RESEARCH PAPER

Gibberellins regulate the transcription of the continuous flowering regulator, *RoKSN*, a rose *TFL1* homologue

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Abstract

The role of gibberellins (GAs) during floral induction has been widely studied in the annual plant *Arabidopsis thaliana*. Less is known about this control in perennials. It is thought that GA is a major regulator of flowering in rose. In spring, low GA content may be necessary for floral initiation. GA inhibited flowering in once-flowering roses, whereas GA did not block blooming in continuous-flowering roses. Recently, *RoKSN*, a homologue of *TFL1*, was shown to control continuous flowering. The loss of *RoKSN* function led to continuous flowering behaviour. The objective of this study was to understand the molecular control of flowering by GA and the involvement of *RoKSN* in this inhibition. In once-flowering rose, the exogenous application of GA₃ in spring inhibited floral initiation. Application of GA₃ during a short period of 1 month, corresponding to the floral transition, was sufficient to inhibit flowering. At the molecular level, *RoKSN* transcripts were accumulated after GA₃ treatment. In spring, this accumulation is correlated with floral inhibition. Other floral genes such as *RoFT*, *RoSOC1*, and *RoAP1* were repressed in a *RoKSN*-dependent pathway, whereas *RoLFY* and *RoFD* repression was *RoKSN* independent. The *RoKSN* promoter contained GA-responsive *cis*-elements, whose deletion suppressed the response to GA in a heterologous system. In summer, once-flowering roses did not flower even after exogenous application of a GA synthesis inhibitor that failed to repress *RoKSN*. A model is presented for the GA inhibition of flowering in spring mediated by the induction of *RoKSN*. In summer, factors other than GA may control *RoKSN*.

Key words: Floral initiation, floral repressor, gibberellins, PEBP family, polycarpic plants, rose.

Introduction

Gibberellins (GAs), important plant growth regulators, are involved in different aspects of plant development such as growth promotion, seed dormancy, floral initiation, and development (Fleet and Sun, 2005). GAs are cyclic diterpenoid molecules, with GA₁, GA₃, GA₄, and GA₇ being the most biologically

active in plants. The floral transition from vegetative growth to flowering is an important period in the life of a plant. In long-day (LD) monocarpic plants, it was reported that GA is a floral stimulus and could be substituted for LDs (Lang, 1965) whereas, in most woody plants, GA inhibits flowering (Zeevaart, 1976).

Abbreviations: CF, continuous flowering; DAB, days after the beginning of treatment; GA, gibberellin; OF, once-flowering; PCB, paclobutrazol.

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For example, GA activates flowering in *Arabidopsis thaliana*, whereas it represses flowering in woody fruit trees (Wilkie *et al.*, 2008).

The molecular basis of floral transition and, particularly, the response to GA, was elucidated in the model plant *A. thaliana*, a facultative LD vernalization-mediated plant. For this transition to be successful, *Arabidopsis* must integrate different endogenous and environmental signals (reviewed by Srikanth and Smith, 2011). Flowering is controlled by four major genetic pathways: photoperiod, autonomous, GA, and vernalization. These different pathways converge to activate the floral integrators, *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF CONSTANS1 (SOC1)*, and to repress *FLOWERING LOCUS C (FLC)*, a floral repressor involved in the vernalization response (Kim *et al.*, 2009). The floral integrators then activate the meristem identity genes, *LEAFY (LFY)* and *APETALA1 (API)*, leading to activation of the floral organ identity genes known as ABCE model genes.

In *Arabidopsis*, GA was required for flowering under short days (SDs) and LDs, with a weaker effect under LDs. *gal-3*, a GA-deficient mutant due to a mutation in a key GA metabolism gene (Koornneef and van der Veen, 1980), led to a delay in flowering under LDs and was unable to flower under SDs. This phenotype was reversed by exogenous application of GA₄ (Wilson *et al.*, 1992). Under SDs, GA₄ was accumulated in the shoot apical meristem (SAM) prior to floral transition. GA₄ may be synthesized in the leaves and then move to the apex (Eriksson *et al.*, 2006).

GAs induce flowering through two different pathways. In leaves, the GA response was FT dependent. Under LDs, GA activated *CONSTANS (CO)* that in turn induced *FT* (Hisamatsu and King, 2008). In the apex, GA acted in an FT-independent manner to induce *SOC1* and *LFY* (Hisamatsu and King, 2008). Failure of the *gal-3* mutant to flower under SDs was due to the absence of up-regulation of the floral integrator *SOC1* (Moon *et al.*, 2003) and the meristem identity gene *LFY* (Blazquez *et al.*, 1998). In *gal-3* mutants, *SOC1* was accumulated after exogenous GA₄ application under SDs (Moon *et al.*, 2003). GA₄ regulation of *SOC1* expression could be mediated by the down-regulation of *SHORT VEGETATIVE PHASE (SVP)*, a floral repressor involved in the vernalization and ambient temperature pathways (Li *et al.*, 2008). The GA effect on *LFY* expression is mediated by a transcription factor, AtMYB33, which is able to link the GAMYB-binding site in the *LFY* promoter (Gocal *et al.*, 2001). AtMYB33 expression corresponds to GA₄ accumulation in the shoot apex during floral transition (Gocal *et al.*, 2001; Eriksson *et al.*, 2006).

