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ORIGINAL PAPER

A survey of flowering genes reveals the role of gibberellins in floral control in rose

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Abstract Exhaustive studies on flowering control in annual plants have provided a framework for exploring this process in other plant species, especially in perennials for which little molecular data are currently available. Rose is a woody perennial plant with a particular flowering strategy—recurrent blooming, which is controlled by a recessive locus (*RB*). Gibberellins (GA) inhibit flowering only in non-recurrent roses. Moreover, the GA content varies during the flowering process and between recurrent and non-recurrent rose. Only a few rose genes potentially involved in flowering have been described, i.e. homologues of ABC model genes and floral genes from EST screening. In this study, we gained new information on the molecular basis of rose flowering: date of flowering and recurrent blooming. Based on a candidate gene strategy, we isolated genes that have similarities with genes known to be involved in floral control in *Arabidopsis* (GA pathway, floral repressors and integrators). Candidate genes were mapped on a segregating population, gene expression was studied in different organs and transcript abundance was monitored in growing shoot apices. Twenty-five genes were studied. *RoFT*, *RoAP1* and *RoLFY* are proposed to be good floral markers. *RoSPY* and *RB* co-localized in our segregating population. GA metabolism genes were found to be regulated during floral transition. Furthermore, GA signalling genes were differentially regulated between a non-recurrent rose and its recurrent mutant. We propose that flowering gene networks are conserved between *Arabidopsis* and rose. The GA pathway appears to be a key regulator of flowering in rose. We postulate that GA metabolism is involved in floral initiation and GA signalling might be responsible for the recurrent flowering character.

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Introduction

Flowering, which is the vegetative to reproductive transition phase, is a critical developmental stage in a plant's life. In monocarpic plants, this transition happens only once, whereas polycarpics have several flowering cycles throughout their life. Monocarpics have four growth phases. First, the juvenile phase followed by the adult vegetative phase, then floral transition leads to the floral phase, ending with senescence (Baurle and Dean 2006). In comparison, the polycarpic life cycle is more complex. Flowering represents only a transitory state before new vegetative growth in polycarpic plants—they must maintain an equilibrium between vegetative and reproductive development throughout their life. Flowering generally occurs once a year. However, some polycarpics can reproduce several times a year. This process is known as recurrent blooming in rose or day neutral in strawberry, for instance (Battey et al. 1998).

Flowering has been widely studied in monocarpic plants at the genetic and molecular levels. In the model plant *Arabidopsis thaliana*, four different pathways controlling floral

initiation have been described: photoperiod and light quality, the autonomous pathway, vernalization and gibberellins (GA; reviewed in Araki 2001; Boss et al. 2004; Mouradov et al. 2002; Simpson and Dean 2002). These four pathways converge to activate the floral integrators, *FT* (*FLOWERING LOCUS T*; Kardailsky et al. 1999; Kobayashi et al. 1999) and *SOC1* (*SUPRESSOR OF CONSTANS*; Onouchi et al. 2000), and repression of a central repressor, *FLC* (*FLOWERING LOCUS C;* Michaels and Amasino 1999; Sheldon et al. 2000b). In turn, the floral integrators activate the meristem identity genes, *LFY* and *AP1* (reviewed in Araki 2001; Simpson and Dean 2002). Organ identity genes are the targets of these floral integrators and meristem identity genes. These genes, known as ABC model genes, are in charge of floral development (Jack 2001; Weigel and Meyerowitz 1994).

Plants have to perceive and respond to photoperiod and light quality. Light is perceived by phyto- and cryptochromes (Quail 2002). Changes in daylength are detected by the intrinsic circadian system, which in turn under long day conditions, activates the transcription factor CONSTANS (Suarez-Lopez et al. 2001). *FT* and *SOC1* are direct targets of the photoperiod and light quality (Onouchi et al. 2000; Samach et al. 2000). CO induces *FT* in the leaves (Samach et al. 2000). FT protein moves from leaves to the shoot apical meristem (SAM) (Corbesier et al. 2007) and interacts with the FD transcription factor that is only expressed in SAM (Abe et al. 2005; Wigge et al. 2005). *FT* activates flowering and is a primary candidate for encoding florigen (Zeevaart 2008).

FT and *SOC1* are subjected to the repression of *FLC*. *FLC* encodes a MADS domain protein that acts as a repressor of flowering (Michaels and Amasino 1999). This repressor is regulated both by the autonomous pathway (Rouse et al. 2002) and vernalization (Sheldon et al. 2000a). Autonomous pathway genes were first described thanks to mutants that flowered late under all photoperiods (Koornneef et al. 1991). It involves seven genes, as reviewed in Simpson (2004), which prevent the accumulation of *FLC* mRNA. Vernalization involves genes such as *VRN1*, *VRN2* and *VIN3* (Gendall et al. 2001; Levy et al. 2002; Sung and Amasino 2004). These genes quantitatively act in response to cold treatment in an epigenetic manner, principally by downregulating *FLC* expression (Bastow et al. 2004; Sheldon et al. 2006). Other different repressors are also involved in floral initiation control. Unlike *FT*, *TFL1* is a repressor of floral initiation (Bradley et al. 1997). Moreover, *TFL1* may play a role in inflorescence meristem identity by regulating the flower indeterminacy (Ratcliffe et al. 1998). The *EMBRYONIC FLOWER* genes, i.e. *EMF1* and *EMF2*, maintain vegetative development and repress reproductive growth as well (Moon et al. 2003b).

Finally, the GA pathway promotes flowering in *Arabidopsis* during short days (SD) (Wilson et al. 1992) by the activation of *LFY* and *SOC1* (Blazquez and Weigel 2000; Eriksson et al. 2006; Moon et al. 2003a). GA metabolism principally involves GA oxidase enzymes that are encoded by multigenic family genes (reviewed in Hedden and Phillips 2000). GA20OX and GA3OX are the enzymes responsible for active GA synthesis; GA2OX also plays a key role by converting active into inactive GA (Sakamoto et al. 2004). GA signalling operates as de-repressible system that is moderated by DELLA-proteins (Fleet and Sun 2005). DELLA-proteins are represented by five proteins in *Arabidopsis*: RGA, GAI, RGL1, RGL2 and RGL3 (Ikeda et al. 2003). DELLA-proteins are activated by SPINDLY (Silverstone et al. 2007). In the presence of active GA, its receptor, GID1 (GA INSENSITIVE DWARF1), recruits SLEEPY and forms a complex with DELLA (McGinnis et al. 2003; Nakajima et al. 2006). Consequently, DELLA protein is degraded via the ubiquitin-proteasome (Sun and Gubler 2004). Then, the system is derepressed and the de facto plant responds to GA.

The unravelled gene network in *Arabidopsis* for the control of floral transition offers a broad framework for studying flowering in other plants. The network is mainly conserved in monocarpic plants such as rice, but some genes exhibit different regulations (Izawa et al. 2003). In perennials, different studies have shown that homologous genes are also implicated in flowering control. In poplar, homologues of *FT* are involved in floral initiation (Igasaki et al. 2008). However, a new role has been demonstrated in juvenility and seasonal flowering, which are perennial specific traits (Hsu et al. 2006). In tomato, *SFT* and *SP* are described as being the orthologues of the *Arabidopsis FT* and *TFL1* genes, respectively (Lifschitz et al. 2006; Pnueli et al. 1998). SFT and SP interact to control flowering in the sympodial shoot system of tomato (Lifschitz and Eshed 2006). In grass, two sequential signals regulate floral initiation (McDaniel and Hartnett 1996). Cold exposure makes plants to respond to GA, and LD induces GA synthesis, leading to floral transition (McMillan et al. 2005). Finally, in grapevine, GA inhibits floral meristem production (Boss and Thomas 2002). These few examples show that flowering genes are conserved within plants but functions or regulation of these genes can vary between species.

