ORIGINAL ARTICLE

Involvement of cytokinin response regulator *RhRR1* in the control of flowering

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Abstract

Flowering at a suitable time is critical for ensuring reproductive success in the plant life cycle. The transition from vegetative growth to reproduction development is finely tuned by environmental and endogenous signals. To date, control of flowering involves five genetically defined pathways. However, the role of type-A response regulator genes in regulation of this process remains largely unclear. In the present study, we cloned and characterized a type-A response regulator gene (*RhRR1*) in rose. The expression of *RhRR1* significantly increased in axillary bud during the transition from the vegetative growth to the start of floral differentiation, and in rose flowers in response to exogenous cytokinin or 1-methylcyclopropene (1-MCP) treatments, while that expression was markedly repressed by ethylene treatment. RhRR1 has the highest degree of sequence homology to AtARR8 and AtARR9, and is localized in the nucleus. Ectopic expression *RhRR1* in *Arabidopsis* promoted early flowering, accompanied with the less rosette leaf number at bolting, and shorter bolting time after transferring the plants into pots. In addition, the expression of flowering regulatory genes in *RhRR1* transgenic *Arabidop*sis, including *FLOWERING LOCUS D*, *GA REQUIRING 1*, *LUMINIDEPENDENS*, *LEAFY*, and *TWIN SISTER OF FT* clearly increased. These results allow us to infer that *RhRR1* plays a key role in the control of flowering.

Keywords Type-A response regulator gene · *RhRR1* · Flowering · *Rosa hybrida*

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Introduction

The timing of flowering is critical for reproductive success in the plant life cycle. Premature flowering restricts vegetative growth and as a result that plants accumulate insufficient resources (Johansson and Staiger 2015). On the other hand, delayed flowering ultimately delays seed development resulting in progeny that may be affected by the harmful environmental conditions exhibited during the autumn and winter seasons (Johansson and Staiger 2015). Accordingly, the transition from vegetative growth to reproduction development is finely tuned by environmental and endogenous signals (Iwata et al. 2012). To date, five pathways controlling flowering have been defined, including: vernalization, photoperiod, gibberellin pathway, autonomous, and plant age pathways (Srikanth and Schmid 2011). In addition, key genes involved in regulation of flowering time have been identified. *FLOWERING LOCUS D* (*FD*) in photoperiod pathway positively regulates flowering through activation of downstream targets, such as *APETALA1* (*AP1*) (Wigge et al. 2005). *GA REQUIRING 1* (*GA1*) catalyzes the first step in gibberellic acid biosynthesis, and is an activator

of flowering in gibberellin pathway (Achard et al. 2007). *LUMINIDEPENDENS* (*LD*) belonging to autonomous pathway functions in promotion of flowering (Koornneef et al. 1991). *VERNALIZATION 2* (*VRN2*) in vernalization pathway plays a critical role in regulation of flowering via affecting of the methylated state of *FLOWERING LOCUS C* (*FLC*) (Gendall et al. 2001). In addition, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) in age-regulated pathway is also involved in flowering regulation (Wang et al. 2009).

Rosa hybrida (rose) is one of the most valuable ornamental flowers worldwide, with their wide use as cut flowers and landscaping plants (Debener and Linde 2009). Roses are also utilized in several commercial products, such as rose oil, perfume, and food (Kawamura et al. 2011). In China, more than 4 billion stems of cut roses were produced every year (Luo et al. 2013). As a woody perennial plant, roses have the ability to continuously flower under favorable environment conditions; however, understanding of the molecular mechanism that controls this important ornamental characteristic is not well characterized (Iwata et al. 2012). One regulator of this characteristic in rose is *TERMINAL FLOWER 1* (*TFL1*) which plays a role in maintaining vegetative growth and modifying flowering seasonality in roses (Iwata et al. 2012). To identify other genes involved in this mechanism, 13 genes were screened in rose that may function in the transition between the vegetative and floral bud stages, and were implicated in either the gibberellic acid (GA) signaling pathway, control of photoperiod, or floral development (Foucher et al. 2008). However, the role of genes involved in the cytokinin signaling pathway in the regulation of flowering is unknown.

