

# An Ethylene-Induced Regulatory Module Delays Flower Senescence by Regulating Cytokinin Content<sup>1[OPEN]</sup>

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In many plant species, including rose (*Rosa hybrida*), flower senescence is promoted by the gaseous hormone ethylene and inhibited by the cytokinin (CTK) class of hormones. However, the molecular mechanisms underlying these antagonistic effects are not well understood. In this study, we characterized the association between a pathogenesis-related PR-10 family gene from rose (*RhPR10.1*) and the hormonal regulation of flower senescence. Quantitative reverse transcription PCR analysis showed that *RhPR10.1* was expressed at high levels during senescence in different floral organs, including petal, sepal, receptacle, stamen, and pistil, and that expression was induced by ethylene treatment. Silencing of *RhPR10.1* expression in rose plants by virus-induced gene silencing accelerated flower senescence, which was accompanied by a higher ion leakage rate in the petals, as well as increased expression of the senescence marker gene *RhSAG12*. CTK content and the expression of three CTK signaling pathway genes were reduced in *RhPR10.1*-silenced plants, and the accelerated rate of petal senescence that was apparent in the *RhPR10.1*-silenced plants was restored to normal levels by CTK treatment. Finally, RhHB6, a homeodomain-Leu zipper I transcription factor, was observed to bind to the *RhPR10.1* promoter, and silencing of its expression also promoted flower senescence. Our results reveal an ethylene-induced RhHB6-RhPR10.1 regulatory module that functions as a brake of ethylene-promoted senescence through increasing the CTK content.

The terminal phase of flower development is senescence, which is generally characterized by time to petal wilting, or withering, or by the time to turgid petal abscission (van Doorn, 2001). Flower senescence is a highly regulated process that exhibits many of the structural, biochemical, and molecular changes that are hallmarks of programmed cell death. These include a loss of membrane permeability, increase in reactive oxygen species, and decreased levels of protective enzymes, followed by protein degradation, fatty acid breakdown, and degradation of nucleic acids (Wagstaff et al., 2002; Jones et al., 2005; Xu et al., 2006; Tripathi and

Tuteja, 2007; Yamada et al., 2009; Shahri and Tahir, 2011; Rogers, 2013).

The initiation of flower senescence can be triggered by external or internal cues (Gan and Amasino, 1997; Rogers, 2013; Zhang and Zhou, 2013), and phytohormones have been shown to play a pivotal role in triggering and modulating the progression of senescence, often through combinatorial interactions (Beaudoin et al., 2000; van Doorn and Woltering, 2008; Zhang and Zhou, 2013). Among these hormones, ethylene is considered a major regulator of flower senescence (van Doorn, 2001), and in ethylene-sensitive species, flower senescence is associated with a burst of ethylene production, and the coordinated expression of ethylene-responsive genes, including those with regulatory functions (Ichimura et al., 2009; Lerslerwong et al., 2009). For example, in *Arabidopsis* (*Arabidopsis thaliana*), the expression of microRNA164 was shown to be repressed by ethylene, thereby promoting senescence (Li et al., 2013), while in rose (*Rosa hybrida*), a reduction in transcript abundance of a homeodomain-Leu zipper (HD-Zip) I transcription factor gene, *RhHB1*, was observed to delay senescence (Lü et al., 2014). In contrast, the expression of another member of the HD-Zip I transcription factor family, *HaHB-4*, was reported to be up-regulated in response to ethylene in sunflower (*Helianthus annuus*), consequently delaying the senescence process (Manavella et al., 2006), suggesting that

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antagonistic mechanisms influence ethylene-induced senescence.

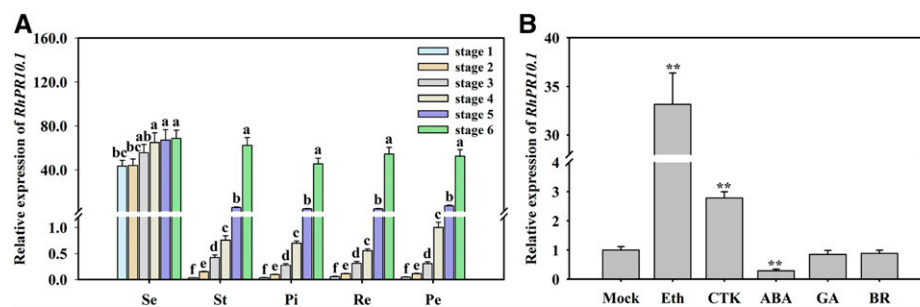
Members of another class of hormones, cytokinins (CTKs), have been proposed to act as antisenesescence factors in flowers (Mayak and Halevy, 1970; Eisinger, 1977). Accordingly, it has been reported that rose varieties with long flower longevity contain higher levels of CTKs than those with shorter lived flowers (Mayak and Halevy, 1970), and CTK application has been shown to delay flower senescence in several plant species, including carnation (*Dianthus caryophyllus*), petunia (*Petunia hybrida*), and rose (Mayak and Halevy, 1970; Mor et al., 1983; Taverner et al., 1999). Conversely, an increase in transcript abundance of two genes encoding CTK oxidase/dehydrogenase during carnation petal senescence accelerated CTK degradation and promoted corolla senescence (Hoerberichts et al., 2007).

In addition to the individual effects of ethylene and CTK on flower senescence, these two hormones have been reported to interact in senescence. For example, in carnation, declining CTK levels act as a trigger for ethylene production (Eisinger, 1977), while treatment of carnation petals with CTK blocked the conversion of exogenously supplied 1-aminocyclopropane-1-carboxylic acid to ethylene (Mor et al., 1983; Taverner et al., 1999). In petunia, driving the overexpression of the CTK biosynthesis gene *IPT* with the *Senescence-associated gene12* (*SAG12*) promoter resulted in a significant delay in flower senescence, accompanied by reduced ethylene sensitivity (Chang et al., 2003). In addition, it has been reported that the Arabidopsis KNOTTED-like homeodomain protein *KNAT2* acts synergistically with CTKs and antagonistically with ethylene to delay senescence (Hamant et al., 2002). However, despite such observations exemplifying the antagonistic effects of ethylene and CTKs on flower senescence, the underlying molecular mechanisms and the many details of the genes in the associated regulatory networks are still largely unknown.