Although the whole sequenced genome of grapevine (Jaillon et al. 2007) and poplar (Tuskan et al. 2006) are now sources of knowledge for flowering in polycarpic plants, a lot remains to be unravelled. In rose, the top-ranking ornamental plant produced worldwide, flowering has been studied at the physiological level in modern recurrent cultivars of cut flower roses (Al-Humaid 2003; Chakradhar and Khiratkar 2004; Horridge and Cockshull 1974). In recurrent rose, flowering is self-inductive, i.e. environmental triggers

are not required to initiate flowering (Havely 1972). In non-recurrent rose, flowering is under environmental control via vernalization. (Foucher F., personal communication). As in other woody plants, GA inhibits flowering in rose (Roberts et al. 1999; Zeevaart 1983). Exogenous application of GA inhibits flowering in non-recurrent rose, whereas it has no effect on flowering in recurrent rose (Roberts et al. 1999). Moreover, the analysis of GA content in a non-recurrent rose and its spontaneous recurrent mutant showed that the GA concentration varies during floral transition and within the mutant (Roberts et al. 1999). Little information on genetic and molecular flowering control is currently available. The recurrent blooming trait is inherited as a single recessive gene, i.e. *RB* for *RECURRENT BLOOMING* (Crespel et al. 2002; Semeniuk 1971). Recently, a QTL for the flowering date was shown to be located in the vicinity of the *RB* locus (Hibrand-Saint Oyant et al. 2008).

Only a few genes have been characterized in rose. These genes are potentially involved in floral control and development. A candidate gene approach has been implemented and led to the isolation of ABC model genes (Hibino et al. 2006; Kitahara et al. 2001; Kitahara and Matsumoto 2000). In a previous study, we sequenced ESTs and thus identified new genes with significant similarities to floral genes (Foucher et al. 2008).

The aim of this study was to explore the molecular basis of rose flowering and more particularly the molecular control of recurrent blooming. According to our knowledge on flowering in rose, we postulate that the GA pathway and floral inhibitors might be key factors. We thus used degenerate primers to isolate genes involved in floral repression, GA signalling and metabolism and floral integrator activation. Genetic mapping and gene expression analysis were performed for genetic and molecular characterization. We propose that GA is a key hormone in the control of flowering in rose; GA metabolism seems to be involved in floral initiation and GA signalling might play a role in recurrent blooming.

Materials and methods

Plant material

Rosa hybrida cv Félicité&Perpétue (FP) and *Rosa hybrida* cv Little White Pet (LWP) were obtained from the Loubert nursery (http://www.rosesloubert.com, Les Rosiers sur Loire, France). *Rosa hybrida* cv Little White Pet resulted from a spontaneous vegetative mutation of *Rosa hybrida* cv Félicité&Perpétue (Lewis 1994). LWP is a recurrent blooming, sterile dwarf rose whereas FP is a non-recurrent, fertile climbing rose (Lewis 1994). A segregating population (HW) of 91 interspecific hybrids derived from a cross between H190 and a *Rosa wichurana* (Rw) hybrid was used as the mapping population (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008). All of these rose genotypes are diploid. Plants were outdoor grown on their own roots on KLASMANN RHP 15® (provided by RIPPERT) substrate in 5 l pots at INRA in Angers, France, and fertilized with LD10® (provided by COMPO France SAS).

Growth measurement and sampling

In spring, terminal parts of growing shoots of FP and LWP were regularly taken off at different developmental stages: from lateral bud outgrowth to the floral bud stage. After the first flowering, plants were pruned (beginning of July), then new shoots arose from buds. Autumn samples were harvested when two leaves were fully opened: in FP, shoots remained vegetative (non-recurrent genotype) whereas LWP shoots flowered rapidly (recurrent genotype). Developmental stages were defined as the number of visible leaves per new shoot. Morphological observations were performed using a binocular microscope to detect floral initiation. Floral initiation corresponds to the first morphological changes leading to an inflorescence meristem according to Foucher et al. (2008). Each sample corresponded to the three most distal buds from five plants per genotype.

Isolation of candidate genes: degenerate primer strategy

To design degenerate primers, conserved domains were identified from protein sequence alignments using the BLOCKMAKER online application (http://bioinformatics.weizmann.ac.il/blocks/blockmkr/www/make_blocks.html). Based on these conserved domains, CODEHOP was used to design degenerate primers (Rose et al. 2003).

All primers used for gene cloning are listed in Table S1. For a few genes, full-length cDNAs were obtained using 3′ and 5′ rapid amplification cDNA ends (Jain et al. 1992) according the manufacturer's recommendations (Clontech, Moutain View, USA).

Sequencing, database searches, alignments and phylogenetic analysis

Sequencing was subcontracted to Genome Express (Grenoble, France).

Sequences of putative rose homologue genes were blasted against the *Arabidopsis* genome (The Arabidopsis Information Resources; http://www.arabidopsis.org) using tBLASTx and tBLASTn searches. Validated sequences were translated (http://bioinfo.hku.hk/services/analyseq/cgi-bin/traduc_in.pl) and protein alignments were performed with MultAlin online software (Corpet 1988).

CLUSTALW (Thompson et al. 1994) and TreeViewX Version 0.5.0 by Roderic D. M. Page (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) were used for phylogenetic analyses and phylogenetic tree edition, respectively.

DNA and RNA extraction

Genomic DNA was isolated from young leaves of rose using the NucleoSpin® Plant kit (Macherey-Nagel) according to the manufacturer's recommendations.

Total RNA isolations were performed on growing apices from FP, LWP and Rw. Tissues were ground in liquid nitrogen with 10% (w/w) PVP40 (PolyVinylPyrrolydone), and total RNA was extracted using the NucleoSpin® RNA plant kit (Macherey-Nagel) according to the manufacturer's recommendations. Nucleic acids were quantified using a Nanodrop (Nanodrop Technologies Inc., Wilmington, USA) and their quality was checked either by electrophoresis on agarose gel or using the Agilent capillary electrophoresis system (Agilent Technologies).

Reverse transcription (RT)

Three microgram of total RNA was DNase-treated for 15 min at 25°C with 1 U of RQ1 RNase-free DNase (Promega, Madison, USA) in a final volume of 10 µl containing the appropriate buffer followed by enzyme inactivation by adding 2 mM of EGTA and incubation for 10 min at 65°C. RNAs were denatured for 5 min at 70°C with 0.5 µg of oligo(dT)15 (Promega) and then subjected to reverse transcription with 200 U of MMLV-RT (Promega), 0.5 mM of each dNTP, 1 U of RNasin® Ribonuclease Inhibitor (Promega) in a final volume of 25 µl for 1 h at 42°C. After RT, the reaction volume was adjusted to 100 µl. The RT efficiency and the absence of genomic DNA in the cDNA were checked by PCR with specific *EF1α* primers surrounding an intron. All primers used for RT-PCR are listed in Table S4.

PCR amplification of genomic DNA and cDNA

Specific PCR primers were designed using the PRIMER3 software available online (Rozen and Skaletsky 2000). Desalted oligonucleotides were synthesized by Sigma-Genosys (Saint Louis, USA). PCR reactions were carried out in 15 µl with GoTaq® flexi DNA Polymerase according to the manufacturer's recommendations (Promega). The general amplification conditions were as follows: 94°C, 2 min; 35 × [94°C, 30 s; annealing temperature, 30 s; 72°C, 1 min]; 72°C, 10 min; 10°C, 10 min. Amplifications were performed in a DNA thermal cycler (PTC-200-MJ Research, Biorad).

