Gibberellin and spermidine synergistically regulate polyamine metabolism during the development of *Rhododendron* flowers

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Abstract

Polyamines (PAs) are involved in various developmental processes, especially plant flowering. Their significant influences have been established; however, the exact mechanism by which PAs regulate flowering remains unclear. To explore PA metabolism in plant flowering, gibberellic acid (GA3, 0–2400 mg L−1) and spermidine (Spd, 0–1 mM) were applied alone or in combination during the early stage of flower bud formation in *Rhododendron simsii*. The application of GA3 alone advanced initial flowering, while that of Spd alone delayed initial flowering. Interestingly, GA3 and Spd applied in combination advanced initial flowering by 2 days. Furthermore, from stage 1 to 2, endogenous PA levels and the soluble conjugated and insoluble bound fractions of PAs and key enzymes (e.g., diamine oxidase, arginine decarboxylase, ornithine decarboxylase and S-adenosylmethionine decarboxylase) increased, and the level of PA oxidase decreased. These findings revealed that exogenous GA3 and Spd delay flower senescence by improving PA biosynthesis and preventing PA degradation. Moreover, exogenous GA3 and Spd enhanced the levels of endogenous PA and GA3, while the conversion of free PAs to soluble conjugated and insoluble bound forms delayed *Rhododendron* senescence. Overall, our findings reveal a potential positive feedback mechanism by which higher endogenous PA contents and the combined effects of exogenous GA3 and Spd synergistically delay *Rhododendron* senescence by enhancing PA biosynthesis and converting free PA to soluble conjugated and insoluble bound forms, thus reducing PA degradation during flower senescence.

Keywords Flowering dynamics · Exogenous gibberellin · Exogenous spermidine · Flower senescence · Polyamine metabolism

**Abbreviations**

ADC  Arginine decarboxylase  
Arg  Arginine  
DAO  Diamine oxidase  
GAs  Gibberellins  
ODC  Ornithine decarboxylase  
Orn  Ornithine  
PAO  Polyamine oxidase  
PAs  Polyamines  
PCA  Perchloric acid  
Put  Putrescine  
SAMDC  S-adenosylmethionine decarboxylase  
Spd  Spermidine  
SPDS  Spermidine synthase  
Spm  Spermine  
SPMS  Spermine synthase

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Introduction

*Rhododendron simsii* Planch. ‘Zichedian’ is widely used as an ornamental plant. This plant is frequently employed to attract tourists and as decoration in festivals; however, high sales of this variety are limited by the season, which reduces its potential benefits. Controlling flowering and delaying senescence are key strategies for planting rhododendrons. *Rhododendron* exhibits dormancy characteristics, and a low temperature is needed for plants to break dormancy and then flower (Black et al. 1990; Chang and Sung 2000). However, the global temperature increase is a potential risk to flowering at the appropriate time, as *Rhododendron* plants are highly sensitive to temperature variation (Yu et al. 2017). The vegetative cycle resumes in summer, with plants undergoing simultaneous leaf drop and bud break when the maximum temperature exceeds 10 °C; thereafter, the plant system is unresponsive to any reproductive stimuli (Choudhary et al. 2019). This dormancy mechanism is the main problem that limits *Rhododendron* production under global warming. Recent studies have shown that *Rhododendron* flowering/senescence is associated with the homeostasis of phytohormones, such as gibberellins (GAs), polyamines (PAs) and ethylene. For instance, Meijon et al. (2011) verified that high levels of free PAs and GAs were involved in cell division during the early stage of vegetative growth and flower bud development in *Rhododendron*. However, low-molecular-weight PA conjugates play crucial roles in floral bud differentiation and maturation processes and correlate with advanced flowering. Exogenous GA synthesis inhibitors change the levels of PAs and GAs, suggesting a significant role of GA in *Rhododendron* development (Meijon et al. 2011). Indeed, biochemical, transcriptomic and proteomic approaches have demonstrated that the genomic/proteomic profile of the respective genes/proteins associated with PAs during anthogenesis and flower development is correlated with the endogenous levels of PAs, GAs and ethylene (Liu et al. 2014; Alagna et al. 2016; Chen et al. 2016; Ning et al. 2019).