In the past decade, genes which control the critical steps in cytokinin biosynthesis and signaling have been categorized in *Arabidopsis thaliana* (*Arabidopsis)* (Uraoa et al. 2000). Histidine kinases (HKs), histidine phosphotransfer proteins (HPs), and response regulators (RRs) constitute the cytokinin signal transduction pathway (Hutchison and Kieber 2002). In *Arabidopsis*, cytokinin is sensed by the cytokinin-binding CHASE domain of HKs (AHKs), whereby HPs (AHPs) mediate His-to-Asp phosphotransfer from the cytoplasm to the nucleus (Brandstatter and Kieber 1998; Müller and Sheen 2007; Rashotte 2003). Type-B *Arabidopsis* RRs (ARRs) transcription factors (ARR1, 2, 10–14, and 18–21) then directly modulate expression of type-A ARRs (ARR3-9, 15–17), which are known as the primary cytokinin target genes (Hutchison and Kieber 2002), while the type-A ARRs function to negatively regulate type-B ARRs expression, as a negative feedback loop to control cytokinin response (Hutchison and Kieber 2002). Research has shown that type-A RR genes are involved in regulation of plant growth and development, including the control of the circadian period in *Arabidopsis*, alteration of rice morphology and cytokinin metabolism, and regulation of cytokinin-modulated rhizoid organogenesis (Hirose et al. 2007; Gao et al. 2013; Salome et al. 2006).

Here, we identified a rose homologue of the type-A RR gene, *RhRR1*, which plays a critical role in the regulation of flowering in rose by conducting both physiological and molecular analyses, including: (1) measuring the expression pattern of *RhRR1* in axillary buds during the transition from vegetative growth to the start of floral differentiation and in rose flowers by exogenous phytohormone treatments, (2) phylogenetic tree construction and protein sequence alignment of RhRR1 with analogues, (3) localization and biological function analysis of RhRR1, and (4) dissection of regulatory mechanism of RhRR1. These findings provide further insight into the role of *RhRR1* in regulation of flowering.

Materials and methods

Plant materials and growth conditions

To investigate the expression patterns of *RhRR1*, cut rose (*Rosa hybrida* 'Samantha') flowers were harvested from a local commercial green house in Beijing. The flowers were transported to the laboratory within 1 h after being harvested. The floral bud initiation stages (stage − 4 to − 1), cut flower opening stages (stage 1–4), and flower senescence stages (stage 5–6) in rose were defined as described previously (Horridge and Cockshull 1974; Ma et al. 2005; Moe and Kristoffersen 1968; Wu et al. 2017). Petal samples were collected from the same middle whorl of the flowers (stages 1–6), and the floral bud samples (stage − 4 to − 1) were collected at different stages of bud development.

For exogenous phytohormone treatments, cut rose flowers at floral stage 2 were used. Flowers were placed in vases with 0.1% dimethyl sulfoxide, and extra supplement with 100 μM abscisic acid (ABA), 100 μM 6-Benzylaminopurine (6-BA), 100 μM jasmonic acid (JA), or 100 μM 1-naphthylacetic acid (NAA) for 24 h. Mock samples were only placed in 0.1% dimethyl sulfoxide. For the ethylene and 1-MCP treatments, rose flowers were treated with 10 μl L^{-1} ethylene, or 2 μl L^{-1} 1-MCP in an airtight chamber for 24 h, and flowers exposed only to air were used as controls. To prevent CO_2 accumulation, 1 mol L^{-1} NaOH was placed in the chamber (Lü et al. 2014).

Arabidopsis seeds were sterilized, plated on Murashige and Skoog medium, and stratified in the dark for 2 d at 4 °C. The plates were transferred to a growth chamber with temperature maintained at 22 ± 2 °C with 60% relative humidity and a 16 h light/8 h dark photoperiod. After 7 days, the seedlings were grown in pots containing a 1:1 mixture of vermiculite and peat moss (Clough and Bent 1998).

RNA extraction and cloning of *RhRR1*

Total RNA was extracted from rose petals and floral bud samples as described previously (Wu et al. 2017). Total RNA was extracted from *Arabidopsis* leaves using an EasyPure Plant RNA kit (Huayueyang Biotechnology Co., Ltd., China). Cloning the full length of *RhRR1* was performed by the SMART™ RACE cDNA Amplification Kit (Clontech, USA). Briefly, Two primers, RhRR1-3′-F1 and RhRR1-3′-F2, were designed based on the sequence of *RU47281* as two forward primers and were used together with the 'Universal Primer A Mix' provided by the Kit to amplify potential *RhRR1* 3′-sequences. In addition, RhRR1-5′-F1 and RhRR1-5′-F2 were designed as reverse primers, and were used for individual reactions with the 'Universal primer A Mix' to amplify the *RhRR1* 5′-sequence. Finally, the complete sequence of *RhRR1* was assembled by the 5′ and 3′ sequences. All of the amplified DNA fragments were inserted into the pMGE-T Easy vector (Promega, USA), and then which were transformed into *Escherichia coli* DH5α cells before sequencing. All primers mentioned above are listed in Table S1.