Except for *Arabidopsis*, few data were available about the molecular control of flowering by GAs in other species. In *Lolium temulentum*, an obligate LD plant, GA activated flowering (Evans *et al.*, 1990). The leaf GA content increased after LD exposure with transcriptional regulation of GA biosynthesis genes (Gocal *et al.*, 1999). GA moves from leaves to the shoot apex where the increase in GA content corresponds to floral induction (King *et al.*, 2001). LD treatment can be mimicked by GA application, especially GA₅ and GA₆ (Evans *et al.*, 1990; King *et al.*, 2001). *Lolium perenne*, a perennial near-relative of *L. temulentum*, requires both LDs and vernalization for flowering. LDs activate GA biosynthesis, which then stimulates the flowering of vernalized plants, whereas non-vernalized plants are unable to respond

to GA for flowering (MacMillan *et al.*, 2005). In grapevine, the *vvGAI* mutant, a gene encoding a DELLA protein, a central repressor of GA signalling, was dwarf, GA-insensitive, and produced inflorescences along the length of the shoot instead of tendrils. GA represses the formation of inflorescences and promotes the initiation of tendrils (Boss and Thomas, 2002).

Rose is an economically important ornamental crop worldwide. Flowers represent the major value of roses, and control of flowering is a major issue. Rose is a perennial woody plant with different modes of flowering. Once-flowering (OF) roses have a single annual flowering period, whereas continuous-flowering (CF) roses have the ability to flower continuously during the season. In rose, the sensitivity of plants to GA was different between OF and CF roses. Exogenous application of GA inhibited flowering in OF roses and had no effect on flowering in CF roses (Roberts *et al.*, 1999). GA signalling genes such as *GID1*, a GA receptor, are regulated differently between OF and CF roses (Remay *et al.*, 2009). Furthermore, GA metabolism was involved in floral initiation. In OF and CF roses, the amount of GA in shoots before floral initiation was low and increased after floral initiation (Roberts *et al.*, 1999). This GA content was correlated with GA metabolism gene regulation during floral transition. In OF and CF roses, the *RoGA20ox* gene, encoding an enzyme involved in GA synthesis, was transiently repressed before floral initiation. In contrast, the expression of *RoGA2ox*, encoding an enzyme involved in GA inactivation, increased before floral initiation and decreased later (Remay *et al.*, 2009). Recently, the gene encoding the continuous flowering locus was shown to correspond to a floral repressor homologue of *TFL1*, *TERMINAL FLOWER1*, and was designated as *RoKSN* (Iwata *et al.*, 2012). CF roses had a retrotransposon inserted in the second intron of *RoKSN*. The presence of a retrotransposon blocked the transcription of *RoKSN* in CF roses, allowing continuous blooming. In OF roses, *RoKSN* is weakly accumulated in spring to permit blooming. Later in the season, *RoKSN* transcripts accumulate and shoots remain vegetative (Iwata *et al.*, 2012).

The objectives of this study were to understand the molecular control of flowering by GA in rose and to decipher the link between the continuous flowering gene, *RoKSN*, a TFL1 homologue, and GA. In this study, it was demonstrated that GA inhibited floral initiation in rose by up-regulating the CF gene at the transcriptional level. The promoter of *RoKSN* was further analysed, and GA-responsive elements were found.

Materials and methods

Plant material

Rosa×wichurana (RW), an OF rose, was obtained from the rose garden, Jardin de Bagatelle (Paris, France). The mutant pair, *R. hybrida* 'Félicité et Perpétue' (FP), an OF rose, and 'Little White Pet' (LWP), its CF mutant, were obtained from 'Loubert Nursery' (Rosier sur Loire, France). Plants were cultivated as described (Remay *et al.*, 2009). *Nicotiana benthamiana* were maintained in a greenhouse (20 °C, 16 h light), and 6- to 8-week-old plants were used for agroinfiltration.

GA and paclobutrazol treatments on plants

In December, stems were pruned at six internodes and maintained outside for vernalization (Fig. 1A). At the beginning of the treatment (end

of January), plants were placed in a tunnel. Different GA₃ concentrations (0, 30, 70, and 140 µM) were sprayed on plants three times a week during different periods and for different durations (Fig. 1B). Paclobutrazol (PCB) treatment (Bonzi[®], at 42, 70, or 112 µM) was performed every 2 weeks. After blooming, a new set of plants was used for GA₃ and PCB treatments. The inflorescences were removed and growing shoots were pruned at six nodes. Plants, maintained in a greenhouse, were sprayed with GA₃ (30 µM) three times a week for 34 d and with PCB (Bonzi[®], at 14, 56, or 112 µM) three times during the experiment [0, 15, and 29 days after the beginning of treatment (DAB)].

Phenotypic observations

Phenotypic observations were carried out on 10 plants with approximately five pruned stems, 2 months after the end of the treatment. Data were gathered from the three new emerging shoots placed on the distal part of the pruned stem (Fig. 1A). For each new emerging shoot, the percentage of flowering that represents the ratio between the number of

flowering shoots and the number of shoots was determined. To calculate the average internode size, three consecutive internodes placed on the apical part of the stem were measured. Statistical analyses were done using the R software, version 2.13.1.

Histological observations

After dissection (removal of leaves), samples were fixed and sections of 2–3 µm were observed under a microscope (Foucher *et al.*, 2008). Observations were made on four different dates (42, 56, 63, and 70 DAB).