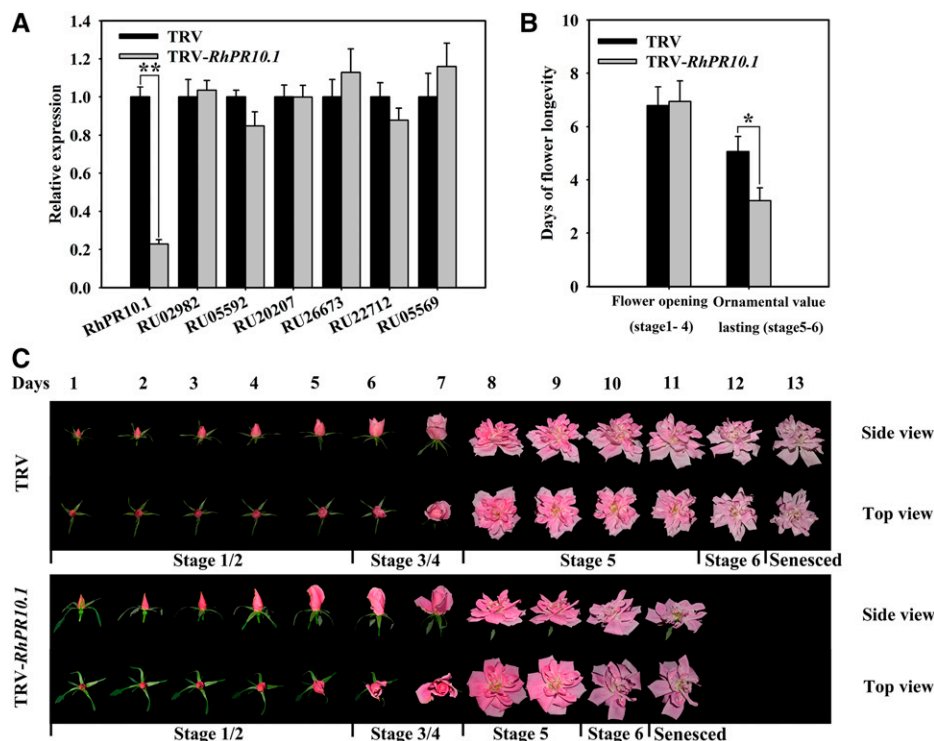
In this study of rose flower senescence, we identified a member of the pathogenesis-related (PR) PR-10

family as being involved in the cross talk between ethylene and CTK signaling. PR proteins, which function in a wide range of processes related to signal transduction and antimicrobial activity (Zubini et al., 2009), have been classified into 17 families based on structural features; of these, the PR-10 protein family is a large group containing more than 100 members (Somssich et al., 1988; Biesiadka et al., 2002; Hashimoto et al., 2004; Xu et al., 2014), which are typically small and localized in the cytosol (Fernandes et al., 2013). PR-10 proteins play multifunctional roles in defense mechanisms against abiotic and biotic stresses, and in developmental regulation via their RNase activity and/or interaction with ligands (Fernandes et al., 2013; Agarwal and Agarwal, 2014). In addition, CTKs can play a role in biological processes that are mediated by PR-10 proteins (Pasternak et al., 2006; Srivastava et al., 2006, 2007; Fernandes et al., 2008; Zubini et al., 2009). It has been proposed that when PR-10 proteins act as RNases, they may degrade certain types of tRNAs containing a CTK moiety, thereby contributing to the regulation of endogenous CTK content (Zubini et al., 2009). In support of this idea, overexpression of the pea (*Pisum sativum*) PR-10 gene *ABR17* in *Brassica napus* and Arabidopsis enhanced abiotic stress tolerance through degradation of RNA to increase CTK levels (Srivastava et al., 2006, 2007). PR-10 proteins are also capable of binding CTKs; LIPR-10.2 from yellow lupine (*Lupinus arboreus*) has been shown to bind trans-zeatin, thereby acting as a reservoir of CTK molecules (Fernandes et al., 2008). However, the role of PR-10 proteins in modulating CTK content during flower senescence has not been characterized.

Roses are one of the most important ornamental crops worldwide, and their commodity value largely depends on the long vase life of the flowers. It is well known that ethylene promotes rose flower senescence (Ma et al., 2005), while CTKs delay the process (Mayak and Halevy, 1970; Lukaszewska et al., 1994). Here, we report that an ethylene-induced rose PR-10 family gene, *RhPR10.1*, inhibits



**Figure 1.** Expression patterns of *RhPR10.1* in different rose flower organs during various opening stages (A) and in rose petals in response to exogenous hormones (B). A, Se, sepal; St, stamen; Pi, pistil; Re, receptacle; Pe, petal. B, Eth,  $10 \mu\text{L L}^{-1}$  ethylene; CTK,  $100 \mu\text{M}$  6-BA; ABA,  $100 \mu\text{M}$  ABA; GA,  $80 \mu\text{M}$  GA<sub>3</sub>; BR,  $5 \mu\text{M}$  brassinosteroid. The petals at opening stage 2 were used as material. The results are the means of three biological replicates with standard deviations. 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 ( $*P < 0.05$ ,  $**P < 0.01$ ).



**Figure 2.** Silencing of *RhPR10.1* promotes flower senescence. A, Expression of the *RhPR10* genes in *RhPR10.1*-silenced and TRV control flowers was analyzed by qRT-PCR. B, The time of flower opening (stages 1–4) and retention of ornamental value (stages 5 and 6) were recorded. C, Flower phenotypes were recorded and photographed every day. Stages of rose flowering were defined as follows: stage 1, bud with completely opened sepal; stage 2, bud with the outmost petal layer loosened; stage 3, flower with the outmost petal layer opened; stage 4, flower with the inner petal layer loosened; stage 5, fully opened flower; and stage 6, flower with fading color and loss of ornamental value. The results are the means of three biological replicates with standard deviations. Asterisks indicate statistically significant differences (Student's *t* test, \**P* < 0.05, \*\**P* < 0.01).

ethylene-induced flower senescence via increasing the CTK content.