Plasmid construction and plant transformation

For the construction of *35S::RhRR1-GFP* expression vector, the ORF of *RhRR1* was inserted into *35S::GFP* vector. *A. tumefaciens* leaf infiltration and the floral-dip methods were used for tobacco leaves and *Arabidopsis* transformation, respectively (Batoko et al. 2000; Clough and Bent 1998).

Microscopy

To investigate the subcellular localization of RhRR1, transiently transformed tobacco leaves with *35S::RhRR1-GFP* were mounted on glass slides, and were viewed under a confocal laser scanning microscope (CLSM). *35S::GFP* vector was used as a control.

Sequence analysis

Protein sequence alignment of RhRR1 with analogues was done through Clustal X and DNAMAN. And phylogenetic tree analysis was performed through MEGA with the calculation pattern of neighbor-joining algorithm with 1000 bootstrap replicates. The amino acid sequences of receiver domain of RhRR1 were used for phylogenetic tree.

Immunoblot assays

*Arabidopsi*s protein extraction and immunoblot analysis assays were executed as described previously (Meng et al. 2014). Briefly, ground *Arabidopsis* leaves mixed with 2×SDS sample buffer-containing 5% β-mercaptoethanol were boiled at 100 °C for 5 min. The above supernatant was collected after centrifuge at 13,000*g* for 5 min. To detect RhRR1-GFP protein, western blot analysis was executed by probing with Rabbit anti-GFP antibody (diluted 1:2000) and anti-Rabbit HRP (horse radish peroxidase), respectively. Finally, GFP was detected using ECL-detecting reagent (WBKLS0500).

Data analysis

Data were analyzed by one-way ANOVA and SPSS version 16.0 (SPSS Inc., USA). Effects were indicated to be significant if the *p* value was less than 0.05. Three biological and technical replicates were performed.