RNA isolation and gene expression analysis

For RNA isolation, the terminal part of the shoot was harvested from five plants per date and per treatment (Fig. 1A). Samples were dissected (removal of expanded leaves and young leaves) and frozen in liquid nitrogen. Total RNA was isolated using the NucleoSpin[®] RNA plant kit (Macherey-Nagel) according to the manufacturer's instructions.

Fig. 1. Management of *R. x wichurana* (RW) after GA₃ and PCB treatments. (A) In December, vegetative shoots were pruned at six nodes. Plants remained outside for vernalization. At the end of January, plants were transferred to a tunnel. New shoots developed from axillary buds of the previous years. These shoots were mainly terminated by an inflorescence. After floral development, new shoots grew from the base. Since RW is an OF rose, the shoots remained vegetative. In summer, these shoots were pruned at six nodes and the fate of new emerging shoots from axillary buds was studied. Under controlled conditions, these shoots remained vegetative. RW plants were treated with GA₃ (0, 30, 70, or 140 µM) during vegetative growth and floral initiation between January and March (hatched boxes). Another set of RWs was treated with GA₃ (30 µM) and PCB (14, 56, and 112 µM) during vegetative growth between August and September. Arrows and asterisks indicate the sampling carried out for RNA extraction and histological analysis, respectively. (B) Experiments with different durations and periods of GA₃ (30 µM) treatment. In the 'Late treatment', the experiment started at different times and ended at the same time, whereas in the 'Early treatment', the experiment started at the same time and ended at different times. Numbers indicate the days after the beginning of treatment (DAB). Broken lines represent the pruned shoots of the previous years, large arrows represent new indeterminate growing shoots, and black circles represent axillary buds. Open circles represent flowers. (This figure is available in colour at JXB online.)

Elimination of genomic DNA and reverse transcription were performed using 1 mg of total RNA; real-time PCRs were performed using 3 µl of reverse transcription product (1/25 dilution), as described by Remay *et al.* (2009). The amount of plant RNA in each sample was normalized using *TCTP* and *UBC* genes (Klie and Debener, 2011), and the relative expression level was calculated according to Pfaffl (2001), from two biological replicates and three technical repetitions per replicate.

DNA constructs

The upstream genomic sequence of *RoKSN* (promoter region) was obtained using the Genome Walker Universal Kit (Clontech), following the manufacturer's recommendations from genomic DNA libraries of *R. hybrida* 'Knock Out'. Serial nested PCRs were performed using the described reverse primers (Supplementary Table S1 available at JXB online) and the forward primers of the kit. A 1200 bp fragment was amplified and sequenced. Then, using Finnzymes' Phusion® High fidelity DNA Polymerase (Thermo Fisher Scientific), the promoter region was amplified from DNA of *Rosa chinensis* 'Old Blush' and cloned into the pGEM T easy vector (Promega). Next, successive promoter deletions were performed to obtain fragments of different sizes: 200, 400, 800, and 1200 bp. PCR products were cloned using the Gateway® system (Invitrogen) with TOPO isomerase mix into the pENTR™/D-TOPO® entry, followed by LR clonase recombination with pKGWFS7 (VIB, Gent, Belgium) (Karimi *et al.*, 2005). The promoter sequence was fused to a GUS::GFP (β-glucuronidase::green fluorescent protein)-coding gene. Constructions were introduced by electroporation in *Agrobacterium tumefaciens* EHA105 with the plasmid pbbR.

Transient expression procedure

Agrobacterium tumefaciens C58C1 containing p19 (Voinnet *et al.*, 2003) and EHA105 containing the different constructions were grown at 28 °C in LB medium supplemented with 50 mg l⁻¹ kanamycin, 50 mg l⁻¹ rifampicin, and 100 mg l⁻¹ gentamicin (only for EHA105) up to the stationary phase. After centrifugation at 5000 g for 10 min, bacteria were resuspended in Murashige and Skoog (MS) medium supplemented with 5% (w/v) sucrose, acetosyringone (200 µM), pH 5.6, and with or without GA₃ (10 µM) to obtain an optical density (OD₆₀₀) of 0.4. Two days after agroinfiltration of 6- to 8-week-old *N. benthamiana* leaves, GFP fluorescence was observed using a Nikon A1 laser scanning confocal microscope.

Sequence analysis

Sequences of promoters of *RoKSN* (HE863824), *MdTFL1*, *MdTFL1a* (AB366639 and AB366640), *FvKSN* (HQ378595), *PpTFL1* (AB636121), and *TFL1* (At5g03837) were used to perform the alignment with MVISTA online software (<http://genome.lbl.gov/vista/mvista/submit.shtml>) (Frazer *et al.*, 2004). The search for putative binding sites of *cis*-elements was then carried out using Plantpan online software (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) (Chang *et al.*, 2008).

GUS activity

To measure GUS activity quantitatively, MUG assays were conducted on each agroinfiltrated leaf (1–2 cm²) as described by Jefferson *et al.* (1987). The reaction was stopped after 5, 10, 15, 20, and 30 min. Fluorescence was measured at these different times on a FLUO-star plate reader (BMG Labtechnologies Inc.) at 460 nm when excited at 355 nm. Values from the fluorescence assay were standardized by protein concentration, determined as described by Bradford (1976).

