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EFFECTS OF 5-AZACYTIDINE AND GIBBERELLIC ACID ON FLOWER DEVELOPMENT OF AZALEA

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Azalea is one of the most popular ornamental plants in China. On the production of pot azaleas, heating strategy is widely used to force flowering, so the products can meet the needs of Spring Festival market in China. This study was conducted to find another possible way to break flower bud dormancy and promote anthesis through environment friendly ways. Four-year-old azalea 'Hong Shanhu' (Chinese azalea cultivar) were sprayed with different combinations of 5-azacytidine (2.5, 10, 40 and 160 mg L^{-1}) and gibberellic acid (300 mg L^{-1}). These were kept in greenhouse during the winter with no extra heating device. Morphological changes, endogenous hormones, and the degree of DNA methylation were recorded. The results showed that the combination of 40 mg L^{-1} 5-azaC and 300 mg L^{-1} GA_3 can highly promote flower bud growth and brought anthesis 17 days earlier than controls. IAA content increased about twofold (control and T1) and fivefold (T4) after growing for four months. A steep decrease of DNA methylation was observed from November to February and followed by an increase until flowering in all treatments. The foliar application of these chemicals was found to be more effective on bud dormancy breaking by DNA demethylation and decrease of ABA levels.

Keywords: Rhododendron, dormancy, methylation, plant growth regulator, epigenetics, abscisic acid.

INTRODUCTION

Azaleas are among the most important ornamental shrubs in Europe (Scariot, 2007), America and East Asia, which contain thousands of cultivars with various flower morphotypes and flowering patterns. In China, it could be chased back to the Tang Dynasty when our ancestors started to enjoy its beauty (Zhou, 2013a). Nowadays, the areas of commercial production for azaleas have been expanded to more than 2500 hectares in China and over 350 million cuttings are propagated every year to meet the needs of landscaping and home cultivation (Zhou, 2010). In natural conditions, flower buds of azalea enter dormancy in winter to overcome the low temperature and usually bloom in April to May (Meijon, 2009). However, almost 80% to 85% floral products are sold during the Chinese New Year (Wang, 2014), which is mainly in January or February, the coldest month in a year. Thus, it is crucial to regulate the timing of flowering in azalea.

It has been studied previously to find various ways to regulate dormancy and bud break by using different chemicals in the last few decades (Arora, 2003). Among these chemicals, gibberellic acid (GA) applications were proved to be efficient on dormancy-breaking by partly substituting the cold requirements in azaleas and other woody plants (Bodson, 1986; Chang and Sung, 2000; Yeo *et al*., 2012; Christiaens *et al*., 2012).

It is also reported that DNA demethylation is involved during vernalization (Demeulemeester, 1999). The usage of 5-azacytidine (5-azaC) could lower the DNA methylation level by merging analogue in place of cytosine (Jones, 1985) and inhibiting the action of methyltransferase enzyme (Bouchard, 1983). In Burn's (1993) experiment, *Arabidopsis thaliana* and *Thlaspi arvense* treated with 5-azaC turned up with an early flowering on nonvernalized plants, just reacted the same as those treated with cold. In some short-day plants, DNA demethylation caused by 5-azaC could also induce flowering (Kondo, 2006, 2007, 2010). As far as our understanding, 5-azaC has never been used to manipulate flowering in azaleas.

In China, on a regular basis, flowering of azaleas is usually forced by temperature control (high temperature) or photoperiod manipulation. When the flower buds become dormant, only be released if their chilling requirements are satisfied (Christiaens, 2015). In view of global warming and expansive costs, cold storage is hard to accomplish in most of the production areas. We tried different combinations of 5-azaC and GA_3 in this study to 1) document the morphological changes and flowering patterns of azalea under different treatments; 2) evaluate the effect of each treatment on promoting flowering; 3) analyze the endogenous hormones and global DNA methylation during

Table 1. Treatments applied to 'Hong Shanhu' to promote flowering.

	Treatments									
	Control	T1	T2	T3	T4	T5	T6	T7	T8	T9
GA_3 (mg L^{-1})	0	300	300	300	300	300	0	0	0	0
5-azaC (mg L^{-1})	0	0	2.5	10	40	160	2.5	10	40	160

floral development.