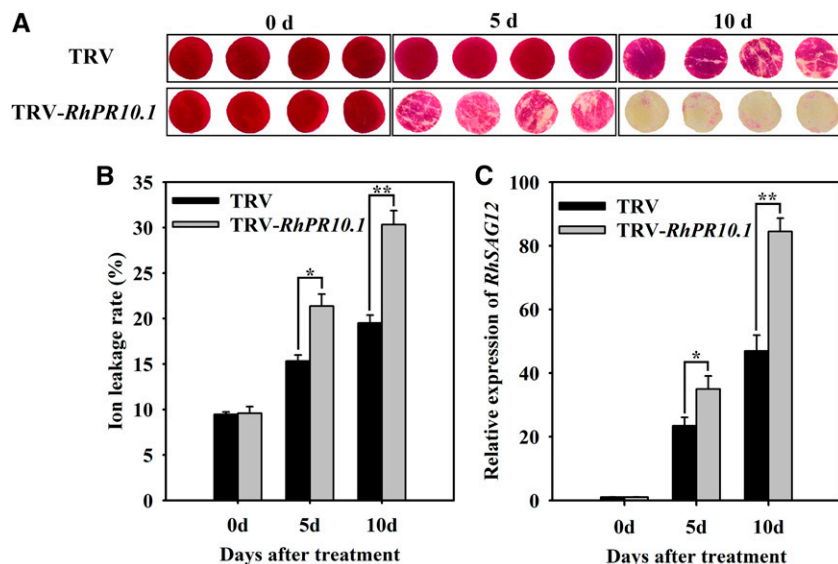
## RESULTS

### The Expression of *RhPR10.1* Is Induced following Senescence and Exogenous Ethylene Treatment

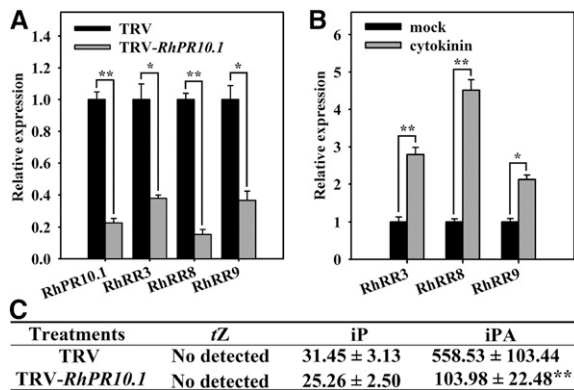
To investigate the function of the PR-10 family genes during flower senescence, seven transcripts encoding putative PR-10 family proteins were identified in an in-house ethylene-treated rose (*R. hybrida* 'Samantha')

petal transcriptome database ([http://bioinfo.bti.cornell.edu/cgi-bin/rose\\_454/index.cgi](http://bioinfo.bti.cornell.edu/cgi-bin/rose_454/index.cgi)): RU05569, RU05592, RU06312, RU26673, RU20207, RU22712, and RU02982 (Supplemental Fig. S1). Expression analysis by quantitative reverse transcription PCR (qRT-PCR) showed that, of these genes, *RU06312* exhibited a trend of increasing expression and the highest accumulation of transcripts during petal opening (Supplemental Fig. S2), and so we targeted this gene for further analysis.

We isolated a 766-bp *RU06312* cDNA sequence with a 477-bp predicted open reading frame by rapid amplification of cDNA ends, encoding a deduced protein



**Figure 3.** Silencing of *RhPR10.1* promotes senescence in rose petal discs. A, The color-fading phenotypes of petal discs were recorded. B and C, Ion leakage rate (B) and expression of *RhSAG12* (C) were analyzed in *RhPR10.1*-silenced and TRV control petals. The results are the means of three biological replicates with standard deviations. Asterisks indicate significant differences (Student's *t* test, \**P* < 0.05, \*\**P* < 0.01).



**Figure 4.** *RhPR10.1* silencing reduces CTK signaling and levels in rose petals. A and B, Expression of genes related to the CTK signaling pathway in petals of *RhPR10.1*-silenced and TRV control flowers (A), and in rose wild-type petals treated with CTK (B). C, CTK contents in petals of *RhPR10.1*-silenced and TRV control flowers; tZ, trans-zeatin. Values are means ( $\mu\text{g g}^{-1}$  fresh weight)  $\pm$  SD. The results are the means of three biological replicates with standard deviations. Asterisks indicate statistically significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

of 159 amino acids with a conserved P-loop motif and Bet v 1 motif (Lebel et al., 2010; Supplemental Fig. S3). Sequence alignment showed that RU06312 had a high degree of sequence homology to FaPR10-4 from *Fragaria*  $\times$  *ananassa* (Supplemental Fig. S3), and the gene corresponding to RU06312 was named *RhPR10.1*. Phylogenetic analysis showed that *RhPR10.1* belongs to the major pollen and food allergens subclass of PR10 protein family (Fernandes et al., 2013; Supplemental Fig. S3).

The expression of *RhPR10.1* was evaluated in different organs during flower opening using qRT-PCR. The opening process of cut rose flowers can be divided into six stages (Ma et al., 2005). The expression patterns of *RhPR10.1* in all the floral organs, including sepal, stamen, pistil, petal, and receptacle, showed similar trends, with transcript levels increasing progressively from the partially opened flower bud (stage 1) to the onset of petal wilting (stage 6; Fig. 1A). The expression was higher in sepals in all the flower opening stages (stages 1–6) than in the other floral organs (Fig. 1A). When the petals treated with hormones were tested, we observed that exogenous ethylene and CTK significantly induced *RhPR10.1* expression, while an abscisic acid (ABA) treatment resulted in a decrease in expression. Gibberellic acid (GA) and brassinosteroid treatments did not alter *RhPR10.1* transcript levels (Fig. 1B).