Marker development and genetic mapping

Mapping was performed on the HW population (Hibrand-Saint Oyant et al. 2008). Version 4.0 of JoinMap® (Van Ooijen, J.W., Kyazma B.V., Wageningen, Netherlands) was used for construction of the integrated genetic map. In a first step, the parental maps were determined using LOD 5.0 and the Kosambi function. The mapping parameters were independence LOD, regression mapping as mapping algorithm and in regression mapping a Jump of 5.0, linkages with a recombination frequency of less than 0.3 and a LOD of more than 2.0, and no third round was performed. The integrated map was built with the join function (combined groups for map integration) using homologous parental LGs.

Polymorphisms were sought between the parents in the mapping population to develop PCR-based markers such as SSCP, CAPS and dCAPS. For SSCP analysis, 6 µl of PCR product was mixed with 4 µl of formamide dye [98% deionized formamide (Sigma-Aldrich), 10 mM ethylenediamine tetra-acetic acid (pH 8.0), 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue], denatured for 5 min at 95°C and quickly cooled on ice. Products were separated on 8–12% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1, 0.5× TBE) by electrophoresis at 40 W for 4–6 h in 0.5× TBE. Gels were pre-run for 30 min under the same electrophoretic conditions, and the improved procedure described by Creste et al. (2001) was used for silver staining of gels. The SSCP markers are listed in Table S2.

For CAPS and dCAPS, restriction enzymes that could generate polymorphisms were identified using online software (http://genoweb.univ-rennes1.fr/Serveur-GPO/outils_acces.php3?id_syndic=2 or dCAPS Finder 2.0: http://helix.wustl.edu/dcaps/dcaps.html (Neff et al. 1998). Five microliter of PCR product was digested with the appropriate restriction enzyme in a 10 µl final volume according to the manufacturer's recommendations (Promega). Enzymes and PCR conditions for CAPS and dCAPS are listed in Table S3.

Quantitative real-time PCR analysis

Primers were designed with Beacon Designer 7 (PREMIER Biosoft International) and listed in Table S5. Real-time PCR reactions were performed in triplicate using 3 µl of RT product (1/50 dilution) in a final volume of 15 µl containing 1× IQ SYBR Green Supermix (Bio-RAD), and 0.3 µM of each primer. Amplifications were performed using an Opticon 4 RealTime PCR detector (Bio-RAD) as follows: 95°C, 3 min; 40 × [95°C, 15 s; 60°C, 1 min]. The amplification specificity was verified by a final dissociation curve ranging from 60 to 95°C. Amplification and dissociation curves

were monitored and analysed with Opticon Monitor (Bio-RAD). The amount of plant RNA in each sample was normalized using the *TCTP* gene as reference after checking the homogeneity of the Ct variation with a second housekeeping gene, i.e. *EF1α*. The relative expression level calculation was done according to Pfaffl (2001). The standard error was calculated from three repetitions per sample. Q-PCR experiments were performed according to the recently published recommendations (Gutierrez et al. 2008; Udvardi et al. 2008).

Sequence data from this article have been deposited with the EMBL data library under accession numbers (in parentheses) *RoEMF1* (*FM999793*), *RoEMF2* (*FM999794*), *RoLHP1* (*FM999795*), *RoTFL1* (*FM999796*), *RoGA20OX* (*FM999797*), *RoGA3OX* (*FM999798*), *RoGID1* (*FM999799*), *RoSPINDLY* (*FM999800*), *RoFT* (*FM999826*), *RoLFY* (*FM999801*), *RoAP1a* (*FM999802*) and *RoAP1b* (*FM999803*).

Results

Isolation of flowering-related genes in rose

Based on our knowledge on rose, we mainly focused on isolating genes involved in the GA pathway and floral repressors in *Arabidopsis*. Some of these genes (such as *RoDELLA*) were present in the rose EST database and previously described by Foucher et al. (2008). The other genes were isolated using a degenerate primer strategy. Degenerate primers were designed from conserved sequence blocks. Rose sequences obtained with these degenerate primers were subjected to tBLASTx searches in turn against *Arabidopsis* nucleic-acid database (TAIR). BLAST hits were visually assessed for degree of amino-acid conservation in order to identify relationships between *Arabidopsis* and rose sequences (Table 1). Phylogenetic analyses were performed for genes belonging to multigenic families.

Floral integrators

MADS-box gene family MADS-box genes encode a family of transcription factors that control a diverse range of processes in flowering plants (Becker and Theissen 2003). MADS-box proteins, represented by more than 100 members in *Arabidopsis*, are characterized by a highly conserved N-terminal domain. Among these proteins, 39 are sub-classified in the MIKC class (Parenicova et al. 2003). They control flowering time (*FLC*, *SOC1*, *SVP*), floral meristem identity (*AP1*, *FUL*, *CAL*), floral organ identity (*AP3*, *PI*, *SEP*), fruit formation and ovule identity (*AG*). We identified *RoAP1a* and *RoAP1b* genes. The predicted proteins from *RoAP1a* and *RoAP1b* sequences, respectively, showed 77 and 58% identity with the *Arabidopsis AP1* gene (Table 1). By phylogenetic analysis, the two genes were found in the S*QUAmosa* clade (Fig. 1), as defined by Becker and Theissen (2003). This clade contains *Arabidopsis* AP1, FUL and CAL proteins.

The *FLC* gene was sought for rose in the rose EST database or by the degenerate primer strategy, but no sequence presenting significant similarities with *FLC* was retrieved.

FT/TFL1 gene family *FT* and *TFL1* are members of the six gene PEBP family in *Arabidopsis* (Kobayashi et al.

Table 1 Floral genes isolated in rose using degenerate primers

Arabidopsis thaliana			*Rosa wichurana*			Gene family
Pathway	Gene name	TAIR Ref.	Name	Accession nb.	% identity	
Repressors and vernalization	*EMF1*	*AT5G11530*	*RoEMF1* (44%)	*FM999793*	27	Monogenic
	EMF2	*AT5G51230*	*RoEMF2* (45%)	*FM999794*	45	Monogenic
	LHP1	*AT5G17690*	*RoLHP1* (73%)	*FM999795*	42	Monogenic
	TFL1	*AT5G03840*	*RoTFL1* (74%)	*FM999796*	77	Multigenic (PEBP)
Gibberellic acid	*GA20OX*	*AT5G07200*	*RoGA20OX* (85%)	*FM999797*	64	Multigenic (Gibberellin oxidase)
	GA3OX	*AT1G15550*	*RoGA3OX* (67%)	*FM999798*	60	
	GID1	*AT3G05120*	*RoGID1* (100%)	*FM999799*	80	Monogenic
	SPINDLY	*AT3G11540*	*RoSPINDLY* (100%)	*FM999800*	79	Monogenic
Floral integrators and meristem identity genes	*FT*	*AT1G65480*	*RoFT* (97%)	*FM999826*	74	Multigenic (PEBP)
	LFY	*AT5G61850*	*RoLFY* (32%)	*FM999801*	82	Monogenic
	AP1	*AT1G69120*	*RoAP1a* (62%)	*FM999802*	77	Multigenic (MADS box)
	AP1	*AT1G69120*	*RoAP1b* (70%)	*FM999803*	58	

