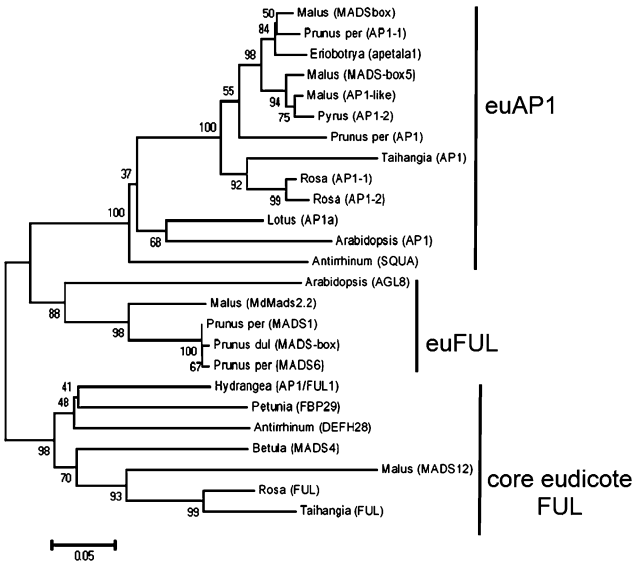

Fig. 3 Phylogenetic analysis of RhAP1-1, RhFUL, and RhAP1-2 from *Rosa hybrida* and AP1 homologous proteins of other species. Sequence alignment analysis was performed using ClustalW. The proteins and accession numbers are as follows: *Taihangia rupestris*: TrFUL (ABB59991), TrAP1 (ABB59990); *Malus x domestica*: MdAP1-like (AAL61543), MdMADS-box (CAA04321), MdMADS12 (AJ320187), MdMADS2.2 (ABB22023), MdMADS-box5 (ABG85297); *Prunus persica:* PpAP1-1 (ABU63953), PpMADS1 (CAJ28929), PrMADS6 (AAU29514); *Prunus dulcis*: PdMADS-box (CAJ28929); *Pyrus pyrifolia*: PpAP1-2 (ABP93402); *Eriobotrya japonica*: EjAPETALA1 (AAX14152); *Lotus corniculatus* var. *japonicus*: LcAP1a (AAX13296); *Hydrangea macrophylla*: HmAP1/FUL1 (AZ77749); *Betula pendula*: BpMADS4 (CAA67968); *Petunia x hybrida*: PhFBP29 (AAK21258); *Antirrhinum majus*: AmDEF28 (AAK72467), AmSQUA (CAA45228); *Arabidopsis thaliana*: AtAP1 (CAA78909), AtAGL8 (NP_568929). Labelling of the groups according to conserved C-terminal motif: euAP1, euFUL and core eudicote FUL (Litt and Irish 2003). Bootstrap confidential values (%) generated from 1000 replicates are indicated at the nodes