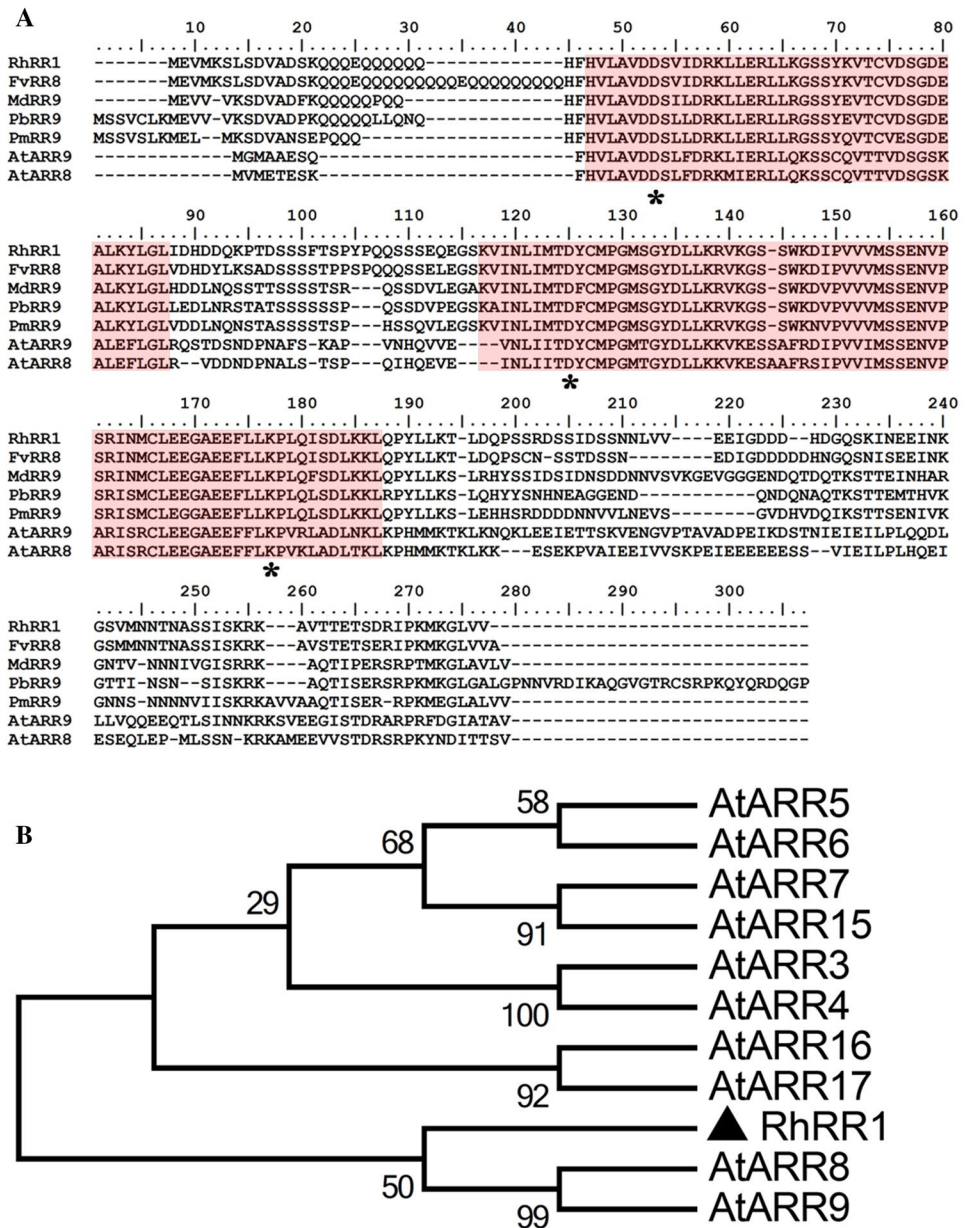## Results

Cloning and characterization of *RhRR1*

To investigate the function of type-A response regulator genes in rose, four putative type-A response regulator transcripts, including *RU28916*, *RU12149*, *RU47281*, and *RU02862,* were identified from an ethylene-treated rose petal transcriptome database (Wu et al. 2017). Transcripts of these genes in petals during various floral development stages (stages 1–6) were evaluated by RT-PCR. As shown in Fig S1, among four genes, the expression pattern of *RU47281* exhibited a significant decrease following floral stage 4. Therefore, we selected *RU47281* for further analysis.

To clone the full-length cDNA of *RU47281* from rose, the RACE amplification method was applied. *RU47281* is 1292 bp in length with a 738 bp predicted open reading frame (ORF), encoding a deduced protein of 246 amino acids (27.39 kDa, pI 4.88). The *RU47281* sequence was submitted to GenBank under the accession number KY014463. Sequence alignment showed that RU47281 contained a conserved receiver domain with three invariant residues 'DDK' and a variable region of 11–21 amino acids. Subsequently, the gene corresponding to *RU47281* was named *Rosa hybrida RR1* (*RhRR1*). RhRR1 has a divergent C-terminus, less than 100 amino acids long, and lacks the GARP domain and a Glu- and Pro-rich region (Fig. 1a). To assess the similarity between RhRR1 and *Arabidopsis* type-A RR proteins, we constructed a phylogenetic tree by the neighbor-joining (NJ) method. RhRR1 had a high degree of sequence homology to AtARR8 and AtARR9 (Fig. 1b).

Localization of RhRR1

To investigate the characteristics of RhRR1, the subcellular localization of RhRR1 was tested in *Nicotiana benthamiana*

Fig. 1 A phylogenetic tree and protein sequence alignment of RhRR1 with analogues. **a** Protein sequence alignment of RhRR1 with analogues; the red highlighted region indicates the conserved receiver domain. The three invariant residues 'DDK' among all type-A RRs are marked with asterisk. Amino acid sequences are from *Fragaria vesca* FvRR8 (GenBank accession number XP_004290880.1), *Prunus mume* PmRR9 (XP_008219624.1), *Malus domestica* MdRR9 (XP_008382445.1), *Pyrus x bretschneideri* PbRR9 (XP_009351080.1), and *Arabiposis thaliana* AtARR8 (1009112986) and AtARR9 (1009119738). **b** A phylogenetic tree of RhRR1 with all type-A response regulator proteins of *Arabidopsis* was constructed by MEGA5 software through the neighbor-joining method (1000 bootstrap replications). The amino acid sequences of receiver domain of RhRR1 were used for phylogenetic tree

Expression pattern of *RhRR1* gene

To investigate the expression pattern of *RhRR1* gene, transcript levels of *RhRR1* were evaluated in rose from floral bud initiation to floral senescence, and in rose flowers after treatment with exogenous phytohormones using qRT-PCR. *RhRR1* expression markedly increased in the axillary bud during transition from vegetative growth stage (stage − 4) to the start of floral differentiation (stage − 3), and then gradually decreased (Fig. 3a). In addition, *RhRR1* expression was clearly induced by cytokinin or 1-MCP treatments, while ethylene treatment significantly decreased its expression. Abscisic acid (ABA), 1-naphthylacetic acid (NAA), and jasmonic acid (JA) treatments did not alter *RhRR1* expression (Fig. 3b).