Arabidopsis genes are classified according to the different pathways. The gene references are from TAIR (http://www.arabidopsis.org). For the new isolated rose genes, the percentage in brackets represents the percentage of the coding sequence isolated according to the coding sequence in *Arabidopsis*. The percentage identity was determined with the corresponding *Arabidopsis* genes

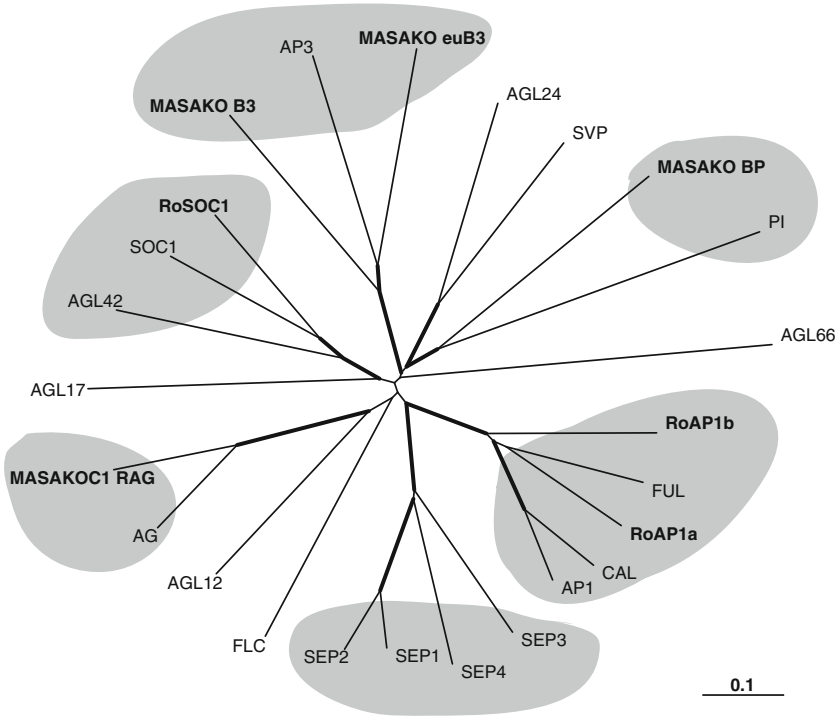


Fig. 1 Phylogenetic tree of different MIKC/MADS-box genes in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded* area. Sequence accession numbers are: *FUL* (*AT5G60910*), *CAL* (*AT1G26310*), *AP1* (*AT1G69120*), *SEP1* (*AT5G15800*), *SEP2* (*AT3G02310*), *SEP3* (*AT1G24260*), *SEP4* (*AT2G03710*), *FLC* (*AT5G10140*), *AGL12* (*AT1G71692*), *AG* (*AT4G18960*), *AGL17* (*AT2G22630*), *AGL42* (*AT5G62165*), *SOC1* (*AT2G45660*), *AP3* (*AT3G54340*), AGL24 (AT4G24540), SVP (AT2G22540), *RoSOC1* (CF349866.1), *RoAP1a* (*FM999802*), *RoAP1b* (*FM999803*), *MASAKOC1/RAG* (*AB025644*), *MASAKO BP* (*AB038462*), *MASAKO B3* (*AB055966*), *MASAKOeuB3* (*AB099875*)

1999). Scant knowledge is available about the four other genes, but they may contribute to flowering time regulation (Mimida et al. 2001; Yamaguchi et al. 2005; Yoo et al. 2004). Using degenerate primers, two genes presenting similarity with *FT/TFL1* genes were isolated. One sequence, named *RoFT*, showed 74% similarity with the *FT* gene. The second sequence, i.e. *RoTFL1*, presented 77% similarity with the *TFL1* gene (Table 1). By phylogenetic analysis, *RoFT* appeared in the *FT/TSF* clade, whereas *RoTFL1* branched with *TFL1* and *ATC* (Fig. 2).

Another floral integrator: LEAFY A partial sequence presenting high similarity (82%) with *LEAFY* from *Arabidopsis* was isolated (Table 1). The partial region isolated coded for six of the seven helix folds necessary for DNA binding as a dimer of the LEAFY protein (Hamès et al. 2008).

GA metabolism and signalling

GA promotes flowering in *Arabidopsis* under SD (Wilson et al. 1992). GA20OX and GA30X are responsible for the synthesis of active GA (as GA4), whereas GA2OX is involved in the degradation of active GA (Hedden and

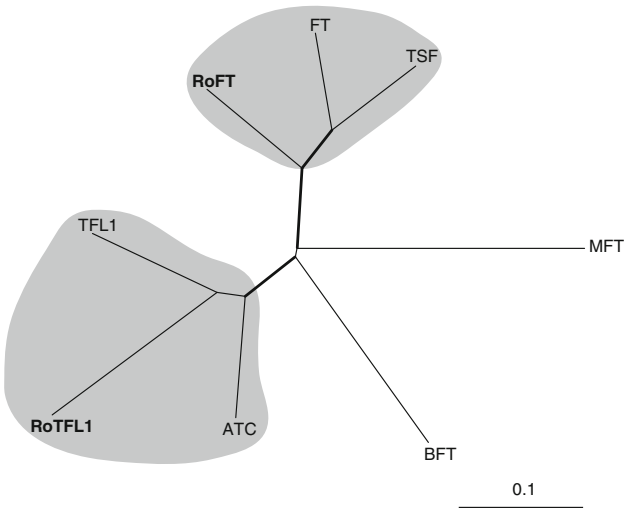


Fig. 2 Phylogenetic tree of FT/TFL1 gene family in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded area*. Sequence accession numbers are: *FT* (*AT1G65480*), *TSF* (*AT4G20370*), *MFT* (*AT1G18100*), *BFT* (*AT5G62040*), *ATC* (*AT2G27550*), *TFL1* (*AT5G03840*), *RoFT* (*FM999826*), *RoTFL1*(*FM999796*)

