Gibberellin and spermidine synergistically regulate polyamine metabolism during Rhododendron flower development

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Abstract

Polyamines (PAs) are involved in various plants developmental processes, especially in flowering. Their significant influence has been established, but the exact mechanisms by which PAs modulate flowering are not yet understood. To understand the PA metabolism during flowering/senescence in *Rhododendron simsii* 'Zichendian' plants, exogenous gibberellin (GA_3, 0-2400 mg L^{-1}) and spermidine (Spd, 0–1 mM) were applied separately or in combination during the early stage of flower bud formation. The application of GA_3 alone advanced the initial flowering by promoting the free Put (F) fraction and decreasing the Spd/Put ratio at the squaring stage, whereas Spd alone delayed the initial flowering by increasing the soluble conjugated (C) form, insoluble bound Put (B) fraction and Spd/Put ratio. When GA_3 plus Spd was applied, the initial flowering advanced by 2 days. Furthermore, endogenous PA levels as well as the C and B fractions of PAs and key enzymes (diamine oxidase, PA oxidase, arginine decarboxylase, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase) of PA metabolism increased, while the Spd/Spm ratio decreased by GA_3 and Spd applications during flowering, resulting in delayed flower senescence. In addition, the structural equation model (SEM) showed that Spd directly participated in PA metabolism, while GA_3 regulated flowering by modulating PA metabolism via Spd (c.f. 0.27). Taken together, our study provides comprehensive evidence regarding the clear relationships between GA_3, Spd and flowering time, supporting the positive effect of PA metabolism on delaying flower senescence, and helps to provide a thorough understanding of the PA interconversions, biosynthesis and catabolism during flowering and senescence.

Introduction

Rhododendron simsii Planch. 'Zichendian', one of the most widely used ornamental plants within gardens, is popular due to its abundance of blooms, brightly colored flowers and ease of propagation. Under natural conditions, the 'Zichendian' cultivar blooms from April to May. As this plant is mainly used for attracting tourists and for festivals, the popular market for the sale of this cultivar is restricted to season, which decreases its potential economic benefits. Controlling flowering and delaying senescence are key strategies for planting *Rhododendron*. Therefore, there have been recent attempts at studying *Rhododendron* flowering characteristics. *Rhododendron* flowering and senescence are associated with phytohormone homeostasis, such as GAs, ethylene and polyamines (PAs). Meijon et al. (2011) verified that high levels of free PAs and GA are involved in cell division during the early stage of vegetative growth and flower bud development in *Rhododendron*. However, low-molecular-weight PA conjugates play a crucial role in floral bud differentiation and maturation processes and correlate with advanced stages of flowering. The use of GA synthesis inhibitors changes the level of PAs and GA, which indicates the significance of GA in *Rhododendron* development (Meijon et al. 2011). Nevertheless, the role of ethylene in the senescence of *Rhododendron* is unclear. The expression levels of the ethylene pathway-associated genes *MYC2*, *TIR1*, *CYCD3*, *COL-1*, and *EIN3* peaked at the *R. pulchrum* flower bud stage, indicating the essential role of ethylene in *Rhododendron* development (Wang et al. 2018). *Rhododendron* with dormancy characteristics needs a low temperature to break dormancy and then flower. However, the

global temperature increase is a potential risk to flowering at the appropriate time, as these plants are highly sensitive to temperature variation and vulnerable to increasing temperature (Yu et al. 2017). The vegetative cycle resumes in summer, exhibiting a simultaneous leaf drop and bud break when the temperature exceeds 10 °C, and the plant system is then unresponsive to any reproductive stimuli (Choudhary et al. 2019). Thus, plant growth regulators are crucial for regulating *Rhododendron* flowering.