Results

Gibberellins block floral initiation in spring in once-flowering rose

To study the effect of GA treatment on plant development, exogenous application of GA₃ at different concentrations (30, 70, and

140 µM) was performed in spring on the OF roses, RW and FP, and its CF mutant LWP (Fig. 1).

On non-treated RW, 80% of the emerging shoots became floral, whereas for the GA₃ treatment (30 µM and 70 µM), almost all new emerging shoots remained vegetative (only 5.5% and 1%, respectively, were floral). At higher GA₃ concentrations, flowering was completely inhibited (Fig. 2B). GA₃ treatment also had an effect on the vegetative development of the stem. The length of internodes increased significantly: without treatment, internodes measured 3 cm, at 30 µM GA₃ they measured 4.3 cm, and they measured 4.5 cm for the higher concentrations of GA₃ (Fig. 2A). The same inhibitory effect on blooming was shown on FP (Supplementary Table S2 at JXB online). Flowering was not inhibited by GA₃ treatment in LWP, whereas internode length was reduced. The effect of the GA synthesis inhibitor PCB was tested in FP and LWP. PCB treatment significantly reduced internode length. Concerning flowering, PCB treatment has no effect on LWP, whereas at high concentrations (70 µM and 110 µM), flowering was significantly reduced in FP. Only 50% of the shoots became floral at 110 µM instead of 79% for untreated plants (Supplementary Table S2).

To evaluate the effect of GA₃ treatment on the fate of the apex, cross-sections of the apex from the vegetative to the floral stage were taken using the OF rose, RW, treated or not with 30 µM GA₃ (Fig. 3). In untreated plants after bud outgrowth (42 DAB), the vegetative meristem was narrow with leaf primordia on its flanks (Fig. 3A). The meristem then emerged and enlarged (56 DAB; Fig. 3C). This stage has been defined as the 'floral initiation' stage. The meristem then became floral and bracts were visible (63 DAB; Fig. 3E). Later, floral organs (sepals, petals, stamens, and pistils) are visible (70 DAB, Fig. 3G). Under untreated conditions, the floral initiation took place 56 DAB. In GA₃-treated plants (Fig. 3B, 3D, 3F, 3H), the meristem was narrow and dome-shaped for all stages. Leaf primordia were visible on the flank of the meristem. This structure was typical of a vegetative meristem. These observations clearly demonstrated that GA₃ blocked floral initiation and that the meristem remained vegetative.

GA₃ is necessary during a short period after bud outgrowth to inhibit flowering.

To characterize the GA response, different durations and periods of GA₃ treatment (30 µM) were tested before blooming in spring. To modify the duration and the period of the treatment, GA₃ application was started and ended on different dates, the 'late' and 'early' treatments, respectively (Fig. 1B). The duration varied from 20 d to 70 d, and the period was from late January to the end of March.

The length of internodes significantly increased between untreated plants (3 cm) and plants treated with GA₃ (from 3.5 cm to 4.6 cm), except for the GA₃ treatment of 20 d (2.3 cm) (Fig. 2A). In general, the longer the treatment, the longer the length of the internodes.

Concerning flowering behaviour, the longer the treatment, the higher the GA inhibiting effect. For GA-treated roses, 5.5, 14.8, 25.5, and 40.5% of the shoots became floral for 70, 63, 47, and 33

Fig. 2. Effect of GA₃ treatment on average internode size in centimetres (A) and percentage of shoots that become floral (B) on *R. xwichurana* (RW). RW plants were treated with GA₃ (0, 30, 70, or 140 μM) for 70 d, and with GA₃ (30 μM) for different durations and periods. Data were the means of 10 plant observations. There was a statistically significant difference (Waerden's test, $P < 0.05$; and the χ^2 test, $P < 0.0033$, for the comparison of percentages) between means with different letters.

d, respectively, of treatment begun on different dates (Fig. 2B). For treatment terminated on different dates ('early' treatment), the same trend was observed: 80% without treatment; 16.5, 3.6, and 3% for 20, 29, and 48 d, respectively. It is interesting to note that for the same duration of treatment, differences are observed if treatments are not carried out in the same period. For 47/48 d of treatment, the percentage of floral shoots was 3% for 'early' treatment, whereas it was 25.5% for 'late' treatment. For 33/29 d of treatment, the percentage of floral shoots was 3.6% for 'early' treatment, whereas it was 40.6% for 'late' treatment (Fig. 2B). 'Early' treatments were more effective at inhibiting flowering than 'late' treatment. For example, an 'early' treatment of 20 d had the same efficiency as a 'late' treatment of 47 d (15% blooming, Fig. 2B). In the 'early' and 'late' treatments, the non-floral shoots remain vegetative and present an indeterminate growth. GA₃ blocked the floral initiation and the meristem remained vegetative. In conclusion, a 1 month treatment in February (during floral transition) is sufficient to inhibit floral transition almost completely.