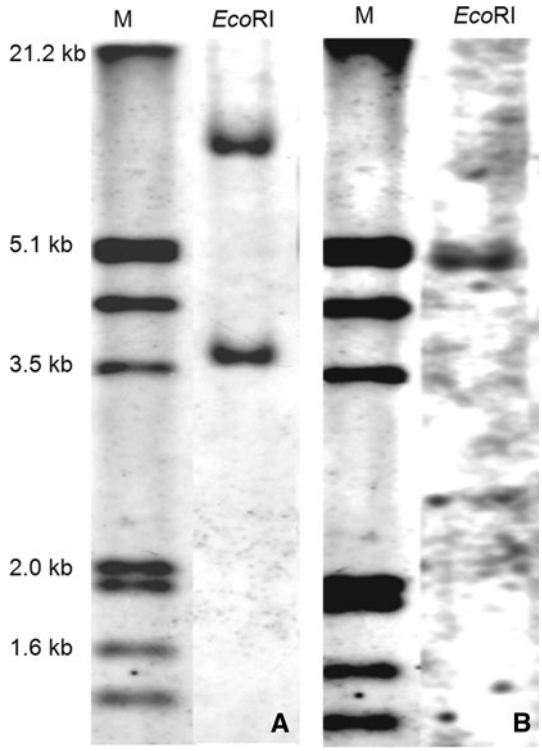

Fig. 4 Southern blot autoradiogram analysis of *RhAP1-1* and *RhFUL* homologs. The genomic DNA of genotype 76/74 was digested with *Eco*RI and probed with 216 bp (*RhAP1-1*) (**a**) or 350 bp (*RhFUL*) (**b**) DIG-labeled probe. M, DIG-labeled DNA molecular weight marker III

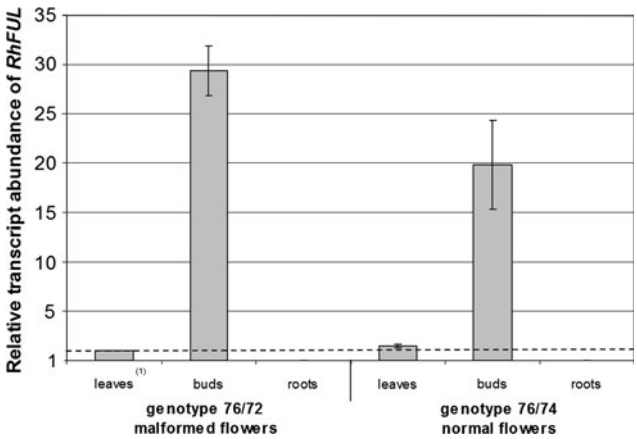

Fig. 5 Relative expression of the *RhFUL* gene in leaves, buds, and roots of genotypes with malformed and normally developed flowers. The fold-change expression of the isolated gene was calculated relative to the lowest expression in leaves from geneotype 76/72(1) after normalization to the ß–actin gene. Vertical bars represent ± standard deviation ($n = 3$)

rugosa showed an identical pattern to *RhB* or *MASAKO BP* (data not shown).

Expression of the C-function gene *RhC1* was detected in stamens and carpels of normally developed flowers, but expression levels in whorls 3 and 4 of malformed flowers was significantly decreased (Fig. 7d). The second C-function gene *RhC2* showed high expression in carpels and stamens of normal flowers (Fig. 7e), but expression intensity was dramatically decreased within petals and sepals (whorls 3 and 4) of malformed flowers. Less than 10% of *RhC2* transcript was detected in whorls 3 and 4 of malformed compared to normal flowers (Fig. 7e).

Discussion

Full-length cDNA sequences of three putative *APETALA1/FRUITFULL*-like genes were cloned and designated *RhAP1-1*, *RhAP1-2*, and *RhFUL* from rose flowers, and Southern blotting indicated that no other genes were closely related in sequence to the homologs *RhAP1-1*, *RhFUL*, and *RhAP1-2* in the rose genome (Fig. 4). All three genes