PAs are aliphatic nitrogen-containing compounds found in all living cells. In plants, the most widely distributed PAs are diamine putrescine (Put), tetraamine spermine (Spm), and triamine spermidine (Spd). In addition, cadaverine (Cad) and thermospermine (tSpm) are also found in higher plants (Handa and Mattoo 2010a; Takahashi 2012). Three major PAs exist as soluble conjugated forms or free soluble forms (Evans and Malmberg 1989). Among these, Put is synthesized via the arginine decarboxylase (ADC) pathway and the ornithine decarboxylase (ODC) pathway. Spd and Spm are generated by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively (Ahou et al. 2014; Majumdar et al. 2016; Moschou et al. 2008). 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 such as nucleic acids, proteins, and lipids (Masson et al. 2017). Hence, they have diverse functions, especially for plant growth and development. PAs impact in vitro shoot regeneration (Nölke et al. 2010), embryogenesis (Baron and Stasolla 2008), root development (Hummel et al. 2002), pollen tube germination (Sorkheh et al. 2011), flowering (Malmberg and Mcindoo 1983; Qin et al. 2019; Tiburcio et al. 1988), fruit ripening (Fortes and Agudelo-Romero 2018)(Fortes et al., 2018) and leaf and flower senescence (Kaur-Sawhney et al. 1980; Sobieszczuk-Nowicka 2017). Biochemical, transcriptome and proteome approaches have demonstrated that the genomic/proteomic profile of the respective genes/proteins associated with PAs during anthogenesis and flower tissue development is correlated with the endogenous levels of PAs, GA and ethylene during flower development. In addition, previous studies have demonstrated the importance of PAs in the flowering process; for example, Put alone can induce flowering of morning glory in the absence of proper induction conditions (Wada et al. 1994). The accumulation of endogenous hormones in flower buds and in vegetative buds exhibits a specific distribution pattern. However, the dynamic changes in PAs differ depending on treatment and plant species. Bagni and Tassoni (2006) reported that the levels of F and C Spd and Put significantly increased in response to their direct application to carnation petals by spraying, whereas the endogenous PA levels did not vary when the PAs were supplied through the vase water. C Put, Spd and Spm increased steadily throughout the entire flowering time of rosa, and the B Spm and F Spd were found to be the highest during the blooming stage in *Rosa damascene* and *Rosa bourboniana*, respectively (Sood and Nagar 2004). Increases in cadaverine and Spd levels in bulbs are a characteristic of flower development in tuberose, and Spd is maintained at a high level during flower development, indicating that Spd participates in flowering and floral development. During carnation senescence, the concentrations of endogenous Spm and Spd do not change with flower senescence, while the concentration of Put rapidly increases (Huang et al. 2004). These studies indicate that the accumulation of PAs is an adaptive mechanism during both flower development and senescence and that PA dynamics are specific and complex.

Gibberellic acid, a dormancy-breaking chemical, has been used for flower initiation and development (Zhao et al. 2009). PAs are also associated with flower bud dormancy and regulate flowering (Naseri et al. 2019; Rey et al. 1994), both activities that have been characterized as modulating PA metabolism, as evidenced by changes in PA concentrations with exogenous applications of GA_3 and Spm during Anthurium senescence (Simões et al. 2018). Dynamic changes in PA levels are considered a response to flower development and senescence. However, the regulation of PA homeostasis is complex and includes biosynthesis, inter-conversion, catabolism and conjugation (Majumdar et al. 2016). The endogenous PAs and GA_3 levels are exogenously altered, whereas the level of endogenous PAs could be converted into GA_3 quickly, and the enzymes involved in biosynthesis and catabolism are also affected by PA/GA_3 application. Due to the role of GA_3 and some PAs in prolonging flowering time, our aim is to characterize PA components and forms (free soluble, insoluble binding and soluble forms) in the flowering and senescence processes of *Rhododendron* to improve our understanding of the potential relationship between GA_3, Spd and flowering. Based on the above background, the following hypotheses were provided in this work: (i) GA_3 and Spd regulate PA interconversions during *Rhododendron* flowering, such as PA components and forms. (ii) GA_3 and Spd regulate PA biosynthesis/catabolism by promoting/inhibiting the synthesis and decomposition of PAs and related enzyme activities. (iii) GA_3 and Spd regulate PA metabolism, and flower senescence is involved in PA metabolism.

Materials And Methods

In mid-October, three-year-old cuttings from the same batch of *Rhododendron* (*Rhododendron simsii* Planch. ‘Zichendian’) were supplied by the Institute of Horticulture, Sichuan Academy of Agricultural Sciences. Uniform cuttings (average height of 32.7 cm, average crown size of 26.7 cm, and free of infection) were removed from the plastic bag, and the mature leaves were removed. The roots of the cuttings were then rinsed with clean water and then placed in plastic nutrient bags. With respect to the basic soil properties of the peat soil used, the contents of alkali-hydrolyzable nitrogen, available phosphorus, and quick-release potassium of the peat soil (pH of 4.85, electrical conductivity [EC] of 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 of the Chengdu Experimental Station of Sichuan Agricultural University (536 m above sea level, 30°71’N, 103°86’E). All plants were grown with 70–80% relative air humidity and 25 ± 3 °C and 9 ± 2 °C average air temperature during the day and night, respectively. Distilled water was supplied at 9:00 a.m. every 3 days (a total of 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 [control]; (2) 800 mg·L^{-1} GA_3 [T1]; (3) 1600 mg·L^{-1} GA_3 [T2]; (4) 2400 mg·L^{-1} GA_3 [T3]; (5) 0.01 mM Spd [T4]; (6) 0.10 mM Spd [T5]; or (7) 1.00 mM Spd [T6]; and (8) 2400 mg·L^{-1} GA_3 + 0.10 mM Spd [T7]. The plants were sprayed during the initial stage of flower bud morphological differentiation