MATERIALS AND METHODS

***Plant materials*:** A late-flowering azalea cultivar 'Hong Shanhu' was used in the experiments supplied by Weitang azalea garden (Jiashan, China). Root cuttings grown outside for four years under standard water and fertilizer management, then were transplanted into one-gallon pots containing peat, pine needles and yellow clay (3:1:1 by volume) and placed in experiment fields belonging to Zhejiang University in Hangzhou (120°11'E, 30°16'N) (China) at a planting density of nine plants per meter square.

***Experimental set-up*:** One hundred plants were subjected to different combinations of 5-azacytidine (5-azaC) and GA_3, as shown in Table 1. A surfactant (Tween-20) at 0.05% was added to each solution. The control plants were sprayed with tap water. The first spray started on 15th November and followed by two more sprays once a week. All the plants were kept outside under natural conditions until 26th December and moved into a greenhouse till blooming with an average temperature above 16°C. Sampling dates for endogenous PGRs analysis and global DNA methylation were selected at least monthly to cover the different stages of flower bud development. In every sampling, three to five apical buds were randomly taken from one plant as one replicate, for a total of five replicates per treatment. All the samples were quick-frozen by liquid nitrogen immediately, and stored at -80°C until extraction.

Observations and measurements: Ten flower buds were randomly picked from each treatment to record the length (L) and diameter (D) every two weeks and their volume were estimated using the following formula:

$V = 1/3\pi(D/2)^2L$

Mark the dates of the buds showing color and anthesis in each treatment. Calculating the necessary growing degree day (GDD) for the plants to bloom using Meijon's method (Meijon, 2011).

***Analysis of PGRs*:** The endogenous plant growth regulators (PGRs) including abscisic acid (ABA), indole acetic acid (IAA) and GA were analyzed from 300 mg buds (frozen weight) using enzyme-linked immunosorbent assay (ELISA) kits for hormones (China Agricultural University, China). The extraction, purification and determination of PGRs were performed as described by Hao (2001).

***Determination of global DNA methylation*:** Genomic DNA was extracted from 200 mg dried frozen buds and purified as described by Zhou (2013b). DNA concentration and quality were determined by electrophoresis and spectrophotometry. Approximately 3 μg DNA was hydrolyzed according to Demeulemeester (1999). After filtered through a 0.45 μm filter membrane, the total hydrolysate was analyzed on an HPLC system (Agilent 1100, USA) with a Hypersil BDS C18 column (4.6 × 250 mm, particle size 5μm, Thermo Fisher, USA) operating at 30°C. The mobile phase consisted of a special solvent (containing 6.25 mM sodium pentanesulfonate, 0.1% triethylamine and 0.4% acetic acid) and methanol in the ratio 20:80 (v/v). The flow rate was set to 1 mL min^{-1} with an injection volume of 20 μL. The detection was carried out at 273 nm using cytosine and 5-methylcytosine (5mC) (Alfa Aesar, USA) as standard. Global DNA methylation was determined by the percentage of 5mC (concentration of 5mC × 100/(concentration of 5mC + concentration of cytosine)).

Statistical analysis: The data were analyzed using SPSS 20.0 software package. The volume of flower buds were subjected to a one-way ANOVA based on dates. For the contents of endogenous hormones, one-way ANOVA analysis was performed both by dates and treatments. The significance level was set to $\alpha = 0.05$ for all the tests.

RESULTS

***Effects of different treatments on flower development*:** Despite we collected the flower buds growth data every two weeks, to get a clear view of the difference between treatments, the results were summarized monthly (Table 2). The last measurement was set at March 5th 2012 due to the trend of blooming. All buds became bigger in the first month of treatment. But during the second month, all the other treatments and control plants hardly showed any increase in flower bud volume except for these in T4. After January 15th, buds in all treatments continued to grow. In the whole flower developing period, buds in T4 (sprayed with 300 mg L^{-1} GA_3 and 40 mg L^{-1} 5-azaC) grew fastest and ended with the largest volume comparing to the rest. As to the effects between different kinds of chemicals, buds sprayed with both GA_3 and 5-azaC were larger than those sprayed only with 5-azaC.

Start dates of buds showing color and blooming are the most direct evidence to evaluate the effects of different chemicals when forcing flowering. Buds in T4 were the earliest to

Table 2. Volume of flower buds measured monthly under different treatments.