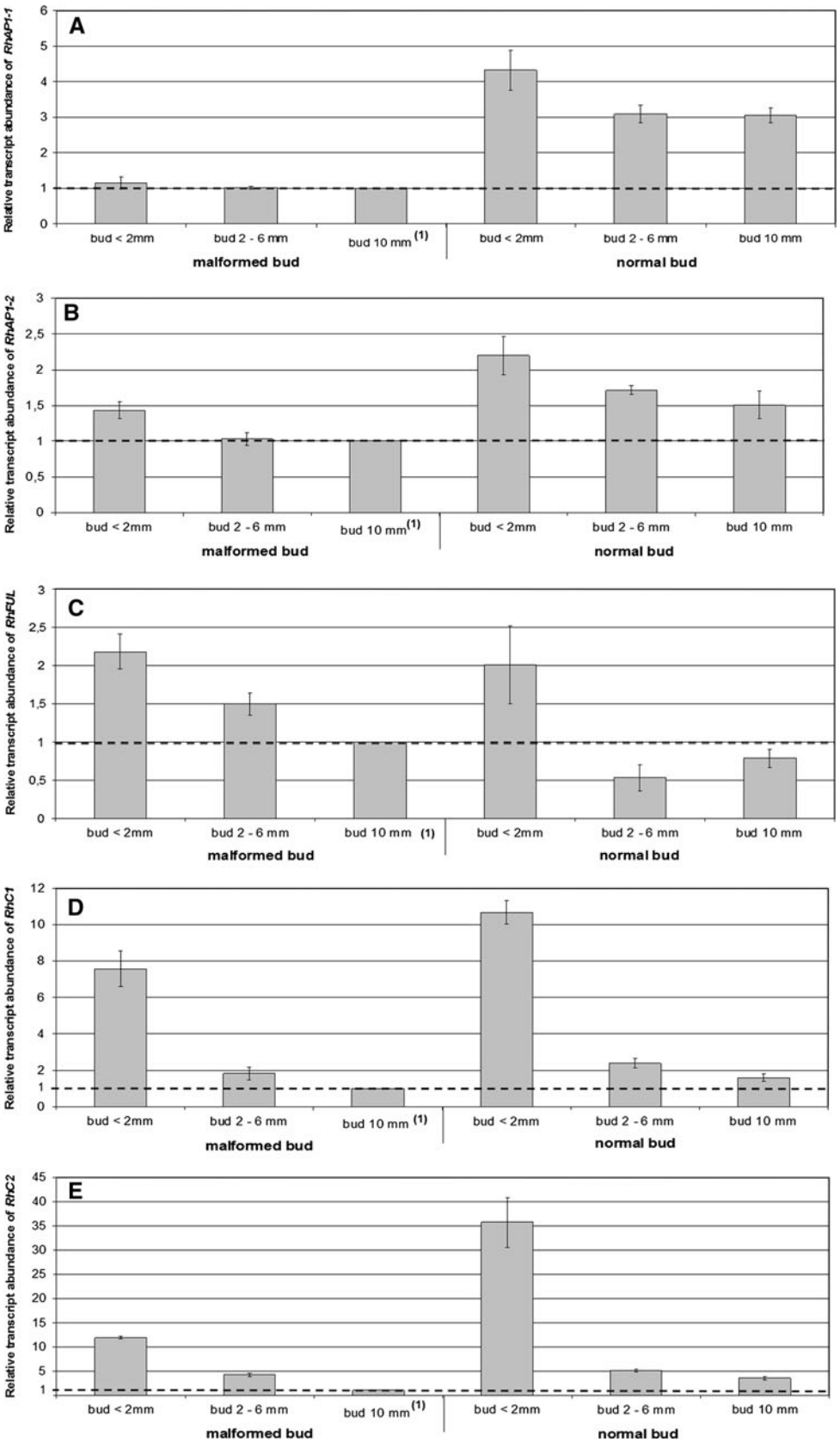

Fig. 6 Relative expression of the genes *RhAP1-1* (**a**), *RhAP1-2* (**b**), *RhFUL* (**c**), *RhC1* (**d**), and *RhC2* (**e**) in three different bud stages (<2 mm, 2-6 mm, and 10 mm) of malformed and normally developed flowers. The fold-change expression of the isolated gene was calculated relative to the lowest expression in 10 mm maformed bud (1) after normalization to the β–actin gene. Vertical bars represent $\pm$ standard deviation ($n = 3$)