beginning 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. Flower samples were collected during the main flowering period (May-June). The flowering process of the plants was observed and recorded, and the flowering period was divided into 4 stages, as shown in Fig. 1.

PA Analysis

The PA extraction method was a modified version of the method of Hu et al. (2012) A total of 0.5 g of fresh petals was weighed and homogenized by the addition of 3.2 mL of 5% (v/v) $HClO_4$ in an ice bath followed by incubation 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 for measuring the insoluble bound PAs. Then, the supernatant was assayed for free and soluble conjugated PAs, as described in the supplementary materials (Methods S1).

Extraction and Quantification of Endogenous GAs

Endogenous GAs were measured according to the method 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 total of 1.0 g of fresh sample tissue 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 method of Zhao et al. (2003) and measured as described in the supplementary materials (Methods S1).

DAO and PAO Activity Assays

The activities of PAO and DAO were determined according to the 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, after which the supernatant was sampled 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. A structural equation model (SEM) was used to explain the influence of related components and types on PA metabolism. Structural equation modeling is a multivariate statistical method that is used for model identification, estimation, and verification of various causal models and clearly gives the influence of each relationship. However, this method requires a previous model that is based on basic theory or practical experience (Doncaster 2007). Linear regression was used to evaluate the relationships between GA_3, PAs and *Rhododendron* flowering.

To ensure that the model operation results are reasonable and reliable, it is usually required that the sample multivariate normality test index value be less than 1.96. AMOS is a statistical software package for structural equation modeling that could not only validate various measurement models and different path analysis models but also optimize multigroup analysis and single-group/multigroup competition models. Moreover, AMOS provides a graphical representation of model diagrams and can quickly draw various hypothetical model diagrams. Moreover, the output report data are easy to interpret. Therefore, the model analysis process was carried out via AMOS 21.0 software.

Results

Flowering Time of Rhododendron

Table 1 illustrates the changes in flowering time after treatment with GA_3/Spd applications. Exogenous GA_3 applications advanced the initial flowering of *Rhododendron* but delayed flowering in the Spd treatments (Table 1). In detail, the initial flowering with 1 600 mg·L^{-1} GA_3 application occurred 6 days earlier than that in the control. Conversely, the initial flowering time was delayed in Spd-treated plants. For example, with Spd applications, the initial flowering time was delayed 7 ~ 12 days. Interestingly, when 1 600 mg·L^{-1} GA_3 was combined with 0.1 mM Spd, the initial flowering occurred just 2 days earlier than it did in the control solution. Moreover, all applications prolonged the flowering lifespan, and the flowering time rose to a greater extent with Spd applications. However, when GA_3 was combined with Spd, the flowering time was only prolonged 1 day compared with that of the control.

Table 1
Effects of GA_3 and Spd on the flowering time of *Rhododendron*

Treatment	Squaring stage (Day/month)	Early flowering stage (Day/month)	Blooming stage (Day/month)	End of flowering (Day/month)	Days of blossoming (Day)
CK	7/5	10/5	12/5	15/5	8
T1	3/5	7/5	10/5	14/5	11
T2	1/5	6/5	13/5	16/5	15
T3	2/5	6/5	10/5	14/5	12
T4	10/5	14/5	19/5	23/5	13
T5	15/5	21/5	29/5	3/6	19
T6	12/5	17/5	21/5	27/5	15
T7	5/5	9/5	11/5	14/5	9

GA_3 Levels

In the current work, GA_3 levels changed appreciably during flower senescence (Fig. 2D). The level of GA_3 demonstrated an upward trend and peaked at stage 3 but then decreased. Applications of flowers with different GA_3 and Spd concentrations resulted in an increase in GA_3. This gradual increase was followed by a drastic rise at stage 3 and then declined rapidly. Moreover, the highest GA_3 level was recorded in response to the GA_3 plus Spd application at all times, and the treatment with GA_3 resulted in a greater accumulation of endogenous GA_3 with respect to the Spd applications.