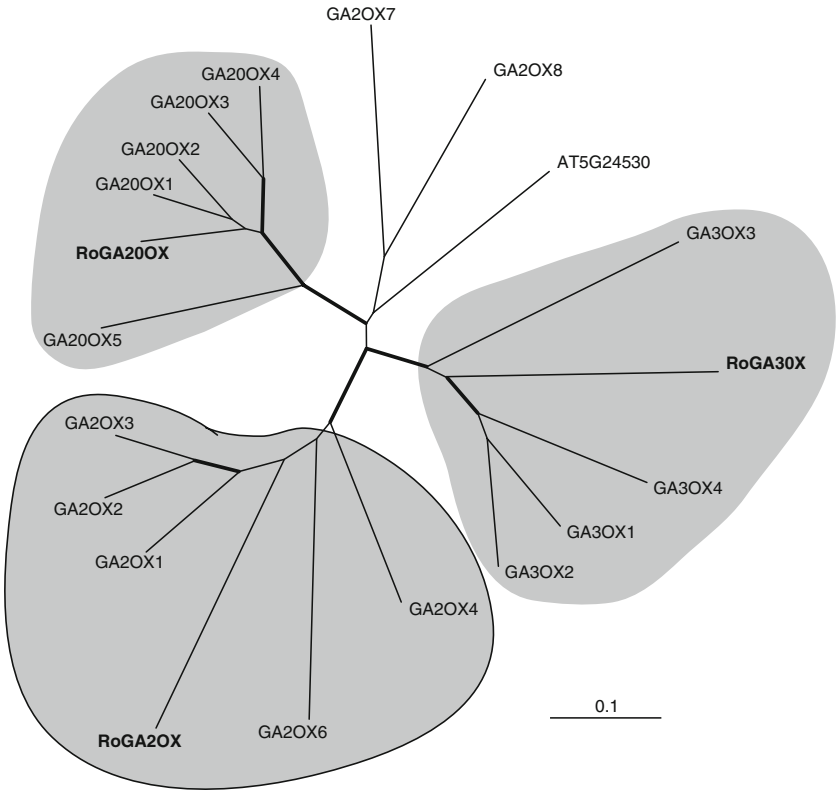


Fig. 3 Phylogenetic analysis of GA oxidase genes in rose and *Arabidopsis*. The tree was constructed using the NJ method with the ClustalW program. Branches with a bootstrap value greater than 700 (of 1,000) are shown with *thick lines*. Sequences belonging to a same clade are grouped together within a *grey shaded area*. Sequence accession numbers are: *GA20OX1* (*AT4G25420*), *GA20OX2* (*AT5G51810*), *GA20OX3* (*AT507200*), *GA20OX4* (*AT1G60980*), *GA20OX5* (*AT1G44090*), *GA3OX1* (*AT1G15550*), *GA3OX2* (*AT1G80340*), *GA3OX3* (*AT4G21690*), *GA3OX4* (*AT1G80330*), *GA2OX1* (*AT1G78440*), *GA2OX2* (*AT1G30040*), *GA2OX3* (*AT2G34555*), *GA2OX4* (*AT1G47990*), *GA2OX6* (*AT1G02400*), *GA2OX7* (*AT1G50960*), *GA2OX8* (*AT4G21200*), *RoGA20OX* (*FM999797*), *RoGA3OX* (*FM999798*), *RoGA2OX* (BQ105545.1)

Phillips 2000). These genes are encoded by a multigenic family and have 20-Fe-oxydoreductase activity (Hedden and Phillips 2000). We identified *RoGA20OX* and *RoGA3OX* that presented more than 60% identity with *AtGA20OX* and *AtGA3OX*, respectively (Table 1). Phylogenetic analysis revealed that the three rose genes belonged to the respective *Arabidopsis* clades (Fig. 3). Moreover, the specific domains described by Sakamoto et al. (2004) were found in rose sequences. RoGA20OX had the NYYPXCXXP and LPWKET domains as well as AtGA20OX. Moreover, His93, His 252 and 308 were conserved in RoGA20OX. Asp242, His248 and His296, which allow cofactor interaction, were present in RoGA3OX. And finally, RoGA2OX was found to have His217, H278 and Asp227, which are supposed to fix Fe++.

Concerning GA signalling, we isolated genes presenting similarities with *GID1* and *SPINDLY* (Table 1). RoGID1 shares 80% identity with the *Arabidopsis* GA receptor GID1 (Nakajima et al. 2006) and presents the two amino acids (Gly196 and Arg251) that are essential for in vitro interaction between GID1 and GA4 (Nakajima et al. 2006). We identified one sequence encoding an O-linked *N*-acetylglucosamine transferase (OGT), i.e. RoSPY, which shared 79% identity with AtSPY (Table 1). SPY contains multiple copies of N-terminal tetratricopeptide repeats (TPR) and two conserved domains (CDI and CDII) in C-termini common to animal OGTs (Silverstone et al. 2007). *RoDELLA*, one homologue of *AtRGA*, and *RoSLEEPY*, a homologue of AtSLY, were previously described by Foucher et al. (2008). Full-length cDNAs were isolated for the four GA signalling genes.

Isolation of floral repressors in rose

In addition to the floral repressor *RoTFL1*, three other sequences presenting similarities with *Arabidopsis* floral repressors were isolated in rose: *RoEMF1*, *RoEMF2* and *RoLHP1*. The *RoEMF1* gene only showed similarity with *EMF1* genes previously isolated in rice and *Arabidopsis* (Table 1, Aubert et al. 2001). The *RoEMF1* partial sequence is predicted to encode the LXXLL motif that is thought to mediate steroid receptor binding. This motif is also found in rice and *Arabidopsis* (Aubert et al. 2001). As previously described for *EMF2* in *Arabidopsis* (Yoshida et al. 2001), the predicted protein for *RoEMF2* contained a

Table 2 Genetic markers, polymorphisms in the HW population and location of rose flowering genes on the genetic map

Name	MM	Polymorphism	LG
RoEMF1	dCAPS	Male	7
RoEMF2	SSCP	Male	5
RoELF8	CAPS	Female	2
RoVIP3	dCAPS	Male	4
RoLHP1	SSCP	Male	1
RoTFL1	dCAPS	Male	1
RoGA20OX	SSCP	Female	1
RoGA3OX	SSCP	Male	2
RoGA2OX	SSCP	Male	6
RoGID1	SSCP	Male	4
RoDELLA	SSCP	Male	4
RoSPINDLY	SSCP	Male	4
RoSLEEPY	SSCP	Male	7
RoGI	SSCP	Male	7
RoCOL1	–	Monomorphic	–
RoCOL2	SSCP	Female	3
RoFT	SSCP	Male/female	3
RoSOC1	CAPS	Male	1
RoLFY	SSCP	Female/male	5
RoAP1a	SSCP	Female/male	5
RoAP1b	SSCP	Female	2
MASAKOB3	SSCP	Female	2
MASAKOeuB3	SSCP	Male	7
MASAKOBP	CAPS	Male	7
MASAKOC1/RAG	dCAPS	Female/male	6

MM molecular markers, *LG* linkage group

single C2H2 zinc finger domain and an acidic W/M domain in the C-term part. LHP1 in *Arabidopsis* showed a structure similar to HETEROCHROMATIN PROTEIN 1 (HP1) from *Drosophila* with the two characteristic HP1 motifs, the chromo domain and the chromo shadow domain (Gaudin et al. 2001). Both domains were found in the predicted protein encoded by *RoLHP1*.

Using degenerate primers (Table 1), we were able to isolate 12 new genes with a potential role in flowering control. Combined with a previous approach based on rose EST database screening (Foucher et al. 2008), we now have a set of 25 genes (Table 2) presenting significant similarities to genes involved in floral initiation and development. To further characterize these genes, we searched for co-localization with previously described loci involved in flowering in rose.

Genetic mapping

Markers (SSCP, CAPS or dCAPS) were developed for mapping candidate genes (Table 2), and were used to expand genetic maps of the interspecific F1 progeny (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008). Of the 25 candidate genes analysed, 24 were assigned on a genetic map at a LOD score of 5.0. *RoCOL1* was monomorphic and could not be mapped. An integrated map consisting of seven linkage groups with 213 markers spanning 482 cM was built (Crespel et al. 2002; Hibrand-Saint Oyant et al. 2008; this work). Candidate genes were distributed throughout the seven linkage groups (Fig. 4). Three of the new mapped genes (*RoFT*, *RoAP1b* and *RoGA3OX*) departed from the expected Mendelian segregation ($P < 0.05$). Three genes involved in GA signalling (*RoSPY*, *RoDELLA* and *RoGID1*) and one involved in the vernalization response (*RoVIP3*) were located on linkage group 4, where the *RECURRENT BLOOMING* locus and a QTL for blooming date were previously localized (Hibrand-Saint Oyant et al. 2008). In the 91 individuals of the mapping population, no recombinant was found between *RB* and *RoSPY*. Furthermore, *RoSPY*, *RoDELLA* and *RoVIP3* were located within the confidence interval of the QTL for blooming date.