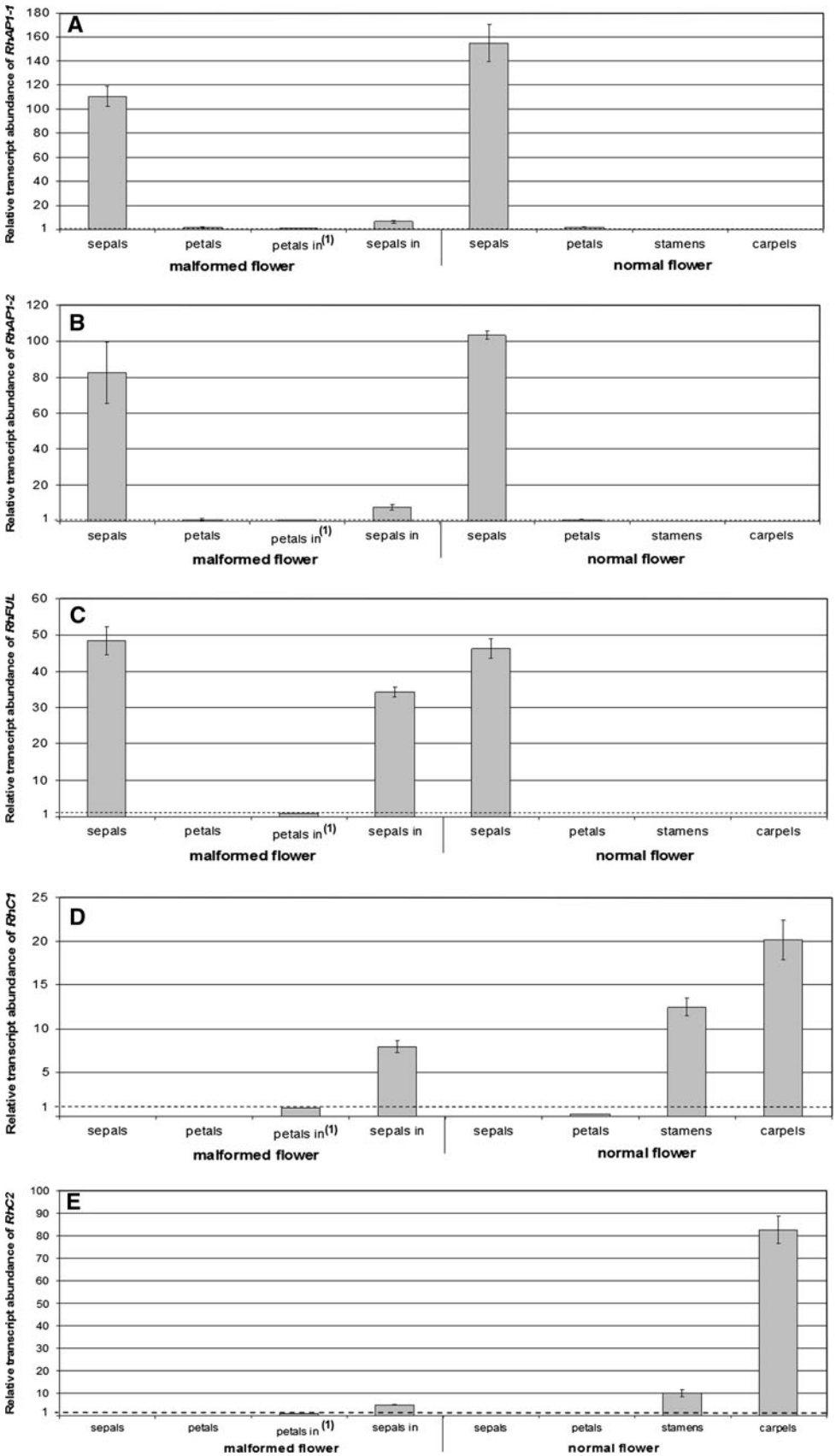

Fig. 7 Relative expression of the genes *RhAP1-1* (**a**), *RhAP1-2* (**b**), *RhFUL* (**c**), *RhC1* (**d**), and *RhC2* (**e**) in four different flower whorls of malformed (sepals = whorl 1, petals = whorl 2, petals in = whorl 3, and sepals in = whorl 4) and normally developed flowers (sepals = whorl 1, petals = whorl 2, stamens = whorl 3, and carpels = whorl 4). The fold-change expression of the isolated gene was calculated relative to the expression in petals inside of malformed flower (1), after normalization to the β-actin gene. Vertical bars represent $\pm$ standard deviation ($n = 5$)