A characteristic of floral development that has been previously shown is that buds at the top of the stem flower much earlier than those further down (Johnson 1924). Therefore, we also evaluated the time course of *RhRR1* expression in five axillary buds of the same stem (named 1–5 from top to bottom) after removal of the terminal flower by qRT-PCR. The expression level of *RhRR1* increased from the fourth
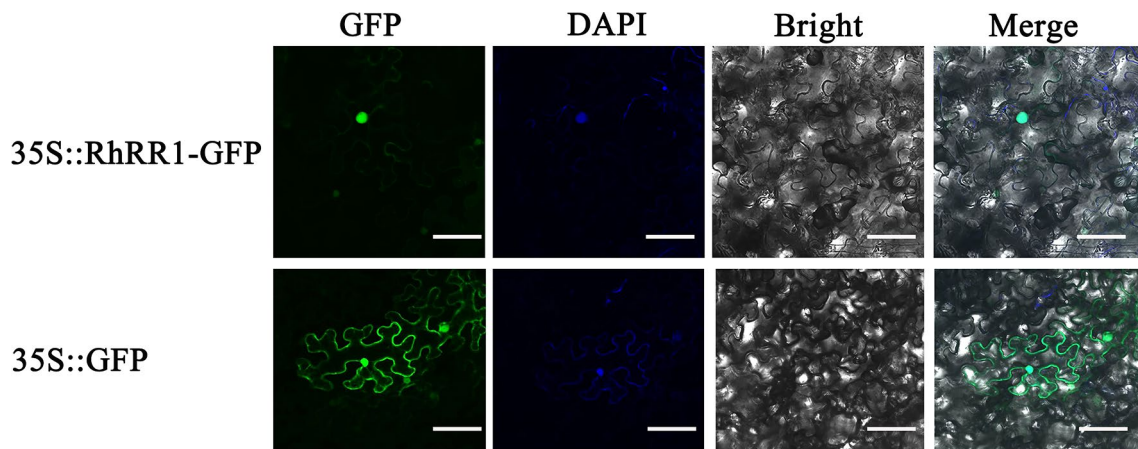
through a transient expression assay. Leaf pavement cells of transiently transformed tobacco showed that RhRR1-GFP was localized in nucleus (Fig. 2). This suggests that RhRR1 acts as a nuclear protein that may function as a transcriptional regulator.

Fig. 2 Subcellular localization of RhRR1. Imaging of the RhRR1-GFP fusion protein was conducted on a laser scanning confocal microscope. Scale bar represents 100 µm
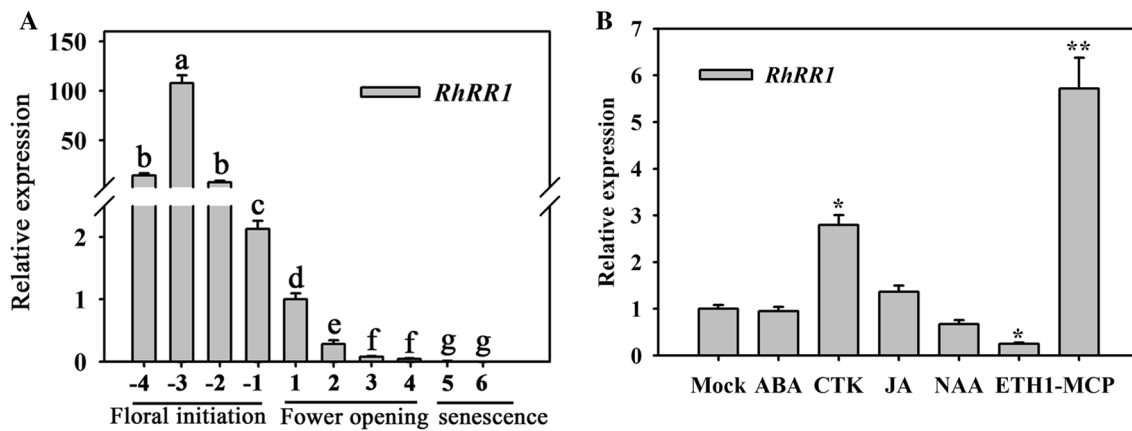