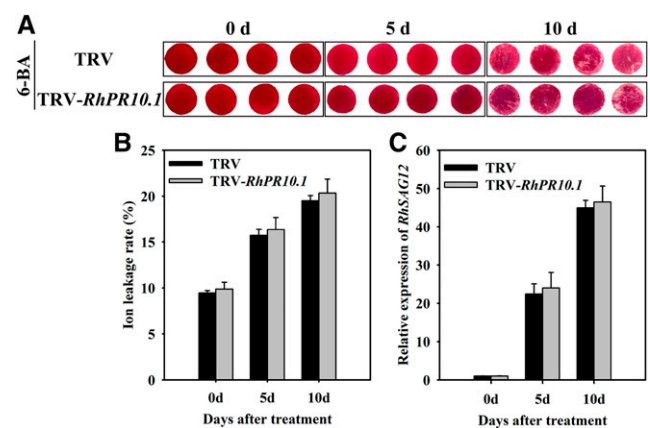
#### Silencing of *RhPR10.1* Accelerates Rose Flower Senescence

To investigate the potential role of *RhPR10.1* in flower senescence, expression of the gene was silenced using virus-induced gene silencing (VIGS). We constructed a tobacco rattle virus vector (TRV-*RhPR10.1*) from the 3' end region of *RhPR10.1* (Supplemental Fig. S1) to specifically silence *RhPR10.1* expression in rose plantlets and petal discs. As shown in Figure 2A, with

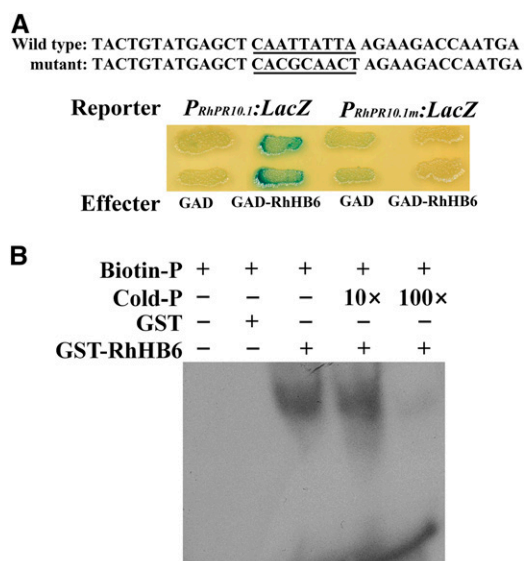
the exception of *RhPR10.1*, none of the *RhPR10* family genes exhibited reduced expression levels in *RhPR10.1*-silenced petals compared with the TRV control, thereby confirming the specificity of the gene silencing.

The flower life span of rose plantlets can be divided into six stages. Stages 1 to 4 are defined as the flower opening phase, where the flowers have not fully opened, while stages 5 and 6 are defined as phases with ornamental value, starting from when the flower fully opens until color fading has occurred (Fig. 2C). The duration of the flower opening phase in the *RhPR10.1*-silenced plant was indistinguishable from that of the TRV control, with stages 1 to 4 lasting  $6.8 \pm 0.6$  and  $6.9 \pm 0.8$  d, respectively (Fig. 2, B and C). The flower sizes were also not statistically significant difference between TRV control and *RhPR10.1*-silenced plantlets. However, in the ornamental value phases, *RhPR10.1* silencing caused an accelerated senescence phenotype, with stages 5 and 6 lasting only  $3.7 \pm 0.4$  d compared with  $5.1 \pm 0.5$  d in the TRV control (Fig. 2, B and C). In addition, we observed that the flower life spans of TRV control plantlets were indistinguishable from that of the wild type (Supplemental Fig. S4), indicating TRV control did not affect the flower opening and senescence.

We further analyzed the effect of *RhPR10.1* silencing on senescence of petal discs. In the TRV control, slight color fading occurred at 10 d (Fig. 3A). In contrast, *RhPR10.1*-silenced discs showed color fading at day 5, and almost all the petal discs were discolored at day 10 (Fig. 3A). In addition, the ion leakage rate and expression of the senescence marker gene *RhSAG12* (Lü et al., 2014) were significantly higher in *RhPR10.1*-silenced petal discs than in the TRV control (Fig. 3, B and C).



**Figure 5.** The effect of exogenous CTK treatment on senescence of *RhPR10.1*-silenced rose petal discs. A, The phenotypes of petal discs treated with  $100 \mu\text{M}$  6-BA were recorded. B and C, The ion leakage rates (B) and transcript levels of *RhSAG12* (C) in *RhPR10.1*-silenced and TRV control petal discs treated by 6-BA. Statistical differences were analyzed by Student's *t* test ( $P < 0.05$ ).



**Figure 6.** RhHB6 binds to a cis-element in the promoter of *RhPR10.1*. A, Transactivation activity of RhHB6 to the *RhPR10.1* promoter in yeast. Wild-type and mutant probes were derived from the *RhPR10.1* promoter. The wild-type cis-element and its nucleotide substitutions in the mutant versions are underlined. B, EMSA was used to analyze the interaction of GST-RhHB6 and a biotin-labeled probe. Purified protein (3  $\mu$ g) samples were incubated with 25  $\mu$ M of the biotin-labeled wild-type probe. Nonlabeled probes at 10- and 100-fold concentrations were added for the competition test. Images are representative of three independent experiments.

### *RhPR10.1* Silencing-Induced Senescence Is Associated with the CTK Pathway

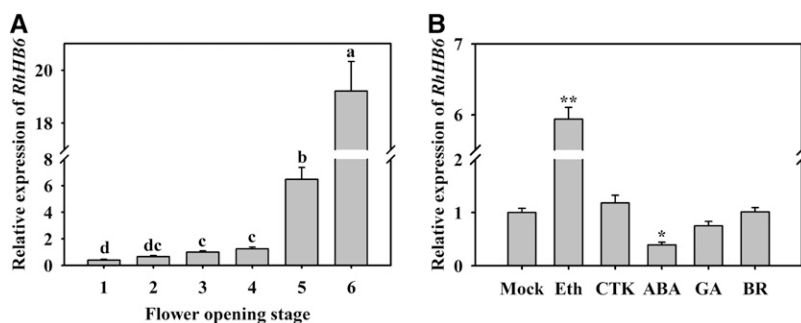
To investigate whether RhPR10.1 influences the CTK pathway during senescence, we measured the expression of three CTK signaling pathway genes, *RhRR3*, *RhRR8*, and *RhRR9*, in *RhPR10.1*-silenced rose petals. qRT-PCR analysis showed that the transcript abundance of all three genes, along with *RhPR10.1*, was reduced in petals of *RhPR10.1*-silenced flowers compared with TRV controls (Fig. 4A). In addition, the expression of the three genes increased in response to exogenous CTK, confirming their involvement in the CTK response pathway (Fig. 4B). We next examined

the contents of the biologically active CTKs trans-zeatin,  $N^6$ -( $\Delta^2$ -isopentenyl)-adenine (iP), and isopentenyl adenosine (iPA) in the petals of the *RhPR10.1*-silenced flowers. iP levels were found to be significantly reduced in the silenced flowers, while trans-zeatin levels were undetectable in both the TRV control and the *RhPR10.1*-silenced petals (Fig. 4C), and no significant difference was observed in iP contents (Fig. 4C).