PA Levels

The PA levels in different forms and their ratios with GA_3 and Spd applications were determined. In general, flower senescence was accompanied by a decrease in the free (F) form from stage 1 to stage 2 and increased thereafter. A steady increase in conjugated (C) and bound (B) form PAs was recorded during flower senescence (Fig. 2A-C). In addition, three PAs increased with all applications to different extents. In detail, at stage 1, compared with the control, F Put content rose 1.02 ~ 1.45-, 0.82 ~ 1.31- and 1.37-fold with GA_3, Spd and GA_3 + Spd applications, respectively. The F fraction of Put rose 0.89 ~ 1.03-fold with GA_3 but decreased 0.81 ~ 0.89- and 0.85-fold with Spd and GA_3 + Spd applications, respectively. Moreover, all applications increased F Spd and Spm content, increased the F fraction of Spm, but decreased the F fraction of Spd. C PA contents increased with GA_3 and Spd to different extents. In general, the increase in C PA content was as follows: GA_3 + Spd□Spd□GA_3. All applications increased the C fraction of Put and decreased the C fraction of Spm, while the C fraction of Spd decreased 0.95 ~ 0.98-fold with GA_3 but rose 0.97 ~ 1.04- and 1.02-fold with Spd and GA_3 + Spd applications, respectively. In the

case of the B form, GA_3 and Spd increased the B PA contents and B fractions of Put and Spd, but decreased the B fraction of Spm; During stages 3 and 4, three forms of PA contents increased with GA_3 and Spd applications. Both GA_3 and Spd decreased the F fractions of Put and Spd but increased the F fraction of Spm. The C fractions of Put, Spd and Spm increased with all applications at stage 3, except for 0.1 ~ 1 mM Spd and GA_3 + Spd applications. Intriguingly, GA_3 decreased the C fraction of Put, which increased with Spd and GA_3 + Spd applications at stage 4. The opposite behavior observed for the C fraction of Spm. Regarding the B form, all applications increased the F fraction of Put, but decreased the F fractions of Spd and Spm.

In the current work, various GA_3 and Spd concentrations affected PA ratios. In detail, at stage 1, the Spd/Put ratio in the flowers decreased 0.97 ~ 1.07-fold compared with the control with GA_3 application and rose 1.00 ~ 1.16- and 1.06-fold with Spd and GA_3 + Spd applications, respectively. The Spd/Spm ratios in the flowers were 0.78 ~ 0.97-, 0.56 ~ 0.71- and 0.56-fold compared with the control with GA_3, Spd and GA_3 + Spd applications, respectively. During stages 3 and 4, GA_3 significantly improved the Spd/Put ratio at stage 3, while Spd and GA_3 + Spd applications significantly decreased the Spd/Put ratio, and similar behavior was observed for the Spd/Spm ratio at stages 3 and 4. The dynamic PA levels in different forms and ratios indicated that GA_3 and Spd play significant roles in modulating *Rhododendron* flowering time and senescence.

Activities of PA Biosynthetic/Degradative Enzymes

The trend of ODC and SAMDC was quite comparable and showed a rise from stage 1 to stage 3 and then a decline (Fig. 3B-C), the rise was faster in SAMDC than in ODC. However, this behavior was opposite to that found for ADC activity (Fig. 3A). The ADC, SAMDC and ODC activities increased significantly in response to GA_3 and Spd applications. The highest ADC activity was recorded in response to 0.01 mM Spd application during stage 3 to stage 4 and 1 600 $mg{\cdot}L^{-1}$ GA_3 + 0.1 mM Spd application during stage 1 to stage 2, whereas the highest ODC and SAMDC activity was recorded in response to 1 600 $mg{\cdot}L^{-1}$ GA_3 + 0.1 mM Spd application.

Flower senescence was accompanied by a rise of PAO from stage 1 to stage 3 and then a decrease, whereas DAO showed a continuous increase (Fig. 4A-B). GA_3 and Spd improved the activity of DAO and PAO. Notably, the activity declined from stage 1 to stage 2 with GA_3/Spd applications. The highest DAO and PAO activity were recorded in response to 1 600 $mg{\cdot}L^{-1}$ GA_3 + 0.1 mM Spd application.