Floral gene expression in response to GA₃ treatment

To examine whether the inhibition of flowering observed after GA₃ treatment could be associated with the modification of floral gene transcript accumulation, this accumulation in RW shoots that emerged in spring and after blooming using was analysed by qPCR (Figs 1, 4). In spring, in the absence of treatment, the transcripts of the floral genes, *RoFT*, *RoFD*, *RoLFY*, and *RoSOC1*, were accumulated in shoots, which then become floral. *RoFT* transcripts were progressively accumulated in two steps as previously described (Remay *et al.*, 2009). The first accumulation occurred early during the floral transition (15–35 DAB; Fig. 4C), and the second occurred after floral initiation. *RoFD* was transiently accumulated early before floral initiation (15 DAB; Fig. 4B). The accumulation of *RoSOC1* transcripts was progressive during floral transition and occurred before the late accumulation of the *RoLFY* and *RoAPI* transcripts (70 DAB; Fig. 4). The accumulation of *RoKSN* transcripts was low in spring (Fig. 4A).

Fig. 3. Longitudinal cross-section through apices treated with GA₃ (30 μM; B, D, F, and H) or not (A, C, E, and G). In the untreated plant, the vegetative meristem (A: 42 DAB) progressively changed into a floral meristem (C, E, and G; 56, 63, and 70 DAB, respectively), whereas in the GA₃ (30 μM)-treated plants, the meristem remained vegetative for the same stage of development (B, D, F, and H, 42, 56, 63, and 70 DAB, respectively). VM, vegetative meristem; LP, leaf primordia; FM, floral meristem; S, sepal; FB, floral bud; P, petal; St, stamen; Pi, pistil. Scale bar=100 μm. (This figure is available in colour at JXB online.)

When GA₃ was exogenously applied at 30 μM, the expression patterns of floral genes were modified. GA₃ treatment strongly increased the accumulation of *RoKSN* transcripts. After 70 d of treatment, the *RoKSN* transcript level increased 40-fold in treated plants (Fig. 4A). *RoFT* transcript accumulation followed the same expression pattern as in untreated plants, but the accumulation was lower in treated plants than in untreated plants (Fig. 4C). The transient expression of *RoFD* (15 DAB) was not detected in treated plants. *RoSOC1* presented a transient accumulation similar to that of untreated plants, except after 70 DAB when a 2-fold transcript decrease was observed (Fig. 4E). There was no accumulation of *RoLFY* and *RoAPI* transcripts at the end of the experiment.

In FP, *RoKSN* transcripts were accumulated in response to GA₃ after a few hours. *RoLFY* transcripts were reduced 2-fold and GA₃ treatment had no effect on *RoFT* (Supplementary Table S3 at JXB online). To test whether floral gene transcription was *RoKSN* dependent, the expression of these floral genes was studied in the CF rose, LWP. After GA₃ treatment, the expression of *RoFT*, *RoSOC1*, and *RoAPI* was not affected, whereas expression of *RoLFY* and *RoFD* was reduced 2-fold (Fig. 4G). No *RoKSN* transcript could be detected.

GA₃ and PCB treatment do not modify the fate of indeterminate vegetative shoots after blooming.

In OF roses, new emerging shoots were vegetative after the blooming in spring (Fig. 1A), whereas in CF roses new shoots were always terminated by an inflorescence. Since GA₃ inhibits flowering in spring, it was investigated whether a GA synthesis inhibitor, PCB, could allow new blooming later in the season in RW (Fig. 1A). Treatments with PCB were performed at different concentrations (14, 56, and 112 μM), and with GA₃ (30 μM) (Table 1). At 30 μM GA₃, a significant increase in internode length (2.5 cm) was observed compared with untreated plants (2 cm). In contrast to the GA₃ treatment, PCB treatment significantly reduced the internode length at the three concentrations tested in RW and LWP. In RW, the length of internodes was 2 cm for untreated plants and 1.6, 1.5, and 1.4 cm for plants treated with PCB at 14, 56, and 112 μM, respectively. The same trend was observed in LWP (Table 1). Neither of the treatments (GA₃ or PCB) had an effect on flowering. Under all conditions, in RW, the shoots remained vegetative; no flowers were observed. In LWP, all the shoots were floral (Table 1).

In the absence of treatment, transcript accumulation of *RoFT*, *RoSOC1*, *RoFD*, *RoAPI*, and *RoLFY* remained low, and *RoKSN* transcripts were accumulated (Fig. 4). After exogenous application of GA₃, *RoKSN* expression increased 3-fold. GA₃ treatment had no effect on *RoFD* and *RoFT* (Fig. 4B). *RoLFY* transcript decreased 3-fold after 34 d of GA₃ treatment (Fig. 4D), while *RoAPI* was accumulated later (34 DAB). No significant changes were detected after PCB (at 56 μM) treatment, except for *RoFD* and *RoFT*, which were transiently induced at 14 DAB (Fig. 4B).

RoKSN promoter contains GA-responsive cis-elements

The induction of *RoKSN* by GA was further investigated by studying the upstream genomic region of *RoKSN*. A 1200 bp sequence

Fig. 4. Transcript accumulation of floral genes in spring and later in summer in GA₃-treated (squares), PCB-treated (triangles), or untreated RW (diamonds). Transcript accumulation of floral genes was followed by qPCR in axillary shoots for (A) *RoKSN*, (B) *RoFD*, (C) *RoFT*, (D) *RoLFY*, (E) *RoSOC1*, and (F) *RoAP1*. The x-axis indicates the number of days at which apices were harvested after the beginning of the GA₃ or PCB treatment. The transcript accumulation levels are expressed in relation to the first sample, harvested in January, for each gene according to the Pfaffl ratio (Pfaffl, 2001) (base value=1). The grey box represents the floral initiation determined according to the histological analysis (Fig. 2). Kruskal–Wallis test on two biological replicates ($P < 0.05$) was realized, and asterisks represent statistical differences between GA₃-treated and untreated plants; triangles represent statistical differences between PCB-treated and untreated plants. (G) Transcript accumulation of floral genes in GA₃-treated (grey box), and untreated (black box) LWP. The transcript accumulation levels are expressed in relation to the untreated plants. Data with different letters indicate a significant difference (Kruskal–Wallis test $P < 0.05$). nd, not detected

Table 1. Effect of GA₃ and the gibberellin inhibitor (PCB) on floral development and vegetative growth in late summer of OF rose (R.xwichurana, RW) and CF rose ('Little White Pet', LWP).