PAs are aliphatic nitrogen-containing compounds found in living cells. In plants, the most widely distributed PAs are the diamine putrescine (Put), the tetraamine spermine (Spm), and the triamine spermidine (Spd). PAs occur in free or covalently conjugated forms, and the latter can be divided into perchloric acid-soluble covalently conjugated PAs and perchloric acid-insoluble covalently conjugated PAs (Chen et al. 2018). Among these compounds, Put is synthesized via the arginine decarboxylase (ADC) pathway and the ornithine decarboxylase (ODC) pathway. Spd and Spm are generated by Spd synthase (SPDS) and Spm synthase (SPMS), respectively (Moschou et al. 2008; Ahou et al. 2014; Majumdar et al. 2016). PA degradation is catalyzed by diamine oxidase (DAO) and PA oxidase (PAO) (Wang et al. 2019). Moreover, due to the polycationic nature of PAs, they easily interact with negatively charged sites in molecules (Masson et al. 2017), which result in diverse functions, especially during flowering (Malmberg and Mcindoo 1983; Tiburcio et al. 1988; Qin et al. 2019; Kaur-Sawhney et al. 1980; Sobieszczuk-Nowicka 2017). Indeed, previous studies have demonstrated their importance in the flowering process (Wada et al. 1994; Bagni and Tassoni 2006; Sood and Nagar 2004). Previous studies have indicated that the accumulation of PAs is an adaptive mechanism during flower development and that PA dynamics are specific and complex. Based on the above background, our objective was to characterize the PA components (Put, Spd, and Spm) and forms (free, soluble conjugated and insoluble bound forms) during *Rhododendron* flowering by applying exogenous gibberellic acid (GA₃) and Spd to further clarify the relationship between polyamine metabolism and *Rhododendron* flowering.

Materials and methods

In mid-October, 3-year-old cuttings from the same batch of *Rhododendron simsii* Planch. ‘Zichedian’ plants were supplied by the Institute of Horticulture at Sichuan Academy of Agricultural Sciences, Chengdu, Sichuan, China. Uniform cuttings (with an average height of 32.7 cm and an average crown size of 26.7 cm and without infection) were removed from the plastic bag they were delivered in, and the mature leaves were removed. The roots of the cuttings were then rinsed with clean water and placed in plastic nutrient bags. Regarding the basic soil properties of the peat soil used, the contents of alkali-hydrolysable nitrogen, available phosphorus, and quick-release potassium of the peat soil (pH 4.85, electrical conductivity [EC] 0.82~1.02 dS m⁻¹) were 212.46 mg L⁻¹, 97.35 mg L⁻¹, and 92.64 mg L⁻¹, respectively, and the organic matter content was 11.2 g L⁻¹. The pots were then placed in the greenhouse at the Chengdu Experimental Station at Sichuan Agricultural University (536 m above sea level, 30° 71’ N, 103° 86’ E). All plants were grown under 70–80% relative air humidity and an average air temperature of 25 ± 3 °C and 9 ± 2 °C during the day and night, respectively. Distilled water was supplied at 9:00 a.m. every 3 days (500 mL each time), no fertilizer was applied during the experiment, and the plants were treated after their growth had resumed.

The plants ready for treatment were divided into eight groups and treated with (1) distilled water, for the control group; (2) 800 mg L⁻¹ GA₃; (3) 1 600 mg L⁻¹ GA₃; (4) 2400 mg L⁻¹ GA₃; (5) 0.01 mM Spd; (6) 0.10 mM Spd; (7)
1.00 mM Spd; or (8) 2400 mg L⁻¹ GA₃ + 0.10 mM Spd. The plants were sprayed during the initial stage of flower bud morphological differentiation, with spraying initiated on December 25, 2016. The leaves were sprayed 3 times every 7 days. There were four pots in each group, of which 3 pots were used per replication. Flowers were collected during the main flowering period from May to June. Flowering was observed at 9:00 AM each day, and the stage (1–4) was recorded, as shown in Fig. 1.