Where are flowering-related genes expressed in rose?

The presence of transcripts was tested by reverse-transcription PCR in different tissues for all genes studied (Fig. 5). The experiment was performed on seven *Rosa wichurana* tissues: roots, leaves, shoots, floral buds and three types of growing apices (vegetative apices, vegetative pre-floral apices and floral apices). Nine genes (*RoEMF2*, *RoGA20OX*, *RoGA3OX*, *RoGA2OX*, *RoGID1*, *RoSPY*, *RoSLY*, *RoSOC1*, *RoCOL1*) were expressed in all tested tissues (Fig. 5). *RoGI* and *RoDELLA* were expressed everywhere except in floral buds, whereas *RoEMF1*, *RoVIP3* and *RoLHP1* were expressed everywhere except in leaves. Few genes showed more specific expression patterns. *RoELF8* transcripts were detected in apices, shoots and roots. *RoCOL2* was expressed in apices, leaves and floral buds. *RoTFL1* was mainly expressed in roots and vegetative apices. Few genes were expressed only during the floral process. *RoLFY* and *RoAP1b* transcripts were present in induced and floral-induced apices as well as floral buds. *RoFT* transcripts were accumulated in floral apices and floral buds. Finally, the expression of *MASAKO* genes and *RoAP1a* was tissue specific: *MASAKO* genes were only expressed in floral buds and *RoAP1a* was only expressed in floral apices.

Analysis of transcript abundance

To examine whether the floral phenotype could be related to floral gene expression, transcript levels of nine previously isolated flowering-related genes were analysed by qRT-PCR in a couple of mutants diverging by the recurrent

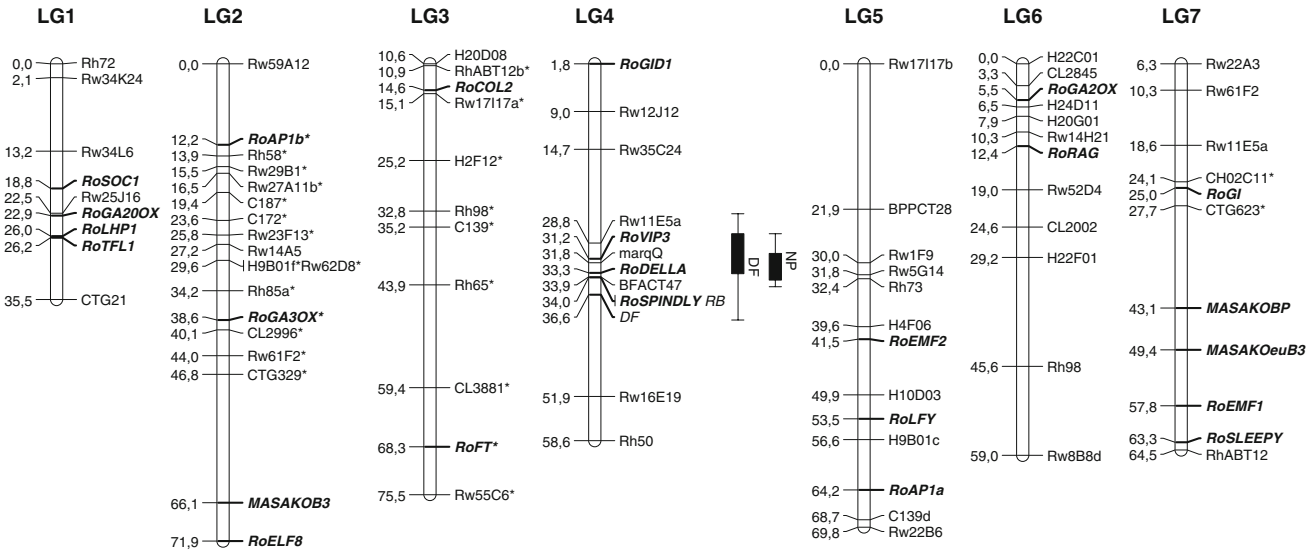


Fig. 4 Integrated genetic map of the HW population. Flowering genes are indicated in bold, SSR markers in normal type. To facilitate reading, AFLP markers are not presented in the figure. Map distances (in cM) are listed on the *left* and loci on the *right* of each linkage group. Distorted markers are indicated with an *asterisk* $*P < 0.001$. Additive QTLs for blooming date (BD) and NP are represented by a box prolonged with lines, which, respectively, span the LOD1 and LOD2 confidence intervals (as previously described by Hibrand-Saint Oyant et al. (2008)

flowering trait. Cv Félicité&Perpétue (FP) is a non-recurrent rose flowering only in spring, whereas its vegetative mutant, cv Little White Pet (LWP) is recurrent and flowers during all favourable seasons. Floral initiation was determined by dissection and observation of the meristem under a binocular microscope. We considered that floral initiation happens before the first morphological changes associated with floral development were observed in the meristem (data not shown). For both cultivars, we considered that floral initiation took place before 5 April (in Fig. 6, floral tissues are indicated by grey boxes). Then, kinetics represent the first floral initiation for LWP and the unique floral initiation for FP in the year. Transcripts were detected in all 144 measurements, showing that the investigated genes were expressed during floral initiation in spring and autumn for both cultivars. Only one gene, i.e. *RoSPY*, showed no variation in transcript abundance among the tested samples. The transcript accumulation of *RoFT*, *RoLFY*, *RoAP1b* and *RoGID1* increased during spring and seemed to be correlated with the flowering time of LWP and/or FP. *RoFT* induction occurred in two waves: the first started on 6 March and the second on 12 April, resulting in an accumulation of 15-fold and 57-fold more transcripts for FP and LWP, respectively. In autumn (1 October), the transcript abundance was comparable to the level in spring before floral initiation. The same pattern was observed for *RoLFY* and *RoAP1b*. For the three genes, the level was low in autumn in vegetative buds. *RoGID1* was also induced in LWP in spring, whereas it remained stable in FP. For *RoDELLA* and *RoSLY*, the transcript level was quite stable for FP. A weak transient increase was detected in spring for LWP (×2 on 13 March for *RoDELLA* and ×2.5 on 23 March for *RoSLY*). The gene encoding one enzyme of active GA synthesis, i.e. *RoGA20OX*, was repressed (4 times) until 13 March, and then increased to 0.6 and 0.7, respectively, on 23 March and 5 April, and then decreased again. In LWP, after a decrease, transient overexpression was detected on 23 March (×4), then the transcript decreased as rapidly as for FP. *RoGA2OX*, encoding a GA inactivation enzyme, showed a complex expression profile. In FP, transcripts accumulated strongly on 6 March, then decreased on 13 March to progressively increase later in spring. In LWP *RoGA2OX*, the transcript abundance was high until 6 March, and then fell on 13 March, and increased again during spring. In October, the *RoGA2OX* transcript abundance was equal to the level of 13 March in FP and LWP.