Genotype	Treatment	Second treatment (August–September)				
		Untreated	GA ₃ 30 μM	PCB 14 μM	PCB 56 μM	PCB 112 μM
RW	Average internode size (cm)	2±0.5 ^b	2.5±0.7 ^a	1.6±0.5 ^c	1.5±0.8 ^c	1.4±0.4 ^c
	Percentage of shoots that become floral	0	0	0	0	0
LWP	Average internode size (cm)	2.3±0.7 ^b	2.6±0.5 ^a	2.1±0.5 ^b	1.7±0.5 ^c	1.5±0.5 ^d
	Percentage of shoots that become floral	99.2 ^a	99.1 ^a	99.1 ^a	100 ^a	100 ^a

Data were the means of five plant observations. There was a statistically significant difference (Waerden's test; *P* < 0.05) between means with different letters. The experimental design is described in Fig. 1.

of *RoKSN* (upstream of the ATG codon) was isolated. By comparing promoters of *TFL1* of other *Rosaceae* (*Malus domestica*, *Prunus persica*, and *Fragaria vesca*) and *Arabidopsis thaliana*, two conserved regions were detected (Fig. 5A). Block A (–350 bp to –281 bp) was specific to the *Rosoideae* subfamily, whereas block B (–177 bp to –61 bp) was common to the *Rosaceae* family. One GAMYB *cis*-element, known to be involved in the GA response (Gocal *et al.*, 1999), was identified in block A. No GA response elements were found in block B. The non-conserved regions of the promoter contained three PIF3, three GAMYB, 10 WRKY71, one CARE, and one GARE element (Supplementary Fig. S1 at JXB online)

To determine the functional region for GA response expression, successive promoter deletions were performed: p200, p400, p800, and p1200, which corresponded to the –200, –400, –800, and –1200 bp of the upstream sequence of *RoKSN*, respectively. No fluorescence or GUS activity was detected with the p200 construct that contains only a TATA box element (Fig. 5C). Weak expression was detected with the p400 promoter in the absence of GA. In the presence of GA, the promoter activity was significantly increased by 14-fold (Fig. 5C). With the p800 and p1200 constructs, the promoter activity was not significantly different between GA-treated and non-treated plants. In both conditions, GUS activity was high (Fig. 5C). The 800 bp fragment may contain a positive *cis*-element, which provokes a constitutive expression of the promoter under the conditions used here (heterologous system). Due to this constitutive expression, GA induction may no longer be detectable.

Discussion

Physiological studies have demonstrated that GAs are floral repressors in woody perennials such as fruit trees (Wilkie *et al.*, 2008). However, how GA mediates its inhibitory effect at the molecular level remains largely unknown. In this study, the role of GAs in flowering on OF roses was investigated at the physiological and molecular level. It was demonstrated that the inhibitory effect of GA in rose is principally mediated by a floral repressor homologue to *TFL1*, *RoKSN*.

GA treatment modified the phenology of flowering in once-flowering rose

Under the experimental conditions without GA treatment, the OF roses (RW and FP) initiated their blooming in mid-March. The

histological analysis showed that the vegetative meristem was progressively converted into a floral meristem (Fig. 3). As previously described by Roberts *et al.* (1999), GA₃ treatment inhibited flowering in OF roses. High levels of GA₃ (>30 μM) completely blocked flowering (Fig. 2). The histological analysis showed that GA₃ treatment inhibited floral initiation. After GA₃ treatment, the apex remained vegetative and none of the morphological modifications observed during floral initiation (such as enlargement and doming of the meristem) was observed (Fig. 3). To specify the effect of GA₃, different times and durations of GA₃ application were used. A 1 month treatment before floral initiation (in February) was sufficient to obtain a strong inhibition of flowering (Fig. 2A). This period was referred to as floral transition, the phase when the meristem is still vegetative but receptive to signals that stimulate or inhibit flowering. During this phase, GA₃ acts as a floral repressor. This is consistent with the role of GA, which is known to promote vegetative growth instead of reproductive development in woody plants. The exogenous application of GA in fruit trees blocked the floral process and partially reduced the intensity of flowering, for example in apple (Tromp, 1982; Bertelsen *et al.*, 2002) or in avocado (Salazar-Garcia and Lovatt, 2000). In peach, this inhibitory effect has been linked to the date of treatment (Painter and Stembridge, 1972). In strawberry, GA treatment induced the initiation of runners and repressed the formation of crown branches (Hytönen *et al.*, 2009). None of these studies explained how GA mediates its inhibitory effect on flowering.