**PA analysis**

PA extraction followed the protocol of Hu et al. (2012) with minor modification. Briefly, 0.5 g of fresh petals was weighed, homogenized by the addition of 3.2 mL of 5% (v/v) cold perchloric acid (PCA) in an ice bath, and then incubated at 4 °C for 1 h. The homogenate was subsequently centrifuged at 12,000×g for 30 min (4 °C), and the pellet was used to measure the insoluble bound PAs after two washes with cold PCA. Then, the supernatant was assayed for free and soluble conjugated PAs, as described in the Supplementary Materials (Methods S1).

**Quantification of endogenous GAs**

Endogenous GAs were measured according to the protocol of Pan et al. (2010). A total of 0.5 g of fresh petals was ground to a fine powder, and 5 mL of extractant (2:1:0.002 v/v/v 2-propanol/water/concentrated HCl) was then added, after which the mixture was shaken for 30 min (4 °C). A total of 1 mL of dichloromethane was added to each sample, which was then shaken for 30 min at 4 °C. The samples were subsequently centrifuged at 13,000×g for 4 min at 4 °C, and the lower phase was collected. Two drops of concentrated ammonia were added at 35 °C until near dryness occurred, after which the sample was redissolved in 0.1 mL of methanol. The sample solution was analyzed by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS).

**Activity of PA biosynthesis enzymes**

A 1.0 g fresh tissue sample and 3.2 mL of potassium phosphate buffer (pH 8.0), which contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 1 mM pyridoxal phosphate (PLP), 5 mM EDTA, 25 mM ascorbic acid (VC) and 0.1% polyvinylpyrrolidone (PVP), were mixed together. The samples were ground in an ice bath and then centrifuged at 12,000×g for 40 min at 4 °C, after which the supernatant and ammonium sulfate were mixed together to a saturation of 40% and subsequently centrifuged at 12,000×g for 15 min at 4 °C. The supernatant and ammonium sulfate were mixed together to a saturation of 60%, incubated at room temperature for 30 min and then centrifuged at 12,000×g for 20 min at 4 °C. The precipitate was then suspended in 3 mL of 100 mM potassium phosphate buffer (pH 8.0; containing 1 mM DTT, 0.1 mM EDTA, and 0.05 mM PLP) and dialyzed at 4 °C for 24 h. The enzyme activity was measured according to the protocol of Zhao et al. (2003) and measured as described in the Supplementary Materials (Methods S1).

**Fig. 1** Four stages of the flowering period. The flower opening of *Rhododendron* was divided into four stages. In stage 1, the squaring stage, 30% of buds display color, and most are tightly closed. In stage 2, the early flowering stage, there are a small number of initial flowers, most of which are still in a semiclosed state (1 cm < flower diameter ≤ 3.5 cm, 2.5 cm < stamen length ≤ 3.0 cm). Stage 3, the blooming stage, is characterized by more than 50% open flowers and flowers presenting a deep color (3.5 cm < flower diameter, 3.5 cm < stamen length ≤ 4.0 cm). In stage 4, the end of flowering, the flower and flower stalk are easy to distinguish, the color is pale, and the flowers appear withered and have begun to fall (3.0 cm < stamen length ≤ 3.5 cm). The dates on which the plants entered the four stages were recorded.
DAO and PAO activity assays

The activities of PAO and DAO were determined according to the previous protocol of Su et al. (2005). A total of 0.5 g of fresh petals was ground in the presence of 1 mL of 0.1 M potassium phosphate buffer (pH of 6.5) and then centrifuged at 10,000×g for 20 min at 4 °C. The resulting supernatant was analyzed for enzyme activity, which was measured as described in the Supplementary Materials (Methods S1).

Statistical analysis

The entire experiment was repeated three times, and the results presented are the averages of three replicates. Differences between treatments were determined by one-way analysis of variance (ANOVA) and Duncan’s test for multiple comparisons. Linear regression was used to evaluate the relationships among GA3, PAs and Rhododendron flowering.