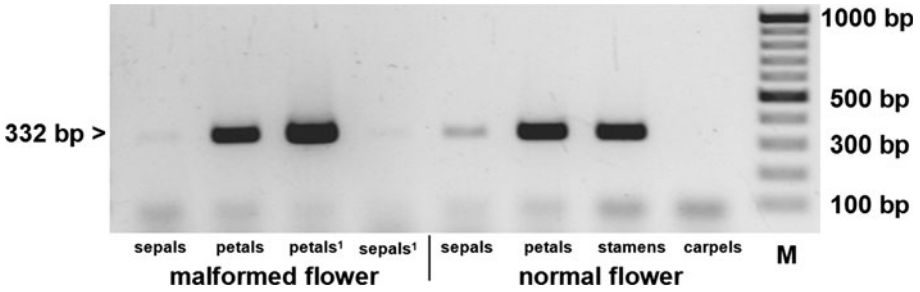

Fig. 8 Expression analyses with RT-PCR of a *RhB* gene (MASAKO B3) in four different flower whorls of malformed and normally developed flowers. Normalization of cDNA concentration was done with the β-actin gene (data not shown). 1, inner petals and sepals of third and fourth whorl; M, 100-bp DNA molecular weight marker

had the expected highly conserved MADS- and K-boxes (Fig. 2). Sequences of the proteins RhAP1-1 and RhAP1-2 were 97.2% identical to each other. The high sequence similarity between RhAP1-1 and RhAP1-2 may be a result of different homologous copies of the gene from the two progenitor species genomes or recent gene duplication already described for MADS-box genes. Alignment analysis demonstrated similarity to several other APETALA1-like proteins from species of the Rosacea family, including *E. japonica*, *T. rupestris*, *M.* × *domestica*, *P. pyrifoli*, and *P. persica*. A phylogenetic tree analysis performed according to Litt and Irish (2003) identified three different clades, designated as euAP1, euFUL, and core eudicote FUL proteins (Fig. 3). AP1/FUL-like proteins from *Rosa* grouped in the first (euAP1) and third (core eudicote FUL like) clades (Fig. 3). The *FUL* gene from *Arabidopsis* clustered in a second clade (euFUL) together with *Malus* MdMADS2. However, no members of the second euFUL group were detected in the rose genome. All euAP1-like proteins, including the novel proteins *RhAP1-1* and *RhAP1-2*, have the conserved C-terminal motif CHLGCFA (Fig. 2), which include the C-terminal prenylation signal CFA, typical for AP1-like proteins (Vandenbussche and others 2003; Litt and Irish 2003).

Comparison of RhAP1-1 and RhFUL proteins indicates an identity of only 58%. However, RhFUL shows 82% identity to TrFUL of *T. rupestris* and has high homology to other FUL-like proteins such as MdMADS-12 of *M.* × *domestica* and BpMADS4 of *B. pendula* (Fig. 3). This clade of FUL-like proteins shares a C-terminal conserved motif MPPVRHL (Fig. 2) and can be designated as an euFUL or a core eudicote FUL protein (Litt and Irish 2003). Differences among the euAP1, euFUL, and core eudicote FUL proteins have been explained by a frameshift mutation in an ancestral *AP1/FUL*-like gene (Litt and Irish 2003; Vandenbussche and others 2003).

Exclusive expression of the *AP1*-like genes *RhAP1-1* and *RhAP1-2* in whorls 1 and 2 of fertile rose flowers (Fig. 7a, b) is similar to that for *AP1*-like genes in other plant species (Yanofsky and others 1990; Mandel and others 1992; Huijser and others 1992). *RhFUL* was expressed in sepals and leaves and thus differs from the typical expression pattern of other *AP1* homologs (Figs. 5 and 7c). Sequence alignment (Fig. 2) demonstrated high sequence identity between RhFUL and TrFUL of *Taihangia rupestris*, but expression analysis is not available (Lu and others 2007).