Relationships of GA_3 and PAs to Rhododendron flowering

Regression analysis illustrated that the flowering time was significantly and positively correlated with the mean contents of soluble conjugated Put, Spd, and Spm, insoluble bound Put, Spd, and Spm, fractions of soluble conjugated Put and Spm, and soluble conjugated Put, Spd and Spm ($r = 0.432 \sim 0.690$, $p = 0.01$; Table 2). The correlations of flowering time with the ratios of Spd/Put ($r=-0.207$, $p = 0.05$), Spd/Spm,

fractions of free Put, Spd and Spm were significant and negative (r=-0.477 to -0.639, p = 0.01), whereas the correlations were insignificant among flowering time with contents of GA_3, free Put, Spd, Spm, and the fraction of soluble conjugated Spd (r=–0.006 to 0.145, p > 0.05).

Table 2

Correlations of the flowering time with the mean contents of free polyamines, soluble conjugated polyamines, insoluble bound polyamines, GA_3 content, ratios of Spd/Spm, Spd/Put, fractions of free polyamines, soluble conjugated polyamines and insoluble bound polyamines during the flowering process of petals of *Rhododendron*

Correlation with	**Flowering time**
free polyamine contents	
Putrescine (Put)	-0.073
Spermidine (Spd)	0.071
Spermine (Spm)	0.145
Content of soluble conjugated polyamines	
Put	0.598**
Spd	0.432**
Spm	0.624**
Content of insoluble bound polyamines	
Put	0.690**
Spd	0.627**
Spm	0.675**
GA_3 content	-0.071
Spd/Spm	-0.503**
Spd/Put	-0.207*
Fraction of free polyamines	
Put	-0.639**
Spd	-0.540**
Spm	-0.477**
Fraction of soluble conjugated polyamines	
Put	0.449**
Spd	-0.006
Spm	0.392**

*,** Correlation significance at $p = 0.05$ and $p = 0.01$ levels, respectively (n = 3). Data used for calculations are from Figs. 1, 2, 3, 4.

Correlation with	Flowering time
Fraction of soluble conjugated polyamines	
Put	0.664**
*,** Correlation significance at $p = 0.05$ and $p = 0.01$ levels, respectively (n = 3). Data used for calculations are from Figs. 1, 2, 3, 4.	

Discussion

GA_3 and Spd Are Associated with Rhododendron Flowering Time

Flowering is a complex process that is regulated by a network of in vitro and in vivo factors. GA is one of the most widely studied hormones for regulating flowering time (Silva et al. 2019; Sven Eriksson and Nilsson 2006). Evidence suggests that GA promotes flowering by suppressing the expression of the DELLA group of proteins and promoting the expression of floral homeotic genes (Mutasa-Gottgens and Hedden 2009). In this work, exogenous GA_3 advanced the initial flowering time, and this finding is consistent with previous observations for *Rhododendron* (Chang and Sung 2000), which demonstrated that GA_3 promotes bud development and advances flowering. Moreover, PA is another important regulator of floral initiation and development (Matsoukas et al. 2012; Srikanth and Schmid 2011). Among previous studies, some observed flowering advanced in *Arabidopsis thaliana* (Molesini et al. 2015), *Dendranthema morifolium* (Dong-Hua et al. 2014) with PA application, while the opposite result was found by Applewhite et al. (2010) and Ahmed et al. (2017). These differences may contribute to plant species, treatment parts and treatment dose. In the present work, the flowering time was delayed for a long time after Spd application (Table 1), which is consistent with previous observations for *S. alba* (Havelange et al. 1996). Notably, advanced or delayed flowering is not accompanied by an increase in the treatment dose, suggesting that flowering requires an appropriate treatment dose in *Rhododendron*; the higher the level, the more the flowering cannot be generalized (Pal et al. 2015). Intriguingly, when GA_3 was combined with Spd, the flowering time advanced just 1 day compared with the control. This finding is similar to previous observations of Alcázar et al. (2005), who demonstrated that the leaves treated with gibberellin reversed the inhibition of flowering. We speculate that there is an interactive effect of GA_3 and Spd on the flowering time of *Rhododendron*, but this hypothesis requires further study.

GA_3 and Spd Delayed Rhododendron Floral Senescence

Floral senescence is the final portion of flower development. Usually, floral senescence is accompanied by dynamic changes in the levels of endogenous hormones, which modulate intricate networks of signaling events that control the senescence program (van Doorn and Woltering 2008; Zhang and Zhou 2013). GA is a crucial hormone that suppresses floral senescence. In the current work, flower longevity significantly improved by GA_3 application (Table 1); this finding is consistent with previous observations for rose (Saks and Staden 1993), *Sandersonia aurantiaca* (Eason and R. 2002), daffodil (Sheikh and

Farooq 1999) and lily (Domenico 2018). Delayed senescence may be attributed to integrated membrane maintenance and reduced electrolyte leakage (Sabehat and Zieslin 1994). However, new evidence has been obtained by Lu et al. (2014) showing that the antagonistic effect of GAs on ethylene during rose petal senescence is mediated by the *RhHB1* gene.