Results

Flowering time

Figure 2 indicates the changes in flowering time after treatment with GA3/Spd. Exogenous GA3 application advanced initial flowering, whereas initial flowering was delayed in the Spd treatments. In detail, with 2400 mg L−1 GA3 application, initial flowering occurred 6 days earlier than that in the control treatment. In contrast, the initial flowering was delayed in Spd-treated plants, by 7~12 days. Interestingly, when 2400 mg L−1 GA3 was applied in combination with 0.1 mM Spd, initial flowering occurred only 2 days earlier than that in the control treatment. Moreover, all applications prolonged the flowering lifespan, with flowering time increasing to a greater extent with Spd application. However, when GA3 was applied in combination with Spd, flowering time was prolonged by only 1 day with respect to the control time.

GA3 levels

GA3 levels changed appreciably over the course of flower senescence (Fig. 6D). The level of GA3 demonstrated an upward trend until peaking at stage 3, decreasing thereafter. Applications of different concentrations of GA3 and Spd to flowers resulted in increases in GA3. The initial gradual increase was followed by a sharp increase at stage 3 and then a rapid decline. Moreover, the highest GA3 level was recorded in response to GA3 + Spd application at all stages, and treatment with GA3 resulted in a greater accumulation of endogenous GA3 than did Spd application.

PA levels

As shown in Figs. 3, 4 and 5A–C, flower senescence was accompanied by a decrease in free form PA from stage 1 to stage 2 and an increase thereafter. A steady increase in soluble conjugated and insoluble bound PAs was recorded during flower senescence. In addition, the three forms of PAs increased with GA3/Spd application to different extents. In detail, at stage 1, compared with the level under control treatment, the free Put content increased 1.02~1.45-fold, 0.82~1.31-fold and 1.37-fold with GA3, Spd and GA3 + Spd application, respectively. All of the soluble conjugated and insoluble bound PA contents and fractions except the insoluble bound fraction of Spm increased under all applications. During stages 3 and 4, the three forms of PA contents increased under all applications. The free fraction of PAs decreased, but the free fraction of Spm increased. The soluble conjugated fraction of PAs increased with GA3/Spd at stage 3. Intriguingly, GA3 application decreased the soluble conjugated fraction of Put, which increased with Spd and GA3 + Spd application at stage 4. The opposite behavior was observed for the soluble...
Fig. 3 Effects of GA₃ and Spd on the levels of free Put (A), Spd (B) and Spm (C) in petals during the flowering of Rhododendron. Vertical bars represent the SDs of the mean (n = 3). Bars within each stage of flower opening with different treatments labeled with the same letters are not significantly different according to Duncan's multiple test (P = 0.05); the same below.
Fig. 4 Effects of GA3 and Spd on the levels of soluble conjugated Put (A), Spd (B) and Spm (C) in petals during the flowering of Rhododendron.
conjugated fraction of Spm. Regarding the insoluble bound form, all applications increased the free fraction of Put but decreased the free fractions of Spd and Spm (Fig. 6 A–C).

**PA biosynthetic/degradative enzymes**

The ODC and S-adenosylmethionine decarboxylase (SAMDC) activities increased during *Rhododendron* flowering and peaked at stage 3. Dose-dependent elevation of ODC and SAMDC activities was observed with the application of GA$_3$/Spd. Exogenous Spd led to higher and more persistent levels of ODC and SAMDC activities than GA$_3$ (Fig. 7B–C). The ADC activity declined from stage 1 to stage 3 and then increased (Fig. 7A). The activity of ADC increased with exogenous application of GA$_3$/Spd, and the highest ADC activity was found with the application of 1600 mg L$^{-1}$ GA$_3$ and 0.01 mM Spd during *Rhododendron* flowering, except for stage 2. Moreover, the highest ADC, ODC and SAMDC activities were observed with 2400 mg L$^{-1}$ GA$_3$ + 0.1 mM Spd application at stages 1 and 2.

During *Rhododendron* flowering, DAO and PAO activities increased gradually, and PAO activity peaked at stage 3 (Fig. 8A, B). A decline in DAO activity was observed with the application of GA$_3$/Spd from stage 1 to stage 2. A rapid increase in PAO activity was observed with the application of GA$_3$/Spd from stage 1 to stage 3. Moreover, the highest DAO and PAO activities were observed with 0.1 mM Spd application at stage 3 and 2400 mg L$^{-1}$ GA$_3$ + 0.1 mM Spd application at stage 4, respectively.