A floral repressor, RoKSN, is transcriptionally regulated by GAs

To look for the molecular targets of GA during the rose floral process, molecular analyses were carried out in spring and after blooming in GA₃-treated and untreated OF rose. During floral transition in untreated plants, floral activators were progressively accumulated (*RoFT* and *RoSOCI*). Later, *RoLFY* and *RoAPI* were accumulated at the end of floral initiation when the meristem started to become floral (Figs 3, 4). *RoKSN* transcripts remained low during floral transition and initiation. Similar patterns of transcript accumulation have already been observed in FP, an OF rose (Remay *et al.*, 2009; Iwata *et al.*, 2012), and in other *Rosaceae*. In apple, where floral initiation takes place in June after blooming, expression patterns of floral activators (homologues of *SOCI*, *FT*, *LFY*, and *API*) and repressors (homologues of *TFL1*) are similar (Hättasch *et al.*, 2008).

Fig. 5. Analysis of GA-responsive elements in the *RoKSN* promoter. (A) Alignment of the four *TFL1* homologue promoters using MVISTA. Conserved regions (>70% similarity on a 100bp window) are in red and surrounded by dotted lines (blocks A and B). Alignment was performed with *TFL1* (*Arabidopsis*), *FvKSN* (*F. vesca*), *MdTFL1* (*M. domestica*), *MdTFL1a* (*M. domestica*), and *PpTFL1* (*P. persica*). (B) Successive promoter deletions fused with a *GFP::GUS* gene. (C) GFP fluorescence and GUS activity. GFP fluorescence observed under a confocal microscope for the four different promoter deletions. Untreated and treated plants are in the left and right panel, respectively. GUS activity for the different deletions in the presence (grey) or absence (black) of GA₃ (10 μ M) by transient expression in tobacco leaves after *Agrobacterium* infiltration. Data are expressed relative to the minimal untreated promoter (p200). Data with different letters indicate a significant difference (Kruskal-Wallis test $P < 0.05$). (This figure is available in colour at JXB online.)

When GA₃ was exogenously applied, the strongest effect was the large accumulation of *RoKSN* transcripts and the absence of the accumulation of *RoLFY* and *RoAPI* transcripts (Fig. 4). In RW, *RoKSN* was accumulated 20 times more in GA₃-treated plants than in untreated plants in spring (Fig. 4). The same pattern was observed in summer. Furthermore, *RoKSN* was induced by GA₃ in two independent OF roses, RW and FP, suggesting a conservation of this regulation in rose (Supplementary Table S3 at JXB online).

To analyse the induction of *RoKSN* by GA₃ further, promoter analysis was performed. A 200 bp fragment (located between -200 bp and -400 bp) is sufficient to induce expression of *RoKSN* in response to GA₃. Indeed, the 200 bp promoter is not responsive to GA₃, whereas the 400 bp promoter revealed a stronger activity in the presence of GA₃ (Fig. 5C). This part of the promoter contained GAMYB and PIF3 *cis*-elements (Supplementary Fig. S1 at JXB online). It is hypothesized that the transcription factor PIF3 (PHYTOCHROME INTERACTING FACTOR 3) integrates different endogenous and environmental factors such as light and GA to induce stem elongation (Feng *et al.*, 2008). In *Arabidopsis*, AtMYB33, a GAMYB transcription factor, is able to link to the GAMYB-binding site in the *LFY* promoter (Gocal *et al.*, 2001). This 200 bp fragment corresponds to block A, conserved between rose and strawberry (Fig. 5A), suggesting that GA regulation might be conserved in strawberry where GA is also known to inhibit flowering (Thompson and Guttridge, 1959).

By studying transcript accumulation and promoter activity, the induction by GA₃ of *RoKSN*, a gene of the *TFL1* family, was clearly demonstrated. Further investigations are necessary to understand the signalling between GAs and *RoKSN*. This study represents the first demonstration of the induction of a floral repressor by GA. In *Arabidopsis*, *TFL1* belongs to a small family (Kobayashi *et al.*, 1999). It was recently demonstrated that other members of the *TFL1* family were regulated by hormones. The paralogues of *FT*, *TWIN SISTER OF FT*, were activated by cytokinin (BAP) to induce flowering under SDs (D'Aloia *et al.*, 2011). In *A. thaliana*, *MOTHER OF FT* and *TFL1* regulated seed germination via abscisic acid and the GA pathway (Xi *et al.*, 2010). During floral initiation, it was hypothesized that *FT* was induced by GA via a positive regulation of *CO* (Hisamatsu and King, 2008; Porri *et al.*, 2012).

RoKSN mediates floral inhibition by GA

GA blocked the floral transition in OF roses (Fig. 2), and *RoKSN*, a floral repressor, is induced in response to GA₃ (Fig. 4). It was uncertain whether this induction by GA might be the principal component in GA floral inhibition. During the floral process, the GA response between CF and OF roses was different: after GA₃ treatment, OF roses did not flower, while CF roses flowered (Roberts *et al.*, 1999; this study). The CF roses had no functional *RoKSN* allele, due to insertion of a retrotransposon (Iwata *et al.*, 2012). As a consequence, CF roses did not accumulate *RoKSN* and were no longer able to respond to GA. No floral repression occurred. In OF roses, during the floral transition, *RoKSN* is weakly accumulated and floral initiation can happen (Fig. 4A). The low accumulation may be explained by the low GA content (Roberts *et al.*, 1999) and the regulation of GA metabolism

genes: transient accumulation of *RoGA2OX*, a gene encoding an enzyme involved in GA inactivation, and low accumulation of *RoGA20OX*, a gene encoding an enzyme involved in the last steps of active GA synthesis (Remay *et al.*, 2009).