PAs are considered to be important growth regulators, especially associated with plant senescence. A decrease in PA content is proven to be a senescence signal (Duan 2006). PAs delay senescence by retarding membrane deterioration, RNase and protease activity (Kaur-Sawhney et al. 2003). Here, the flowering lifespan extended with Spd application, which was comparable to reports in rose (Tatte and Ahlawat 2015), carnation (Lee et al. 1997), anthurium (Simões et al. 2018) and *Nicotiana plumbaginifolia L.* (Domingos et al. 2016). Both GA_3 and PAs were demonstrated to be delayed in plant senescence; hence, the combinations of GA_3 + PAs can postpone senescence synergistically.

GA_3 and Spd Influence Flowering and Senescence by Modulating PA Metabolism

Initially, the PA levels do not always peak during senescence, depending on the type of senescence model and whether PAs are induced by external or natural factors (Del Duca et al. 2014). In the present work, senescence of these petals was accompanied by a rapid decline in F PAs in the petals during the early stages and a continuous increase in C and B PAs, which was comparable to the report of Qin et al. (2019) in apple but different from that of Huang et al. (2004) in *P. tuberosa.* The reason for such a decline may be a decline in the F fraction and a transformation from the F form to the C form at early stages (Fig. 2A-C). In addition, concomitant increases in C and B PAs may be attributed to PA degradation (Fig. 5) and an increase in B and C fraction PAs (Fig. 2A-C), as well as a decrease in F PA levels and an increase in the conjugated pool associated with the initiation of cell expansion (Altamura et al. 1993). On the other hand, the increase in C PA is associated with cell wall organization, extension or rigidity. The B PAs, which result from C PAs (Fig. 5) moving to cell wall hemicelluloses and/or lignin or to cell wall proteins via PA-conjugating enzyme transglutaminase (TGase), may form protein polymers or heteropolymers with polysaccharides, indicating the significance of B PA in the cell walls (Cai et al. 2015b). However, the specific mechanism remains unclear and requires further research.

The homeostatic regulation of cellular PA levels is a dynamic balance of biosynthesis, catabolism, interconversion and conjugation and is important for plant development, such as flowering (Majumdar et al. 2016; Yu et al. 2019), whereas the regulatory effect of GA_3 and Spd remains largely unknown. Thus, we analyzed the key enzymes involved in PA metabolism and the interconversion of PA forms/components with GA_3 and Spd applications. As shown in Fig. 2, after spraying with GA_3 and Spd, the PA and GA levels increased. The reason for such a result may mainly be attributed to the permeation of exogenous Spd in addition to the synthesis of new Spd (Qin et al. 2019). Another direct cause of an increase in PA may be attributed to the effect of GA_3 (Fig. 5), which contributed to a direct effect on Spd (c.f. 0.27). Notably, the GA_3 pathway had to a negative effect on Spm (Fig. 5). Namely, Spm may have

had a positive effect on GA_3 (Fig. 5), which was consistent with previous observations for maize (Li et al. 2018) and sweet corn seed embryos (Huang et al. 2017). During stages 3 and 4, the contents of C and B PAs were significantly improved by GA_3 and Spd applications, which may contribute to longer flower lifespan (Table 2). Intriguingly, when GA_3 was combined with Spd, the highest C and B PA contents were not consistent with the longest flower lifespan, indicating that other factors affect senescence.