**Relationships of GA$_3$ and PA contents with flowering time**

Regression analysis showed that flowering time was significantly positively correlated with soluble conjugated Put, Spd and Spm, insoluble bound Put, Spd and Spm, fractions of soluble conjugated Put and Spm, and insoluble bound Put, Spd and Spm ($R^2 = 0.391–0.678, p = 0.01$; Table 1). The correlations of flowering time with the fractions of free Put, Spd and Spm were significantly negative ($R^2 = –0.462$ to $–0.621, p = 0.01$), while those between flowering time and the contents of GA$_3$, free Put, Spd, and Spm and the fraction of soluble conjugated Spd ($p > 0.05$) were nonsignificant. Notably, flowering time was not significantly correlated with free the PA content but was significantly negatively correlated with the free PA fractions, indicating that PAs are strongly related to plant flowering.

**Discussion**

We found that *Rhododendron* flowering involves PA metabolism. GA$_3$ and Spd promote PA biosynthesis and prevent PA degradation by promoting the synthesis of PA-related enzymes and then regulate PA components (Put, Spd, and Spm) and forms (free, soluble conjugated and insoluble bound forms). However, the explanation for the results is not clear; although GA$_3$ and Spd may synergistically regulate PA metabolism during the development of *Rhododendron* flowers, other factors may also affect PA metabolism.

**PA metabolism is involved in Rhododendron flowering**

Flowering is a complex process and is regulated by many factors. PA metabolism is a key factor affecting flowering (Rey et al. 2006; Naseri et al. 2019). Usually, floral development is accompanied by dynamic changes in the components and forms of endogenous PAs, which modulate intricate networks of signaling events that control the flowering program.
Fig. 5  Effects of GA$_3$ and Spd on the levels of insoluble bound Put (A), Spd (B) and Spm (C) in petals during the flowering of *Rhododendron*
In our study, free PAs decreased from stage 1 to stage 2 and increased thereafter. The free Put and Spd levels at stage 4 were almost equal to those at stage 1 (Fig. 3A–C). Moreover, steady increases in soluble conjugated and insoluble bound PAs were recorded (Figs. 4 and 5A–C); similar behaviors have been described in *Rosa* flowering (Sood and Nagar 2004). Regarding findings in other species, increases in free and soluble conjugated Spd and Put were observed following exogenous Spd application during carnation flowering (Bagni and Tassoni 2006), and concentrations of endogenous Spm and Spd did not change with flower senescence (Huang et al. 2004). These results indicate that the changes in PAs differ depending on the treatment and plant species. The decline in the free fraction could be due to conversion of the free form to the soluble conjugated form at early stages (Figs. 3 and 4A–C) and/or oxidation by PAO (Cvikrová et al. 2013). Conjugation of PA might be a regulatory mechanism that controls the PA level within a nontoxic range for plant survival (Alcázar et al. 2005). The concomitant increases in soluble conjugated and insoluble bound PAs may be attributed to PA degradation (Fig. 8A–B) and increases in their fractions (Fig. 6A–C) as well as a decrease in free PA levels and an increase in the conjugated pool associated with the initiation of cell expansion (Altamura et al. 1993).

One of the interesting findings of this study was the lack of a significant relationship between flowering time and free PA content, whereas the fraction of free PAs was significantly negatively correlated with flowering time (Table 1). Data suggest that the PA ratio plays an important role in *Rhododendron* flowering (Hura et al. 2015). Moreover, significant positive correlations between flowering time and soluble conjugated PA contents, insoluble bound PA contents, soluble conjugated Put and Spm fractions, and insoluble bound PA fractions were observed. The dynamics of PA components and forms indicated that PAs have significant physiological functions during *Rhododendron* flower development.