Since CTK response and content were altered in *RhPR10.1*-silenced flowers, we speculated that *RhPR10.1*-mediated flower senescence may function by regulating CTK pathway. We therefore examined the effect of treating *RhPR10.1*-silenced petals with 6-benzyl aminopurine (6-BA; a synthetic CTK), and found that its application resulted in a slight color fading after 10 d in both the TRV control and *RhPR10.1*-silenced petal discs (Fig. 5A). In addition, similar expression levels of *RhSAG12* and ion leakage rates were detected in petal discs from the TRV control and the *RhPR10.1*-silenced plants (Fig. 5, B and C).

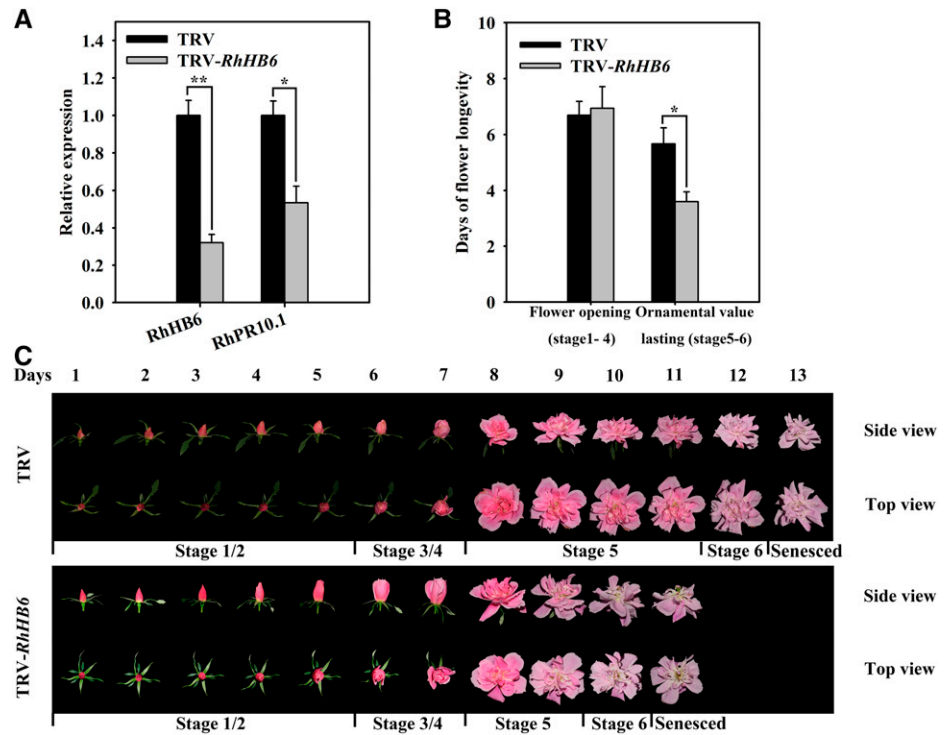
### *RhPR10.1* Is Directly Regulated by Transcription Factor RhHB6

To better understand the regulatory mechanism of *RhPR10.1*, a 1,378-bp promoter region upstream of the *RhPR10.1* coding sequence was identified. Sequence analysis using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) suggested that the cis-elements in the promoter of *RhPR10.1* included 13 homologous sequences of ARR1AT for CTK response regulator, five binding sites for MYB transcription factor, four binding sites for MYC, and three binding sites for WRKY (Supplemental Fig. S5). In addition, a known binding site (CAATTATTA) for the HD-Zip subfamily I member ATHB6 (Himmelbach et al., 2002) was located between positions -706 and -715 (Supplemental Fig. S5). A search of an in-house-generated ethylene-treated rose petal transcriptome database ([http://bioinfo.bti.cornell.edu/cgi-bin/rose\\_454/index.cgi](http://bioinfo.bti.cornell.edu/cgi-bin/rose_454/index.cgi)) revealed three members of the HD-Zip I subfamily, *RhHB1* (RU00522; Lü et al., 2014), *RhHB6* (RU12591), and *RhHB7* (RU00731), as well as a member of the HD-Zip II subfamily, *RhHB2* (RU01217).



**Figure 7.** Expression of *RhHB6* in rose petals at different flower opening stages (A) and in response to exogenous hormones (B). Eth, 10  $\mu$ L L<sup>-1</sup> ethylene; CTK, 100  $\mu$ M 6-BA; ABA, 100  $\mu$ M ABA; GA, 80  $\mu$ M GA<sub>3</sub>; BR, 5  $\mu$ M brassinosteroid. The petals at stage 2 were used as material in B. The results are the means of three biological replicates with standard deviations. 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 (\* $P < 0.05$ , \*\* $P < 0.01$ ).

**Figure 8.** Silencing of *RhHB6* promotes flower senescence. A, Expression of the *RhHB6* and *RhPR10.1* genes in *RhHB6*-silenced and TRV control petals was analyzed by qRT-PCR. B, The time of flower opening and senescence phases was recorded. C, The phenotypes of flowers were recorded and photographed every day. The results are the means of three biological replicates with standard deviations. Asterisks indicate statistically significant differences (Student's *t* test, \**P* < 0.05, \*\**P* < 0.01).



We conducted a yeast one-hybrid assay, using the cis-element in the *RhPR10.1* promoter, CAATTATTA, as bait, to assess the interaction of the HD-Zip family members with the *RhPR10.1* promoter (Supplemental Fig. S6). RhHB1, RhHB7, and RhHB2 did not bind to the *RhPR10.1* promoter (Supplemental Fig. S6); however, RhHB6 bound and triggered expression of the LacZ reporter gene (Fig. 6A), but did not bind a mutated *RhPR10.1* fragment (Fig. 6A). An electrophoretic mobility shift assay (EMSA) was conducted to confirm the interaction of RhHB6 and the *RhPR10.1* promoter, and RhHB6 was found to bind to the biotin-labeled *proRhPR10.1* probe; moreover, binding was gradually attenuated by increasing concentrations of unlabeled probe (Fig. 6B).