Based on sequence alignment and phylogenic analysis, RhFUL is grouped in the clade of FRUITFULL (FUL)-like proteins (Fig. 3) with the typical conserved C-terminus of FUL-like proteins (Fig. 2) (Litt and Irish 2003; Vandenbussche and others 2003). This group of genes is closely related to *AP1*. The *FUL* gene of *Arabidopsis* regulates transcription of genes essential for cellular differentiation during fruit and leaf development and is expressed at high levels in inflorescence meristems (Gu and others 1998). Although single mutants of *FUL* genes in *Arabidopsis* have no impact on meristem identity (Gu and others 1998; Kempin and others 1995; Mandel and Yanofsky 1995), recent results suggested that *FUL* single mutants have a delayed transition to floral meristem (Yamaguchi and others 2009). In *Oryza sativa* and *Lolium temulentum*, duplicated *AP1/FUL* genes show distinct but overlapping expression patterns, suggesting discrete functional roles in the transition to flowering, specification of spikelet meristem identity, and specification of floral organ identity (Preston and Kellogg 2007). Northern analysis showed that *AmDEFH28*, a *FUL*-like gene of *Antirrhinum*, is expressed in inflorescences and older floral buds but not in vegetative organs (Müller and others 2001). Expression of *BpMADS4* from *Betula penula* starts at an early stage of the male and female inflorescence development but is also expressed in shoots and roots. Ectopic expression of *BpMADS4* accelerates flowering, and plants transformed with the *BpMADS4* antisense construct show a delay in flowering (Elo and others 2001, 2007). The *FUL*-like gene in *Spinacia oleracea*, *SpFUL*, is expressed in leaf as well as in floral tissue and shows strong expression late in flower development, particularly in the tapetal layer in males and in the endothecium layer and stigma in females (Sather and

Golenberg 2009). Detection of *RhFUL* transcripts in leaves and sepals indicated that its expression pattern is similar to *BpMADS4* from *Betula penula*.

Expression of class B genes *RhB* (highly similar to *MASAKO B3*) (Fig. 8) and *MASAKO BP* (data not shown) and class C genes *RhC1* and *RhC2* (highly similar to *MASAKO C1* and *MASAKO D*1) (Fig. 7d, e), investigated in normally developed flower organs of genotype 76/74, was consistent with the ABC model. These results confirm expression analysis in the different flower organs of wild rose (*R. rugosa* Thumb. Ex Murray) (Kitahara and Matsumoto 2000; Kitahara and others 2001).

Comparison of expression patterns between malformed and normally developed flowers demonstrated that *RhAP1-1* and *RhAP1-2* were expressed in all flower organs of malformed flowers (Fig. 6a, b). This reflected class A function in the formation of sepals and petals that replace stamens and carpels in whorls 3 and 4 of malformed flowers and supports the hypothesis that *RhAP1-1* and *RhAP1-2* are class A genes.

Expression of *RhFUL* in malformed flowers was restricted to mainly sepals of whorls 1 and 4, except for weak expression in petals of whorl 3 (Fig. 7c). This extended expression of class A genes in all flower whorls, in combination with missing class C gene expression (see below), was described in several plants (reviewed by Ferrario and others 2004).

RhB (homolog to gene *MASAKO B3* of *R. rugosa*) was expressed in petals and anthers of both normal and malformed flowers (Fig. 8). There were differences in expression of class B genes *MASAKO BP*, *B3*, and *euB3* between flowers of 'green rose' (*R. chinesis viridiflora*) and wild rose (*R. rugosa*) (Matsumoto and Kitahara 2005).