Fig. 3 Expression patterns of *RhRR1*. **a** Quantitative RT-PCR analysis of *RhRR1* expression in rose from floral initiation to flower senescence. Floral initiation stages (stage − 4 to − 1), flower opening stages (stages 1–4), and flower senescence stages (stages 5–6). **b** Quantitative RT-PCR analysis of *RhRR1* expression in rose flowers in response to exogenous phytohormones. *RhActin5* was used as an internal control. Error bars represent the standard deviation of three biological replicates. Letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$), and asterisks indicate significant differences according to Student's t test (*$p < 0.05$, **$p < 0.01$)

axillary bud to the third axillary bud, but then decreased significantly (Fig. S2). This *RhRR1* expression trend was similar to that of *RhRR1* in the axillary bud during transition from the vegetative growth stage (stage − 4) to the start of floral differentiation (stage − 3) (Fig. 3a). Based on the results of expression pattern of *RhRR1*, we speculated that *RhRR1* may play a key role in regulation of flowering.

Phenotype of transgenic *Arabidopsis* overexpression *RhRR1*

To evaluate the role of *RhRR1* in development, we generated an *Arabidopsis* transgenic line overexpressing *RhRR1-GFP*. T2 homozygous plants were used for analysis. Protein levels of RhRR1 in both wild-type and overexpression lines were assessed using western blot probed with anti-GFP antibody (Fig. 4b). Line #1, #4, and #6 were used for further characterization. Overexpression of *RhRR1* in *Arabidopsis* did not show any changes in morphogenesis, including formation of roots, stem, leaves, and flowers of adult plants, but showed early or premature flowering time (Fig. 4a). Accordingly, the rosette leaf number at bolting was clearly less in *RhRR1*-ox plants than that in the wild type, with only 7.50 ± 0.41 leaves in line 6# compared with 11.80 ± 0.87 leaves in the wild type (Fig. 4c). In addition, the bolting timing after transferring the plants into pots was also significantly different between wild-type and transgenic lines, with only 7.10 ± 0.36 days
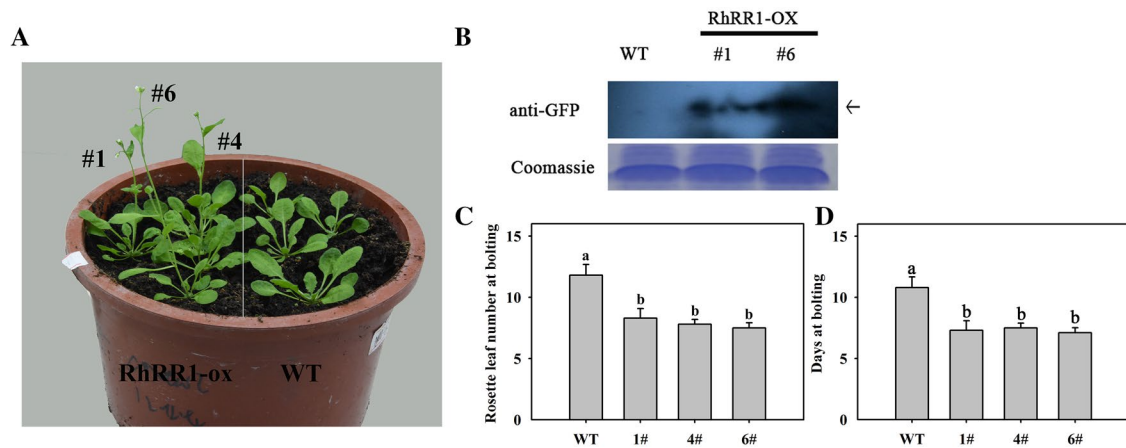
Fig. 4 Ectopic expression of *RhRR1* in *Arabidopsis*. **a** Phenotypes of 20-day-old seedlings of wild-type and transgenic plants. **b** Immunoblot analysis of RhRR1-GFP in *Arabidopsis* leaves. 10 μg of total protein were loaded in each lane. A Coomassie Brilliant Blue-stained blot (CBB) is shown to confirm equivalent sample loading. **c** Rosette leaf number at bolting in wild-type and 35S:: RhRR1-GFP transgenic plants. **d** The bolting timing after transferring the plants into pots. Error bars represent the standard deviation of three biological replicates. Letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$)

in line 6# compared with 10.78 ± 0.61 days in the wild type (Fig. 4d).