#### Silencing of *RhHB6* Accelerates Rose Flower Senescence

To investigate the potential function of *RhHB6* in rose petal senescence, we evaluated its expression in different flowering stages and following various hormone treatments. As shown in Figure 7, the expression of *RhHB6* increased during flower opening (Fig. 7A), and was induced by ethylene but inhibited by an ABA treatment (Fig. 7B). When *RhHB6* was silenced in rose plantlets by VIGS, the expression levels of *RhHB6* and *RhPR10.1* were lower in petals of the silenced flowers than in those of the TRV control (Fig. 8A). The duration of the flower opening phase of the *RhHB6*-silenced flowers was similar to that of the TRV control (Fig. 8, B and C); however, *RhHB6* silencing caused an accelerated senescence phenotype during the ornamental value phases,

with stages 5 and 6 only lasting  $3.6 \pm 0.3$  d compared with  $5.7 \pm 0.6$  d for the TRV control (Fig. 8, B and C).

## DISCUSSION

### *RhPR10.1* Is Involved in Ethylene-Induced Rose Petal Senescence

Flower senescence is accompanied by changes in the levels of endogenous hormones, which regulate networks of signaling events that control the senescence program (van Doorn and Woltering, 2008; Zhang and Zhou, 2013). Ethylene is known to be a crucial accelerant of flower senescence. Based on the results generated in this study, we propose that *RhPR10.1* is a member of the network of genes involved in the ethylene-induced senescence process in rose flowers. This hypothesis is supported by the expression pattern of *RhPR10.1*, which was found to be significantly induced by ethylene treatments (Fig. 1B), and by the accelerated flower senescence phenotype of the *RhPR10.1* VIGS plants (Figs. 2 and 3).

The expression of PR-10 family members is known to increase during senescence, although their function in senescence is not clear. In bean (*Phaseolus vulgaris*), the transcript levels of the PR-10 protein *Ypr10* were reported to increase at the onset of leaf senescence (Walter et al., 1996), and in yellow lupine, the expression of the PR-10 gene *LIPR10.1A* was induced in senescent leaves (Sikorski et al., 1999). Here, we showed that in rose, the transcript levels of several *PR10* members also accumulated during flower senescence (Supplemental Fig. S2). Although we targeted *RhPR10.1* with the highest

accumulation among *RhPR-10* members for analyzing the function in senescence, it is feasible that other *RhPR-10* genes may also play roles in petal senescence, especially those showing trends of increasing expression during flower senescence. Therefore, additional studies are required to determine whether other *RhPR-10* members have functions in flower senescence.

Intriguingly, functional analysis of the *RhPR10.1* gene indicated that the effect of *RhPR10.1* on senescence is antagonistic, with reduced *RhPR10.1* expression promoting flower senescence (Figs. 2 and 3). In *Arabidopsis*, during the last step of petal senescence, abscission, the expression of *BREVIPEDICELLUS* (*BP*)/*KNAT1* is induced, and the accelerated petal abscission phenotype of its knockout mutant, *bp*, indicates that *BP/KNAT1* plays an antagonistic role in the petal abscission process (Shi et al., 2011). It has also been shown that in sunflower, increased expression of *HaHB-4* suppresses flower senescence (Manavella et al., 2006). These represent antagonistic mechanisms that may be important in preventing premature organ senescence.

#### ***RhPR10.1* Inhibits Ethylene-Induced Rose Flower Senescence through Regulation of CTK Signaling and Content**

Altered levels of CTKs and the expression of three CTK signaling pathway genes were observed in rose petals with reduced *RhPR10.1* expression (Fig. 4), suggesting that *RhPR10.1* may play a role in modulating CTK levels. Sequence alignment showed that *RhPR10.1* has a conserved P-loop domain, which functions as a nucleotide binding site correlated to RNase activity (Supplemental Fig. S3A; Liu and Ekramoddoullah, 2006). Three conserved residues related to ribonucleolytic activity of PR-10 proteins also existed in *RhPR10.1* protein, including Glu (E) at amino acid position 96, 148, and Tyr (Y) or His (H) at 150 (Supplemental Fig. S3A; Liu et al., 2006; Lebel et al., 2010). The two conserved features above suggest that *RhPR10.1* may have RNase activity. In addition, *RhPR10.1* belongs to the major pollen and food allergens subclass of the PR10 protein family (Supplemental Fig. S3B). It is known that PR-10 proteins from the major pollen and food allergens subclass acting as RNases can degrade certain types of tRNAs (Fernandes et al., 2013), which have been suggested to be a major source of CTK since isoprenoid CTKs have been identified in tRNA hydrolysates (Sakakibara, 2006). Moreover, although PR-10 protein has been reported to have capable of binding to trans-zeatin (Fernandes et al., 2008, 2009), here we found that the levels of trans-zeatin were undetectable in both TRV and *RhPR10.1*-silenced petals (Fig. 4C), suggesting that *RhPR10.1* might not interact with trans-zeatin during petal senescence.

It has been suggested that PR-10-mediated tRNA degradation may result in the accumulation of different types of CTKs, depending on plant species and environmental conditions (Srivastava et al., 2006, 2007). Ectopic expression of the pea PR-10 family gene *ABR17*

in both *B. napus* and *Arabidopsis* enhanced endogenous CTK levels (Srivastava et al., 2006, 2007), and in *ABR17*-overexpressing *B. napus* seedlings, a significant increase in trans-zeatin riboside, but not iP or cis-zeatin, was observed (Srivastava et al., 2006). However, in *ABR17*-overexpressed *Arabidopsis* seedlings, increases in the concentrations of cis-zeatin and iP were observed in both Murashige and Skoog medium- and soil-grown plants, while trans-zeatin content was only increased in Murashige and Skoog-grown plants (Srivastava et al., 2007). In this study, we detected a significant reduction in the levels of iPA, but not trans-zeatin or iP, in *RhPR10.1*-silenced flowers (Fig. 4C), suggesting that different pathways may function in CTK accumulation in rose petals as a consequence of tRNA degradation. In carnation flower, among *iso*-pentenyladenine derivatives, iPA was most abundant during flower senescence, and application of iPA delayed the onset of flower senescence (van Staden et al., 1990). It suggests that iPA may play an important role in flower senescence.