Discussion

Flowering genes are conserved in rose

Except for *FLC*, all genes we looked for in rose were found among the four different floral pathways, floral integrators and organ identity genes. The mean similarity between rose and *Arabidopsis* sequences was as high as 65% and reached 82% for *RoLFY*, for example. For single genes such as *SPY*, *RoSPY* showed high similarity with the *Arabidopsis* sequence and presented conserved domains. We are thus

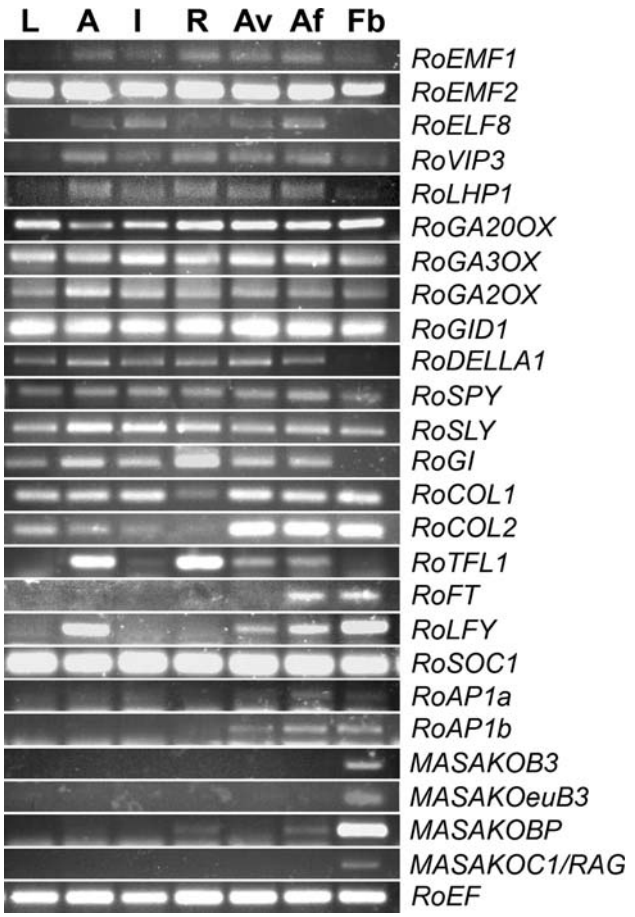


Fig. 5 RT-PCR analysis of rose gene expression. Specific primers for each gene were used to amplify the cDNA. PCR was performed on cDNA obtained from different tissues: leaves (*L*), apices from indoor grown roses (*A*), internodes (*I*), roots (*R*), apices during floral transition (*Av*, before the first floral modification; Af, after the first morphological changes) and floral buds (*Fb*)

confident that *RoSPY* is the homologue of *SPY*. The same conclusions are drawn for *RoLFY*, *RoLHP1, RoEMF2* and *RoGID1*, which were found to be similar to *LFY, LHP1, EMF2* and *GID1*, respectively. For *EMF1*, the percentage similarity was low [27% between *RoEMF1* and *EMF1* (Table 1)]. This value is in the same range as the similarity between *Arabidopsis* and rice sequences (31% between *SPY* in *Arabidopsis* and its homologue in rice). Furthermore, *RoEMF1* showed a conserved domain characteristic of *EMF1* (Aubert et al. 2001). Therefore, we propose that *RoEMF1* is the homologue of *EMF1*.

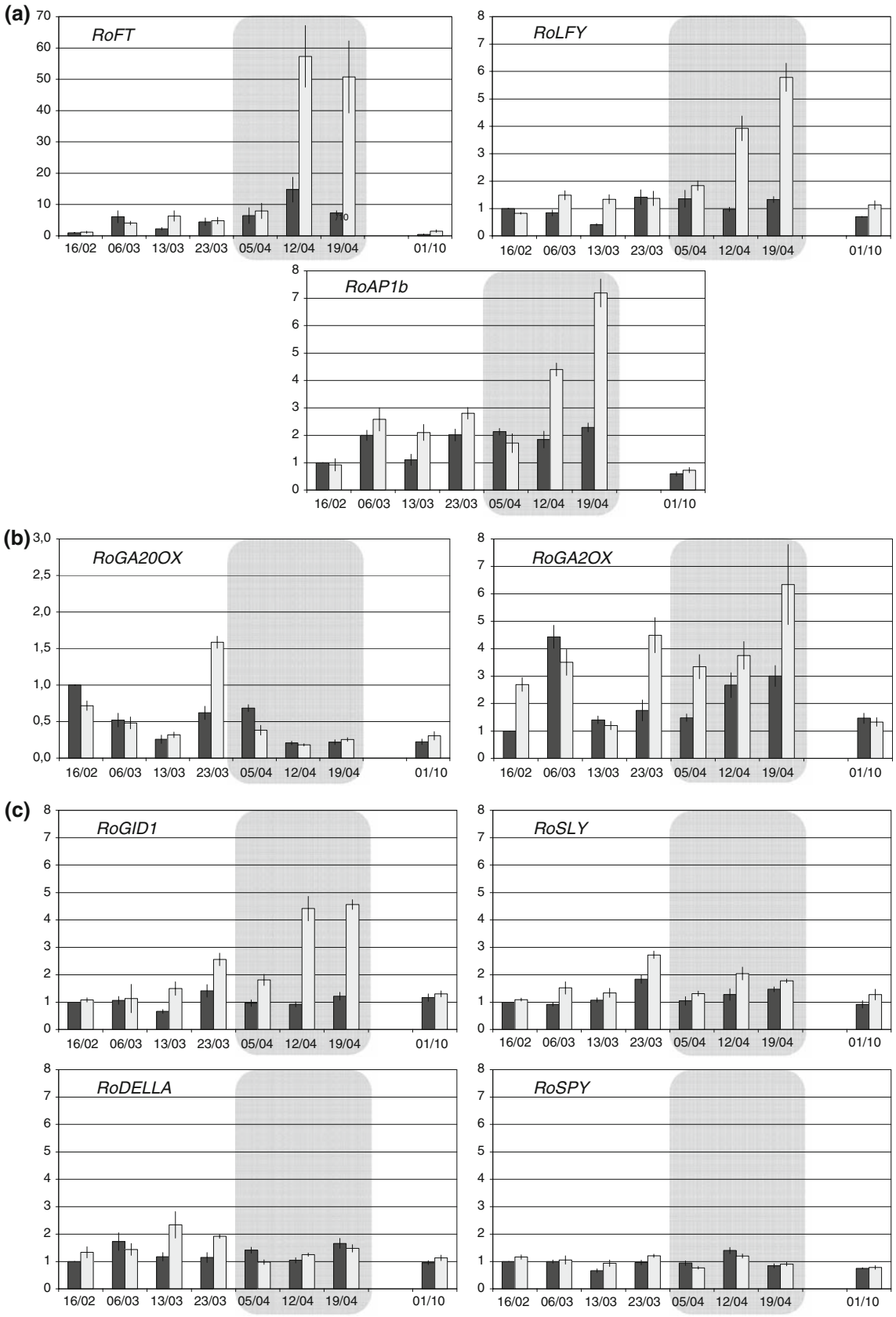
We also performed a phylogenetic analysis for genes belonging to multigenic families such as MADS-box genes. The two MADS-box genes isolated in rose clearly belonged to the AP1 clade (SQUAMOSA; Fig. 1; Becker and Theissen 2003). We were unable to isolate a gene with similarity to *FLC*, which is a major floral repressor in *Arabidopsis* (Michaels and Amasino 1999). *FLC* exists in other

Fig. 6 Expression pattern of nine flowering genes during the floral process determined by qRT-PCR. **a** floral integrators, **b** GA metabolism, **c** GA signalling. The *X*-axis indicates the dates at which apices were sampled in 2007. Data are ratios of expression according to Pfaffl (2001) ±SE (for three replicates). For each gene, transcript levels are expressed relative to the first sample of FP (16 February, base value = 1). Apices were harvested from new shoots grown from budbreak to autumn in the non-recurrent cv Félicité&Perpétue (*black bar*) and the recurrent cv Little White Pet (*grey bar*). Floral initiation was determined according to Foucher et al. (2008) by binocular observation. Floral tissues (after floral initiation) are represented in a *grey box* (sample since 5 April)

Brassicaceae such as *Brassica oleracea* (Okazaki et al. 2007), but no FLC has been detected in rice (Izawa et al. 2003). Recently, Reeves et al. (2007) revealed two *FLC* homologues in sugar beet and assumed that *FLC* homologues likely exist in other Eudicot lineages. Moreover, sequence analysis of the grapevine genome shows two sequence homologues of *FLC* as well (Diaz-Riquelme et al. 2009). Therefore, we cannot exclude the possibility that the absence of a rose *FLC* in our study could be explained by a failure to amplify the rose homologue with degenerate primers. Phylogenetic analysis of FT/TFL1 (Fig. 2) and GA oxidase (Fig. 3) confirmed the similarity between rose and *Arabidopsis* genes. Based on sequence similarities and conserved domains, we conclude that we have isolated rose genes that are putative homologues to genes in *Arabidopsis thaliana*.