**GA3 and Spd regulate PA metabolism**

The homeostatic regulation of cellular PA levels is a dynamic balance of biosynthesis and catabolism and is important for plant development (Majumdar et al. 2016; Yu et al. 2019). In the PA biosynthetic pathway, ornithine or arginine is decarboxylated by ODC or ADC to form Put.
Fig. 7 Effects of GA$_3$ and Spd on the activities of ADC (A), ODC (B) and SAMDC (C) in Rhododendron petals during the flowering process.
In general, the highest levels of endogenous PAs and PA synthetase activity occur in the meristem and growing cells, and the lowest occur in senescent tissues (Chen et al. 2018). The elevated activities of ODC, ADC and SAMDC were responses to the elevation of PA level. In our study, after spraying with GA$_3$/Spd, the PA levels increased. In general, the elevation followed the order GA$_3$ + Spd > Spd > GA$_3$ (Fig. 7A–C) during stages 1 to 3. ODC and SAMDC activity increased following GA$_3$/Spd application, and the pattern of change was consistent with the levels of certain PAs during stage 1 to stage 3. The main reasons for this result may be the permeation of exogenous Spd, the synthesis of new Spd and the increases MdADC1 and MdODC1 expression and MdSAMDC2 transcription (Qin et al. 2019). Another direct cause may be the effect of GA$_3$.

The PA degradative pathway also plays a signaling role in developmental processes (Qin et al. 2019). In this study, despite the rapid increases in PAO and DAO activities and the gradual increase in ODC and SAMDC activities from stage 1 to stage 2 (Figs. 7B–C and 8A–B), little free Put accumulated (Fig. 3A), which was due to the decrease in ADC and the conversion of free Put to conjugated and
bound Put and free Spd and Spm (Figs. 7 A, 3, 4 and 5A–C) (Ndayiragije 2006). Moreover, the DAO and PAO activities increased during stages 1 to 3 (Fig. 8A–B), which resulted in free PAs decreasing at the initial flowering stage and soluble conjugated and insoluble bound PAs increasing throughout flowering (Figs. 3, 4 and 5A–C). The DAO and PAO activities could accelerate the conversion of free PAs to soluble conjugated PAs and insoluble bound PAs (Wang et al. 2019). During stages 3 and 4, the contents of soluble conjugated PAs and insoluble bound PAs were significantly increased by GA3 and Spd applications (Figs. 4 and 5A–C), which may have contributed to the longer flower lifespan (Fig. 2). PA catabolism induces the accumulation of hydrogen peroxide (H2O2) and cytotoxic products, which is considered a possible mechanism of PA association with programmed cell death (PCD) (Yoda et al. 2006; Del Duca et al. 2014; Cai et al. 2015a, b). The highest DAO/PAO activities were observed with 0.1 mM Spd application at stage 3, which resulted in the longest flower lifespan (Figs. 8A–B, 2). When PAs are catabolized, an oxidized product of H2O2 appears; it acts as a signaling compound that activates the signaling pathway and contributes to delayed senescence (Moschou et al. 2008). However, the highest PA levels and DAO/PAO activities were observed with GA3 plus Spd application at stage 4, resulting in only a 1 day increase in the flower lifespan (Figs. 8A–B, 2). This result indicated that when PA–derived H2O2 is not quenched properly, the process may lead to PCD rather than delayed senescence (Moschou et al. 2008). Moreover, the flowering time was longer with Spd application than with GA3 application (Fig. 2). Based on these results, one can infer that Spd directly participated in PA metabolism and that GA3 delayed flower senescence by modulating PA metabolism via Spd.

Our results collectively demonstrate that GA3 and Spd applications enhance the activities of PA biosynthetic enzymes and endogenous PA accumulation. Moreover, the increase in PA levels can cause endogenous GA accumulation. These results suggest that GA3 and Spd are positively correlated with PA concentration. The PA concentration is regulated using a positive feedback mechanism.

Conclusions

In this work, moderate GA3 and Spd concentrations altered the initial flowering time and delayed flower senescence in Rhododendron. GA3 application advanced the squaring stage, and Spd application produced the opposite result. We propose that a potential positive feedback mechanism may be activated, resulting from the increased endogenous PA levels due to the combined effects of exogenous GA3 and Spd application. The results suggest that exogenous GA3 and Spd play significant roles in the delay of flower senescence in Rhododendron by improving PA biosynthesis and preventing PA degradation. Exogenous GA3 and Spd enhanced PA and GA3 concentrations, and the conversion of free PAs to soluble conjugated and insoluble bound forms delayed Rhododendron senescence.

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Declarations

Conflict of interest No potential conflicts of interest were reported by the authors.

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