#### **RhHB6 Acts Upstream of *RhPR10.1* to Regulate Flower Senescence**

HD-Zip transcription factors have been grouped into four different classes (HD-Zip I–IV) based on sequence similarity criteria, and these groupings are further supported by their intron/exon patterns (Henriksson et al., 2005). Previous studies have reported that HD-Zip I transcription factors are involved in abiotic stress responses (Ariel et al., 2010), fruit ripening (Lin et al., 2008), and leaf and flower senescence (Manavella et al., 2006; Lü et al., 2014). Here we showed that *RhHB6* acts as a regulator of *RhPR10.1*, and that its expression was also up-regulated in senescent rose petals and involved in ethylene-induced flower senescence (Figs. 7 and 8). A regulatory action of *RhHB6/RhPR10.1* is suggested based on the observation that *RhHB6* binds to a 9-bp CAATTATTA region of the *RhPR10.1* promoter, as revealed by EMSA and yeast one-hybrid analysis (Fig. 6, A and B). In *Arabidopsis*, *ATHB6* recognized the same 9-bp cis-element present in the promoters of its target genes (Himmelbach et al., 2002). In addition, *ATHB6* specific targeting of this cis-element was revealed by the elimination of the interaction when a single nucleotide was mutated in this cis-element (Himmelbach et al., 2002). The effects of *RhHB6* silencing in rose (Fig. 8) are consistent with those of *RhPR10.1* silencing, in that both accelerated flower senescence (Fig. 2). We conclude that the ethylene-induced *RhHB6-RhPR10.1* regulatory module inhibits ethylene-induced flower senescence through increasing the levels of CTKs (Supplemental Fig. S7).

## **MATERIALS AND METHODS**

### **Plant Materials and Growth Conditions**

For expression pattern analyses of *RhPR10.1* and *RhHB6*, cut rose (*Rosa hybrida* ‘Samantha’) flowers were harvested from a local commercial greenhouse and transported to the laboratory within 1h. Stems were then recut under

water to 25 cm length and placed in vases with distilled water. The cut flower opening stages were defined as described previously (Ma et al., 2005). Floral organ samples were collected at different opening stages, and petal samples were collected from the same middle whorl of the flowers.

For hormone treatments, cut rose flowers at opening stage 2 were placed in vases containing 100  $\mu\text{M}$  6-BA, 100  $\mu\text{M}$  ABA, 80  $\mu\text{M}$  GA<sub>3</sub>, or 5  $\mu\text{M}$  brassinosteroid for 24 h. Mock samples were placed in 0.1% dimethyl sulfoxide. For ethylene treatment, the cut rose flowers at opening stage 2 were exposed to 10  $\mu\text{L L}^{-1}$  ethylene in an airtight chamber for 24 h. NaOH solution (1 M) was also applied in the chamber to prevent the CO<sub>2</sub> accumulation (Lü et al., 2014).

For the VIGS assay, rose plantlets were propagated by in vitro culturing. Rose shoots of at least 2 cm, and including one node, were cultured on propagation medium comprising Murashige and Skoog medium supplemented with 1.0 mg L<sup>-1</sup> 6-BA, 0.05 mg L<sup>-1</sup>  $\alpha$ -naphthalene acetic acid, and 3 mg L<sup>-1</sup> GA<sub>3</sub> for 30 d, before being transferred to rooting medium, comprising half-strength Murashige and Skoog supplemented with 0.1 mg L<sup>-1</sup>  $\alpha$ -naphthalene acetic acid for 30 d. Flower opening stages were defined as follows: stage 1, bud with completely opened sepal; stage 2, bud with outmost petal layer loosened; stage 3, flower with outmost petal layer opened; stage 4, flower with inner petal layer loosened; stage 5, fully opened flower; and stage 6, flower with faded color and, consequently, reduced ornamental value.

## Cloning, Plasmid Construction, and Plant Transformation

The full-length and promoter sequences of *RhHB6* and *RhPR10.1* were amplified using rapid amplification of cDNA ends (Clontech) and thermal asymmetric interlaced PCR (Takara) according to the manufacturer's instructions, respectively. All PCR products were subcloned into the pGEM-T easy vector (Promega) and then transformed into *Escherichia coli* DH5 $\alpha$  cells prior to sequencing.

The *RhHB6* VIGS construct was created by inserting a 365-bp fragment of the *RhHB6* 3'-untranslated region into the pTRV2 vector, and the *RhPR10.1* VIGS construct by inserting a 210-bp fragment (Supplemental Fig. S1) from the 3'-untranslated region into the pTRV2 vector.

To express the *RhHB6* recombinant protein in *E. coli* for the EMSA (see below for details), the open reading frame of *RhHB6* was inserted into the pGEM-4T-2 vector to produce the GST-*RhHB6* fusion protein.

For the yeast one-hybrid assay (see below), the open reading frame of *RhHB6* was cloned into the *EcoRI* and *XhoI* sites of the pJG4-5 vector (Clontech), resulting in the GAD-*RhHB6* construct. To generate a construct expressing the *LacZ* reporter gene driven by the wild-type or mutant motif of the *RhPR10.1* promoter, a 35-bp wild-type or mutant oligonucleotide was synthesized and ligated into the pLacZi2 $\mu$  vector (Lin et al., 2007). All primers used above are listed in Supplemental Table S1.

## RNA Extraction and qRT-PCR

Total RNA was extracted from rose floral organs as described previously (Xue et al., 2008). One microgram of DNase-treated RNA was used to synthesize cDNA in a 25- $\mu\text{L}$  reaction volume by Superscript Reverse Transcriptase (Invitrogen). qRT-PCR reactions were performed using 1  $\mu\text{L}$  cDNA as template and the Step One Plus real-time PCR system (Applied Biosystems) with the KAPA SYBR FAST quantitative PCR kit (Kapa Biosystems). *RhACT5* was used as an internal control (Pei et al., 2013).

## Sequence Analysis

Deduced amino acid sequences were aligned using ClustalX and DNAMAN, and phylogenetic analysis was performed using MEGA. The phylogenetic tree was computed using the Neighbor-Joining algorithm with 1,000 bootstrap replicates.