Aribaud et al. (1998) demonstrated that the relative polyamine proportion and their metabolism are more important than their endogenous content in the development processes. In this study, PA forms and ratios were also altered by GA_3 and Spd applications. GA_3 treatment increased the F fraction of Put and decreased the Spd/Put ratio, Spd and the GA_3 + Spd applications increased the C and B fractions of Put and the Spd/Put ratio at stage 1. The results infer that GA_3 promotes flowering by decreasing the Spd/Put ratio, whereas Spd delayed flowering by increasing the Spd/Put ratio and the C and B fractions of PAs. The Spd/Put ratio decreased with GA_3 + Spd application, and the C and B fractions of PAs increased, which may contribute to the advancement of the initial flowering time by 2 d because flowering time is significantly positively correlated with the C and B fractions of PAs and significantly negatively correlated with the Spd/Put ratio (Table 2). The reasons for the increase in the F Put fraction by GA_3 may be due to the direct effect on Spd (Fig. 5), which is converted into Put and synthesizes new F Put. The decrease in F Put and increase in B and C Put with Spd application may contribute to Spd accelerating the conversion of F into C and C into B forms (Fig. 5). Moreover, the Put level is similar to that of GA_3 and Spd at stage 1 (Fig. 2A), and the Spd level is significantly higher with Spd application compared with GA_3 application (Fig. 2B); thus, the decrease in the Spd/Put ratio with GA_3 application may be attributable to GA_3 accelerating conversion of Spd into Put, and the increase in the Spd/Put ratio with Spd application may contribute to the significant increase in the Spd level. The results infer that the role of the F Put fraction and Spd/Put ratio in flowering time could be in response to GA_3 and Spd, as suggested by Handa and Mattoo (2010b) and Nambeesan et al. (2019) that PA ratios are associated with N:C balance and flower developmental progress. Moreover, flower senescence is accompanied by a decrease in the F fractions of Put and Spd and increases in the F fraction of Spm, the C fractions of Put, Spd, and Spm and the Spd/Spm ratio with GA3 and Spd applications (Fig. 2B-C, Fig. 4D). In general, the increases in the C fraction of Pas and the Spd/Spm ratio and the decreases in the F fractions of Put and Spd were followed by: Spd□GA_3□GA_3 + Spd. It is significant that Spd delaying flower senescence showed the best effect because the F fraction of PAs and Spd/Spm were significantly negatively correlated with flowering time, whereas the C and B fractions of PAs were significantly positively correlated with flowering time (Table 2). A direct cause of the increase in the C Spd fraction and the F Spm fraction is that GA_3 and Spd significantly improved the PAO activity (Fig. 4A), which could accelerate the conversion of F PA into C PA and C PA into B PA (Wang et al. 2019). Despite the significant improvements in the contents of Spd and Spm by GA_3 and Spd applications during flowering, the Spd/Spm ratio decreased (c.f. 0.91) (Fig. 5). It has been reported that higher Spm levels decrease ROS accumulation and induce nitric oxide (NO) production. Spm may be a signaling metabolite that provides protection delay senescence via metabolic conversions involving ascorbate/dehydro-ascorbate redox state modification, changes in sugar and

nitrogen metabolism, cross-talk with ethylene biosynthesis and thereby delayed senescence (Sequera-Mutiozabal et al. 2016). Moreover, the increase in the C Spd fraction and the F Spm fraction may also result in an increase in the flower lifespan. PA interaction with cell wall components may be responsible for changes in cell wall rigidity (Berta et al. 2010). The conjugates are significant not only for their modulation of PA forms inside the cell but also for their interaction with cell wall components (Bagni and Tassoni 2001). In addition, conjugation of PA might be a regulatory mechanism to control its level within a non-toxic range for plant survival (Alcázar et al. 2005). Furthermore, ethylene triggers senescence in plants, and its synthesis pathway directly competes with the synthesis of PAs by the use of S-adenosyl methionine (SAM) in both pathways (Mehta et al. 2002; Pandey et al. 2000; Sobieszczuk-Nowicka 2017). In the present study, flower senescence was accompanied by an increase in the contents of F Spd and Spm (Fig. 2B-C). GA_3 and Spd significantly improved the PA levels during flowering, especially for free Spd and Spm levels and the F Spm fraction. This increase may have contributed to the improvement of SAMDC activity (Fig. 3C, Fig. 5) and expression levels of *SAMDC.* Antisenescence agents in some tissues have been attributed to F PAs, which may be responsible for the reduction in ethylene production (Saftner and Baldi 1990). This result indicates that higher F fractions of Spd and Spm may inhibit ethylene production, as demonstrated by Chen et al. (2013) in rice. Based on these results, one can infer that exogenous Spd and GA_3 regulated PA metabolism by increasing the content and altering the form, components and ratios of PAs.

One possible explanation for the differences in PA content among the treatments is that the responses to flowering of PA biosynthetic/degradative enzymes are different. In the PA biosynthetic pathway, ornithine or arginine is decarboxylated by ODC or ADC to form Put. In general, the highest levels of endogenous PAs and PA synthetase activity were discovered in the meristem and growing cells, and the lowest were in senescent tissues (Chen et al. 2019). Here, flower development was accompanied by an increase in the activities of ODC and SAMDC and a decrease at stage 4 (Fig. 3B-C). Exogenous Spd significantly improves ADC, ODC and SAMDC activities, possibly due to the increased *MdADC1* and *MdODC1* expression and even *MdSAMDC2* transcription (Qin et al. 2019). High activities of ODC and SAMDC contributed to the positive effects on Spd and Spm contents (Fig. 5). However, the level of ADC presented an opposite trend, and a continuous increase in C and B PAs was found, indicating that ADC had a negative effect on PA levels (Fig. 5).