After exogenous application of GA₃, floral genes were down-regulated (such as *RoSOC1*, *RoFD*, *RoLFY*, and *RoAPI*) (Fig. 4). To determine whether these regulations were *RoKSN* dependent, the expression of these genes in response to GA₃ was studied in a CF rose, LWP (Fig. 4G). In the absence of *RoKSN*, transcript accumulation of *RoFT*, *RoSOC1*, and *RoAPI* was not modified after GA₃ treatment. In contrast, *RoLFY* and *RoFD* transcripts were reduced 2-fold after GA₃ treatment. These results suggest that *RoFT*, *RoSOC1*, and *RoAPI* regulation by GA is *RoKSN* dependent, whereas the GA effect on *RoFD* and *RoLFY* is, at least partially, independent of *RoKSN*.

The following model is proposed to explain the floral inhibition by GA in OF roses. During the floral transition, exogenous application of GA₃ leads to a strong accumulation of *RoKSN* that blocks induction of floral genes such as *RoAPI*, *RoFT*, and *RoSOC1* (Fig. 4). As proposed in *Arabidopsis*, it can be hypothesized that *RoKSN* is interacting with *RoFD* to block induction of downstream floral genes such as *RoSOC1* and *RoAPI* (Hanano and Goto, 2011). The *RoFT* repression could be due to a positive feedback loop that is blocked in the presence of *RoKSN*. Such a positive feedback loop has been shown for *TSF*, the paralogue of *FT* in *Arabidopsis* (Yamaguchi *et al.*, 2005). *RoKSN* is not the only target of GA. *RoFD* and *RoLFY* are down-regulated in the absence of *RoKSN*, suggesting an independent pathway for these genes. However this *RoKSN*-independent pathway seems to be minor, as in *roksn* mutants (such as as LWP, a CF rose), no GA floral inhibition happens even at high GA₃ concentrations (Supplementary Table S2 at JXB online). The transient expression of *RoFD* (15 DAB, Fig. 4B) is early and may suggest a role for *RoFD* in processes other than floral initiation. *FD* is interacting with *TFL1* and *FT* (Wigge *et al.*, 2005; Hanano and Goto, 2011). However, a role for *FD* in other developmental process cannot be excluded as *FD* homologues can interact with proteins controlling processes such as growth (Pnueli *et al.*, 2001; Mimida *et al.*, 2011).

This is the first model that focuses on the inhibition of flowering by GA. Previous studies concerning the role of GA in flowering mainly targeted plants where GA had a stimulating effect. In these plants, for example *Arabidopsis* and *L. perenne*, GA acts via activation of floral activators (Blazquez *et al.*, 1998; Gocal *et al.*, 2001; Moon *et al.*, 2003; MacMillan *et al.*, 2005).

GA might not be the only factor that regulates *RoKSN*. After flowering in spring, the *RoKSN* level increases and blooming is blocked (Iwata *et al.*, 2012; this study). Treatment with PCB, a GA synthesis inhibitor, fails to induce new flowering (Table 1). Reduction of GA content is not sufficient to induce flowering. The pattern of floral gene accumulation is not modified after PCB treatment (Fig 4). It cannot be excluded that PCB treatment did not reduce GA content enough to induce blooming (as no GA dosage study has been performed). However, PCB treatment is sufficient to reduce internode length (Table 1; Supplementary Table S2 at JXB online). It is proposed that after the blooming in spring, *RoKSN* could be regulated by GA but also by other pathways. Vernalization is a potentially interesting pathway as OF roses maintained in a greenhouse without a cold period do not

flower the next spring. Control of *TFL1* homologues by another environmental pathway has already been proposed. In diploid strawberry, the expression of *FvKSN*, the orthologue of *RoKSN*, is regulated by the photoperiodic pathway. Under SDs, expression of *FvKSN* is repressed to permit floral initiation, whereas under LDs, expression of *FvKSN* increases to inhibit flowering (Koskela *et al.*, 2012). Furthermore, in *Arabis alpina*, *AaTFL1* is involved in an 'age-dependent response to vernalization' (Wang *et al.*, 2011). In young shoots, *AaTFL1* acts as a floral repressor after vernalization and blocks *LFY* expression. In old shoots, *AaTFL1* increases the duration of vernalization required for flowering (Wang *et al.*, 2011).

In conclusion, a model has been proposed that reveals how GA can inhibit flowering in rose by positively regulating a floral repressor, *RoKSN*. Pathways other than GA such as vernalization may control blooming in rose, and further experiments are required to explore the molecular interactions between these different pathways.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Location of GA response elements in the promoter of *RoKSN*.

Table S1. List of the names and sequences of primers used in the study.

Table S2. Effect of GA₃ and a gibberellin inhibitor (PCB) on OF rose (Félicité et Perpétue; FP) and CF rose (Little White Pet; LWP) on floral development and vegetative growth in spring during floral transition

Table S3. Induction of *RoKSN*, *RoLFY*, and *RoFT* by gibberellins (GA₃) in *R. hybrida* 'Félicité et Perpétue'.

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