Transcripts of key genes involved in flowering regulation

To uncover the mechanism of *RhRR1* in the regulation of flowering, the expressions of six key genes belonging to five flowering pathways in *Arabidopsis* were evaluated by qRT-PCR. Transcripts of *FD* in the photoperiod pathway, *GA1* in the GA pathway, *LD* in the autonomous pathway, and the floral integrators *LEAFY* (*LFY*) markedly increased in the overexpression lines than that in the wild type (Fig. 5a–d), while no markedly changes of a flowering integrator gene *FT* expression were observed (Fig. 5e). Intriguingly, the *TSF* transcripts, the *FT* paralogue, also significantly increased in the overexpression lines (Fig. 5f). Although the expression of *RhRR1* was induced by cytokinin treatment in rose flowers in Fig. 3b, it is unclear whether the early flowering phenotype in *RhRR1* overexpression lines was related to cytokinin regulation. To confirm it, the expressions of two cytokinin signaling pathway genes *ARR4* and *ARR5* were evaluated in transgenic *Arabidopsis*. However, no significant difference of the transcripts of *ARR4* and *ARR5* was measured between wild-type and *RhRR1* overexpression lines (Fig. 3s).

Discussion

Plants adjust the time of floral initiation for successful reproduction in their environment (Iwata et al. 2012; Johansson and Staiger 2015; Srikanth and Schmid 2011). Several genes involved in regulation of flowering have been identified and characterized in detail, but cytokinin response regulator gene function in regulation of flowering has not yet illuminated (Srikanth and Schmid 2011). Our results imply that *RhRR1* is a member of the type-A response regulator family, and is involved in the regulation of flowering in rose. This is supported by the expression pattern of *RhRR1*, which was significantly increased in axillary bud during the vegetative growth stage (stage − 4) transition to the start of floral differentiation (Fig. 3). In addition, the early flowering phenotype exhibited by overexpression of *RhRR1* in *Arabidopsis,* as shown in Fig. 4, which was accompanied by a decreased rosette leaf number at bolting and shorter bolting time also demonstrated the role of RhRR1 in rose floral development. Although we targeted *RhRR1* to analyze its function in flowering, other type-A RRs in rose may also play similar role, especially those showing trends similar to *RhRR1*. Therefore, future experiments are required to determine whether other type-A RRs in rose have functions in controlling flowering.

Although cytokinins (CTKs) act as anti-senescence factors in flowers, cytokinin also promotes flowering in plants, including rose (Wang et al. 2002; Wu et al. 2017; Zeng et al. 2013). The expressions of type-A response regulator genes, including *ARR3*, *ARR5*, *ARR7*, and *ARR8,* are known to increase in response to cytokinin treatment; however, their function in controlling of flowering is not clear (Lee et al. 2007; Li et al. 2013; Osakabe et al. 2002; Salome et al. 2006). In *Rosa hybrida*, the transcripts of *RhRR1* were also induced by exogenous cytokinin treatment (Fig. 3b). In addition, the expression of *RhRR1* significantly decreased by ethylene treatment and induced by ethylene inhibitor 1-MCP treatment (Fig. 3b). This expression pattern of *RhRR1* is similar to the expression patterns exhibited by type-A RRs in *Arabidopsis*, such as *ARR5*, *ARR7*, and *ARR15*, in