The expression of both putative C-function genes *RhC1* and *RhC2* was lower in malformed buds in all developmental stages (<2, 2-6, and 10 mm) compared to normal buds (Fig. 6d, e). and in whorls 3 and 4 of malformed buds (10 mm) compared to those in normal buds (Fig. 7d, e).

The downregulation of the class C gene *RAG* (highly similar to *RhC1*) in malformed rose flowers was shown in the early bud stage (1-3 and 4-5 mm) of *R.* × *hybrida* cv. Motrea and 'green rose' (Chmelnitsky and others 2003). In contrast, no expression differences of class C genes *MASAKO C1* and *D1* (highly similar to *RhC1* and *RhC2*) were detected between 'green rose' and wild rose organs of developed flowers (Matsumoto and Kitahara 2005). Based on these results, it appears that aberration of class C gene expression in the early stages of bud development can lead to phyllody or malformed rose flowers.

In the present study, expression analysis was completed in flower organs from buds (10 mm). However, similar gene expression patterns between abnormal and normally developed flowers were detected in fully open flowers (date not presented). The late expression of *AP1*-like genes in mature flowers may explain the outgrowth of sepals of rose flowers under environmental stress conditions (Mor and Zieslin 1992; Ganelevin and Zieslin 2002). However, only in situ hybridization analysis with all related genes at different developmental bud stages is required to correlate the expression patterns with phyllody or malformed flower type.

To explain the malformed flower type it is necessary to investigate the interaction between C-function and A-function genes that determine organ identity of the flower meristem. The C-function gene *AGAMOUS* is repressed in whorls 1 and 2 by the A-function gene *APETALA2* (Chen 2004), but it represses expression of the other A-function gene *APETALA1* in whorls 3 and 4. One explanation of the downregulation of *AGAMOUS*-like genes in this malformed flower type is a genetic mutation or a change of the methylation of the promoter of the C-function genes. However, such an assumption must be verified by detailed in situ hybridization in early developmental bud stages of all related genes.

On the other hand, the suppressed expression of the C-function genes does not depend exclusively on *AP2*. A number of other genes, like *LEUNIG* (*LUG*), *SUESS* (*SEU*), *STERILE APETALA* (*SAP*), and *AINTEGUMENTA* (*ANT*), also play a role in repression of C-function genes in whorls 1 and 2 (summarized in Jack 2004). Based on these results, a deregulation of at least one of these suppressor genes can lead to an increased suppression of class C genes in whorls 3 and 4 in the malformed rose buds (Fig. 7d, e), However, such assumptions must be verified by further experimentation.

In summary, three novel *AP1*-like genes were isolated and analyzed in *Rosa* × *hybrida* L. Sequences and phylogenetic analysis verified the cloning of two putative *euAP1*-type genes, designated *RhAP1-1* and *RhAP1-2*, and one *paleoAP1*-type gene, designated *RhFUL*. Expression patterns of *RhAP1-1* and *RhAP1-2* and the genes *RhB*, *RhC1*, and *RhC2* in normally developed rose flowers were consistent with the ABC model in *Rosa* × *hybrida* L., but functional studies are still required for confirmation of the model. *RhFUL*, the putative *FRUITFULL* homolog in *Rosa* × *hybrida*, showed unique expression in sepals and leaves. Expression patterns of class A, B, and C genes in phylloid compared to normally developed flowers demonstrated suppression of *RhC1* and *RhC2* in combination with transcript accumulation of *RhAP1-1*, *RhFUL*, and *RhAP1-2* in malformed flowers. The significant reduction of *RhC1* and *RhC2* transcripts observed in whorls 3 and 4 of the malformed flowers may explain the upregulation of *RhAP1-1*, *RhFUL*, and *RhAP1-2*. These data suggest that downregulation of *RhC1* and *RhC2* in the floral tissue is likely responsible for malformed, phylloid flowers. Further

experimentation is necessary to determine the mechanism responsible for downregulation of the genes *RhC1* and *RhC2*.

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