In addition to the PA biosynthetic pathway, increasing evidence suggests that the PA degradative pathway also plays a signaling role in developmental processes (Wang et al. 2019). NO is one of the signals that control flowering (He et al. 2004)(He et al., 2004). DAO and PAO resulted in a high level of PAs and play a role in flowering since PA and NO share some common signal transduction pathways (Wimalasekera et al. 2011). Oxidation of PAs by PAOs contributes to the regulation of PA homeostasis (Angelini et al. 2010). Moreover, PA catabolism induces hydrogen peroxide (H_2O_2) and cytotoxic products, which is considered to be a possible mechanism of PA association with programmed cell death (PCD) (Cai et al. 2015a; Cai et al. 2015b; Del Duca et al. 2014; Yoda et al. 2006). In the present study, senescence of these flowers was accompanied by a minimal decline in the activity of DAO at the initial flowering

stage and then an increase; PAO presented an opposite trend (Fig. 4A-B). F PA decreased at the initial flowering stage, and C and B PA increased throughout flowering. One possible explanation is that the increase in PAO (Fig. 4A) could accelerate the conversion of F PA into C PA and C PA into B PA (Wang et al. 2019). However, the high level of F Spm could function as negative feedback, thus inhibiting the production of Spd (Fig. 5) (Hu et al. 2012). The high level of Spd mainly contributed to Put (c.f. 0.63), SAMDC (c.f. 0.52) and GA_3 (c.f. 0.27) (Fig. 5). Thus, the longer flower longevity of application with Spd may be associated with its capacity to maintain high activities of DAO and PAO. Notwithstanding, the longest flowering time was not associated with the highest concentration of Spd (1 mM). When GA_3 combined with Spd, the highest level of endogenous PA (Fig. 2A-C) was accompanied by a significant increase in DAO and PAO (Fig. 4A-B), but the flowering time was only prolonged 1 day (Table 1). Appropriate hormonal action demands polyamine homeostasis (Carbonell and Blazquez 2009). One possible explanation is that the increase in endogenous PA concentration was accompanied by an increase in DAO (Fig. 4B). When PAs are catabolized, an oxidized product of H_2O_2 is a signaling compound that activates the signaling pathway and contributes to delaying senescence (Moschou et al. 2008). However, when PA-derived H_2O_2 is not quenched properly, the process may lead to PCD rather than delay senescence (Moschou et al. 2008). Moreover, the flowering time was longer with Spd application than with GA_3 application (Table 1). Based on these results, one can infer that Spd directly participated in PA metabolism and that GA_3 delayed flower senescence by modulating PA metabolism via Spd (Fig. 5). Our results collectively demonstrate that endogenous PA contents, forms, and ratios and PA biosynthetic/degradative enzymes, as well as developmental stages of flowering, were altered markedly and regularly after exogenous Spd and GA_3 applications, thereby delaying flower senescence.

Conclusion

In conclusion, moderate GA_3 and Spd concentrations altered the initial flowering time and delayed flower senescence in *Rhododendron*. GA_3 accelerated flower development was closely associated with an enhanced F Put fraction and a decrease in the Spd/Put ratio at stage 1, whereas Spd slowed flower developmental progress and contributed to an increase in the C and B Put fractions and Spd/Put ratio. There was a potential antagonistic effect of GA_3 and Spd on flowering time. Moreover, GA_3 and Spd prolonged flowering time by decreasing the Spd/Spm ratio. Another reason could be the increases in the C and B fractions of PAs. In addition, all applications promoted the PA levels and key enzymes involved in PA metabolism. A potential metabolic interaction or competition between higher free PAs (Spd and Spm) and ethylene biosynthesis mediated the effects of GA_3 and Spd on flower senescence, but further study of the mechanism is needed. For many physiological processes, it is not the presence of “high” PAs that matters, but the concentration of “adequate” PAs. Furthermore, the PA fraction and ratio are indeed significant for flowering in *Rhododendron*.

Declarations

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Compliance with Ethical Standards

No potential conflict of interest was reported by the authors.

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