## VIGS

Silencing of *RhPR10.1* and *RhHB6* in petal discs and plantlets by VIGS was conducted as described previously (Ma et al., 2008; Dai et al., 2012; Tian et al., 2014), but with some modifications. The pTRV1, pTRV2, pTRV-*RhPR10.1*, and pTRV-*RhHB6* vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. The transformed *A. tumefaciens* lines were cultured in Luria-Bertani medium supplemented with 10 mM MES, 20 mM acetosyringone, 50  $\mu\text{g mL}^{-1}$  kanamycin, and 50  $\mu\text{g mL}^{-1}$  gentamycin sulfate. The cultures were harvested by centrifugation at 4,000 rpm for 10 min, and resuspended in infiltration buffer

(10 mM MgCl<sub>2</sub>, 200 mM acetosyringone, 10 mM MES, pH 5.6) to a final OD<sub>600</sub> of approximately 1.5. Mixtures of cultures containing an equal ratio (v/v) of pTRV1 and pTRV2, pTRV1 and pTRV-*RhPR10.1*, or pTRV1 and pTRV-*RhHB6*, were used as TRV control, TRV-*RhHB6*, and TRV-*RhPR10.1* experiments, respectively. The culture mixtures were placed at room temperature in the dark for 3 to 6 h before vacuum infiltration into the rose petal discs or plantlets.

For VIGS in rose petal discs, the petals were collected from the same middle whorl at stage 2 of flower opening, and 1-cm diameter discs were excised from the center of the petals using a hole punch. For VIGS in rose plantlets, plantlets were propagated by in vitro culturing as described above. The plantlets were washed in deionized water, and the roots were placed in water for 2 d to equilibrate. Vacuum infiltration was performed by immersing the discs or plantlets in the bacterial suspension and infiltrating under a vacuum at 0.7 MPa. After release of the vacuum, the discs or plantlets were washed in deionized water. Discs were placed in petri dishes, and plantlets were transplanted into a mixture of vermiculite and nutritive soil (1:1). The discs and plantlets were then placed in the dark at 8°C for 3 d. For RNA extraction, discs were kept in deionized water at 23°C until sampling. The phenotypes of the discs were observed daily until the onset of necrosis. Three independent experiments were performed with 40 petal discs in each experiment. The flowers of the rose plantlets were observed from stages 1 to 6. Three independent experiments were performed with 30 plantlets in each experiment.

## Extraction and Quantification of Endogenous CTKs

The extraction and quantification of endogenous CTKs was performed as previously described (Pan et al., 2010). Rose petals (100 mg) were frozen in liquid nitrogen, ground to a powder in a mortar, and transferred into 2-mL tubes. Extraction solvent (2-propanol/H<sub>2</sub>O/concentrated HCl [2:1:0.002, v/v/v]) was added to each tube, keeping the ratio of sample:solvent at 1:10 (mg  $\mu\text{L}^{-1}$ ). Samples were shaken at 100 rpm for 30 min at 4°C. One milliliter dichloromethane was added to each sample, and then samples were shaken at 100 rpm for 30 min at 4°C. After centrifugation at 13,000g for 5 min (4°C), the solvent (approximately 1.5 mL) from the lower phase was transferred into a new 2-mL tube and concentrated (not completely to dryness) using a concentrator (Eppendorf), and redissolved in 0.1 mL methanol. Quantitative analysis of endogenous CTKs in crude rose flower extracts was performed by high-performance liquid chromatography electrospray ionization tandem mass spectrometry.

## Measurement of Electrolyte Leakage Rates

Electrolyte leakage rates were measured as described previously (Lee et al., 2015), with minor modifications. Briefly, membrane leakage was determined by measurement of electrolytes leaking from rose petal discs. Sixteen rose petal discs from each treatment were immersed in 15 mL of 0.4 M mannitol at room temperature with gentle shaking for 3 h, and initial conductivity of the solution was measured with a conductivity meter (DDBJ-350; LeiCi). Total conductivity was determined after sample incubation at 85°C for 20 min. The electrolyte leakage rates were calculated as the percentage of initial conductivity divided by total conductivity.

## Yeast One-Hybrid Assay

The yeast one-hybrid assay was performed by transferring *LacZ* reporter gene constructs into yeast strain EGY48, as described in the Yeast Protocols Handbook (Clontech). Transformants were grown on synthetic dextrose plates lacking uracil and Trp, but containing X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) to observe the color development of yeast colonies.

## Purification of GST-*RhHB6* Recombinant Protein and EMSA

The EMSA was performed as described previously (Dai et al., 2012). Expression of the GST-*RhHB6* fusion protein was induced in 100-mL cultures of transformed *E. coli* BL21 cells (see above) by addition of isopropylthio- $\beta$ -galactoside to a final concentration of 0.2 mM, before the cultures were incubated at 20°C for 6 h. The recombinant proteins were extracted from the cells and purified using glutathione Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). EMSA was performed using a Light Shift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. Biotin-labeled DNA fragments (wild-type fragment in Fig. 6A) were synthesized and used as probes, while unlabeled DNA of the same sequence was used as a competitor.



## Accession Numbers

The GenBank (<http://www.ncbi.nlm.nih.gov>) accession numbers for rose genes used in this study are as follows: RhPR10.1 (KX462848) and RhHB6 (KX462849).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Alignment of seven rose PR-10 family cDNA sequences.

**Supplemental Figure S2.** qRT-PCR analysis of the expression of seven PR-10 family genes in rose petals at different flower opening stages.

**Supplemental Figure S3.** Alignment of deduced amino acid sequences (A) and a phylogenetic tree (B) of the RhPR10.1 protein and representative PR-10 members from other species.

**Supplemental Figure S4.** Flower phenotypes of wild-type and TRV control plantlets.

**Supplemental Figure S5.** A pictorial representation of the promoter region of *RhPR10.1* with potential cis-element binding sites.

**Supplemental Figure S6.** Transactivation activity of four HD-Zip transcription factors to the *RhPR10.1* promoter in yeast.

**Supplemental Figure S7.** Proposed model for how the RhHB6-RhPR10.1 module regulates ethylene-induced flower senescence.

**Supplemental Table S1.** Primer list.

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