Fig. 5 The expression levels of flowering regulatory genes in *Arabidopsis*. Quantitative RT-PCR analysis of expression of *FD* (**a**), *GA1* (**b**), *LD* (**c**), *FT* (**d**), *LFY* (**e**), and *TSF* (**f**) in 10-day-old *Arabidopsis* leaves. *TUB2* was used as an internal control. Error bars represent the standard deviations of three biological replicates. Asterisks indicated significant differences according to Duncan's multiple range test ($p < 0.05$)
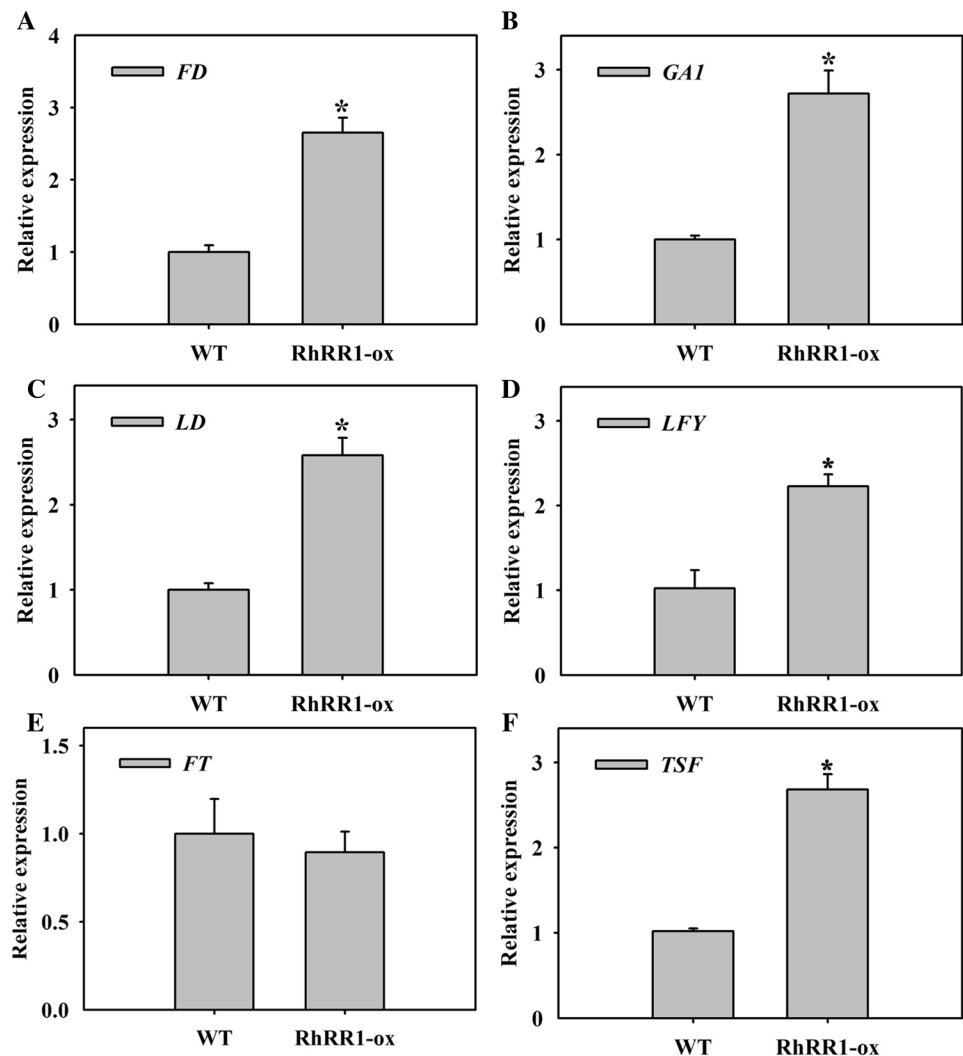
response to ethylene treatment (Shi et al. 2012). Ethylene is an important plant hormone, involved in the regulation of various developmental processes including seed germination, flowering, and organ senescence (Pei et al. 2013). It was also reported that ethylene is related to flowering in pineapple (*Ananas comosus*) (Burg and Burg 1966). Based on the expression patterns of *RhRR1*, we speculated that *RhRR1* may function as a key node to integrate ethylene and cytokinin signals in regulation of plant flowering.

Altered expression of five flowering time activator genes including *FD*, *GA1*, *LD*, *LFY*, and *TSF* was observed in *Arabidopsis* leaves with overexpression *RhRR1*, suggesting that *RhRR1* may play a critical role in modulating floral initiation via affecting the photoperiod pathway, GA pathway, and autonomous pathway (Fig. 5). Phylogenetic analysis revealed that the determined amino acid sequence of RhRR1 showed high similarity to those of AtARR8 and AtARR9 in *Arabidopsis*, containing a conserved receiver domain with three invariant residues 'DDK' (Fig. 1a).

In addition, a subcellular localization assay showed that RhRR1 is localized in the nucleus and may act as a transcriptional regulator (Fig. 2a). The localization may underlie the regulation of flowering by *RhRR1* via regulation of genes involved in several flowering pathways.

Interestingly, the expression of *FLOWERING LOCUS T* (*FT*), a flowering time activator, was not significantly difference between *RhRR1* overexpression lines and wild type (Fig. 5e). However, the expression level of the paralogue of *FT*, *TWIN SISTER OF FT* (*TSF*), was significantly increased in *RhRR1* overexpression lines (Fig. 5f). This may be because the promotion of flowering by cytokinin in *Arabidopsis* does not require *FT*, but rather activates its paralogue, *TSF*, as well as *FD*, which encodes a partner of TSF (D'Aloia et al. 2011). Our findings provide a new insight that a type-A response regulator gene in rose, *RhRR1*, plays crucial role in regulation of flowering through controlling of expression of *FD*, *GA1*, *LD*, *LFY*, and *TSF*.

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