However, in our study, for most of the genes, we only managed to isolate one homologue per gene and other paralogues may exist. As paralogues can act in specific processes, a role of the isolated genes in flowering cannot be ascertained. For example, there are five DELLA proteins in *Arabidopsis* that play redundant and specific roles in different processes: RGL2, RGA and RGL1 in flower development (Cheng et al. 2004; Tyler et al. 2004; Yu et al. 2004), RGA and GAI in stem elongation and root growth (Fu and Harberd 2003), RGL2, RGL1 in seed germination (Cao et al. 2006; Lee et al. 2002). In rose, we have only isolated one gene, i.e. *RoDELLA*, but additional DELLA proteins may occur as previously shown in apple, where six DELLAs were isolated by Foster et al. (2007).

We performed expression analyses to further study the homologue genes in rose and tried to obtain evidences of their involvement in the floral process. RT-PCR experiments (Fig. 5) showed that each gene was expressed during the floral process. Moreover, some genes such as organ identity genes (*MASAKO* genes) were only expressed in floral tissues, as previously shown (Hibino et al. 2006; Kitahara et al. 2001; Kitahara and Matsumoto 2000) (Fig. 5). Furthermore, *RoFT*, *RoLFY* and *RoAP1* are induced during the floral process, as previously shown in *Arabidopsis* (Lee et al. 2006; Wigge et al. 2005) (Fig. 6c).


(a)
RoFT
RoLFY
RoAP1b
(b)
RoGA20OX
RoGA2OX
(c)
RoGID1
RoSLY
RoDELLA
RoSPY
16/02 06/03 13/03 23/03 05/04 12/04 19/04 01/10

They could therefore be useful as markers of floral initiation in rose. Key floral genes (floral integrators, floral identity genes and organ identity genes) seemed to be expressed in a similar way as in *Arabidopsis*, thus suggesting that the floral gene network might be conserved in rose. The same conclusions were drawn in pea (Hecht et al. 2005), grapevine (Carmona et al. 2007) and rice (Izawa 2007).

In summary, by combining the EST search (Foucher et al. 2008) and the degenerate primer strategy (this study), we studied 26 genes putatively homologous to genes involved in floral initiation and development in *Arabidopsis*.

Genetic linkage between floral traits and floral genes

To further investigate the role of these genes in flowering control processes in rose (floral initiation as well as recurrent blooming), we looked for co-localization with loci controlling flowering in rose and the analysed transcript abundance of candidate genes in a non-recurrent rose and in its spontaneous vegetative recurrent mutant.

Interestingly, three genes, i.e. *RoVIP3*, *RoSPY* and *RoDELLA*, were found in the vicinity of two important loci: a QTL governing the flowering date (or precocity) and the *RECURRENT BLOOMING* locus. No recombination was found between *RB* and *RoSPY* when 91 individuals were analysed. These genetic data indicate a possible role of GA signalling in flowering control in rose (flowering precocity and recurrent blooming). Roberts et al. (1999) already proposed that GA may be involved in flowering in rose. Exogenous application of GA inhibits flowering in non-recurrent roses, whereas it has no effect on flowering in recurrent roses (Roberts et al. 1999). As *RoVIP3*, a homologue of *VIP3* (*VERNALIZATION INDEPENDENCE 3*), is also a putative candidate for the QTL of precocity, we cannot exclude a role of vernalization in the control of flowering in rose. Indeed, recurrent roses are self inductive, whereas vernalization is necessary for flowering in non-recurrent roses (Foucher F., personal communication). Therefore, we hypothesise that the recurrent blooming mutation could affect GA signalling as well as the vernalization response. In *Lolium temulentum*, a perennial plant, vernalization affects GA signalling, i.e. without vernalization, plants are unable to respond to GA (McMillan et al. 2005). After vernalization, a long-day treatment is necessary for GA synthesis, which in turn induces flowering (King et al. 2003).

Gibberellins control rose flowering

To further explore the role of GA in flowering in rose, we analysed the expression of GA genes isolated in rose during the floral process in a pair of rose mutants for recurrent blooming, i.e. FP and LWP.

GA metabolism and floral initiation

In early March, before morphological changes, we observed a peak of *RoGA2OX* expression in both FP and LWP (Fig. 6b) that might be responsible for greater GA degradation activity, whereas RoGA20OX was repressed (no GA synthesis). Indeed, a correlation between the GA metabolism gene transcript level and the active GA content has been reported in other plants (Oh et al. 2006; Yamaguchi et al. 1998). Repression of *RoGA20OX* and induction of *RoGA2OX* might cause a decrease in GA content. In the same genotypes (FP and LWP), low GA content was already observed in early March (Roberts et al. 1999). Therefore, we proposed that *RoGA2OX* induction and *RoGA20ox* repression lead to low GA content, which enable floral initiation to occur in early spring in recurrent and non-recurrent roses. It would be interesting to determine what signals control GA degradation in rose (link with vernalization/photoperiod). Photoperiodic control of GA metabolism could thus be an interesting focus of further studies. Indeed, in *Lolium perenne,* LD induces GA metabolism genes and then induces flowering in GA responding plants (King et al. 2006; McMillan et al. 2005).

GA signalling and recurrent blooming

We detected co-localization between a GA signalling gene, *RoSPY,* and the *RECURRENT BLOOMING* locus (*RB*, Fig. 4). Furthermore, a gene potentially involved in the early steps of GA signalling (*RoGID1*) showed different expression patterns when FP and LWP (recurrent blooming mutation) were compared. In FP, GA signalling genes were expressed at the same level during the floral process, whereas in the mutant (LWP), *RoGID1* was upregulated after the floral initiation (Fig. 6c). The present analysis was based on only 91 individuals: further studies using a larger sample are needed for a closer investigation of the linkage between *RB* and *RoSPY*. Moreover, Roberts et al. (1999) showed that an exogenous application of GA has no effect on flowering in recurrent roses whereas it inhibits flowering in non-recurrent roses. From these different results, we hypothesise that *RB* mutation could affect GA signalling and *RoSPY* could be a candidate for GA signalling disruption. Nevertheless, *RoSPY* remained stable at the transcriptional level in both recurrent and non-recurrent rose. Further experiments are therefore needed to clarify the link between *RoSPY* and the recurrent blooming phenotype.

To conclude, the flowering gene network seems to be conserved in rose. We isolated and characterized genes involved in each of the four floral pathways, floral integrators, meristem identity genes and organ identity genes. The GA pathway appears to be a key regulator of flowering in rose; GA metabolism might be responsible for floral

initiation and GA signalling might be involved in recurrent blooming control.

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