	Flower bud volume (mm^3)				
	15 Nov.	**15 Dec.**	**15 Jan.**	**14 Feb.**	**5 Mar.**
Control	87.60±12.20a	126.02±18.89a	114.73±17.18b	137.07±18.33c	188.20±22.58e
T1[a]	87.60±12.20a	122.07±12.95a	124.63±12.03b	218.04±44.78ab	360.54±77.52ab
T2	87.60±12.20a	127.83±13.20a	131.65±15.36ab	195.63±63.94abc	316.90±69.56bc
T3	87.60±12.20a	125.30±8.13a	121.37±14.59b	186.31±51.13bc	325.68±73.32bc
T4	87.60±12.20a	129.56±18.74a	150.16±35.47a	256.13±81.50a	410.18±56.64a
T5	87.60±12.20a	118.70±10.83a	132.70±13.68ab	177.61±38.65bc	343.19±79.65abc
T6	87.60±12.20a	125.88±16.58a	130.72±14.24ab	147.21±47.46bc	235.18±41.85de
T7	87.60±12.20a	126.32±12.99a	128.18±10.78a	165.38±18.10bc	276.39±54.94cd
T8	87.60±12.20a	114.66±17.64a	124.36±18.46a	160.19±47.54bc	346.61±91.62abc
T9	87.60±12.20a	123.59±11.01a	124.23±12.12a	174.09±70.18bc	350.93±20.66abc

[a] for abbreviation of treatments please refer to Table 1. Means (n = 10) and standard deviation followed by different letters are significantly different ($P = 0.05$, Duncan's test) between treatments based on the same date.

Table 3. Start date of buds showing color, flowering and growing degree day in different treatments (Control, T1 and T4).

	Control	**T1[a]**	**T4**
Start date of buds showing color	21 Mar, 2012	5 Mar, 2012	25 Feb, 2012
Start date of flowering	27 Mar, 2012	13 Mar, 2012	10 Mar, 2012
Growing degree day	1199	1038	1008

[a] for abbreviation of treatments please refer to Table 1.

show color and anthesis with an advance of 3 and 17 days as compared to the plants in T1 and control respectively (Table 3). Buds in T9 showed color 9 days later but bloomed at the same day as buds in T4 (results not listed here). It took the plants 14 days from showing color to start bloom in T4, while the time is shortened to 8 and 6 days in T1 and control. The later the buds showing color, the more quickly it bloomed (Table 3). In addition, the minimum temperature required for flowering under our experimental set-up was determined by growing degree day (GDD). It was observed that the controls needed to accumulate more GDD to bloom than plants with chemical treatments. We chose plants in T4 (with the best effect of bud break), control and T1 (only with GA_3) to measure the changes of endogenous hormones and DNA methylation as the flower buds grow up.

***Effects of 5-azaC and GA_3 on endogenous hormones in flower buds*:** IAA content increased about twofold (control and T1) and fivefold (T4) after growing for four months (Fig.1a). In controls, it reached a minimum value on the second sampling and followed by a continuous growth. T4 triggered a violent increase of IAA content in flower buds immediately and significantly almost two times higher than in the buds of T1 and control except for the fifth sampling. Buds treated only with GA_3 showed no significant difference on IAA content compared with controls except for the suddenly drop on 28th February. The ABA content peaked at 1st February and returned to the similar level as the beginning in all plants (Fig. 1b), especially for controls and buds from T1 the maximum content of ABA increased up to 236.21% and 179.48% of its original value. However, ABA content increased only 13.55% at the top in T4 and remained almost extremely lower than in the other two groups. The ratio of IAA content to ABA content in buds rose about 7.6 times in T4, which was dramatically higher than in T1 and control (not shown in this Figure). The ratio change of GA content to ABA content (GA/ABA) during flower development showed a double peak figure (Fig. 1c). It increased up till January and dropped in February, and finally reached the top when the plants were about to bloom. The ratio change (GA/ABA) was found higher in T4 earlier than in T1 and control.

***Effects of 5-azaC and GA_3 on global DNA methylation*:** A steep decrease in the percentage of 5mC was observed from November to February and followed by an increase until flowering in all treatments (Fig. 1d). Plants sprayed with both 5-azaC and GA_3 (T4) had the lowest level of DNA methylation and methylation were faster after the turning point in all treatments. After the flowering started, DNA methylation level decreased to normal at 13th March (T4). While, it took longer for the plants in T1 and control to accomplish this process. Control plants were demethylated much slower than the others and the off-peak value occurred about one month later. However, the dynamics of methylation during flower development were similar for all treatments.

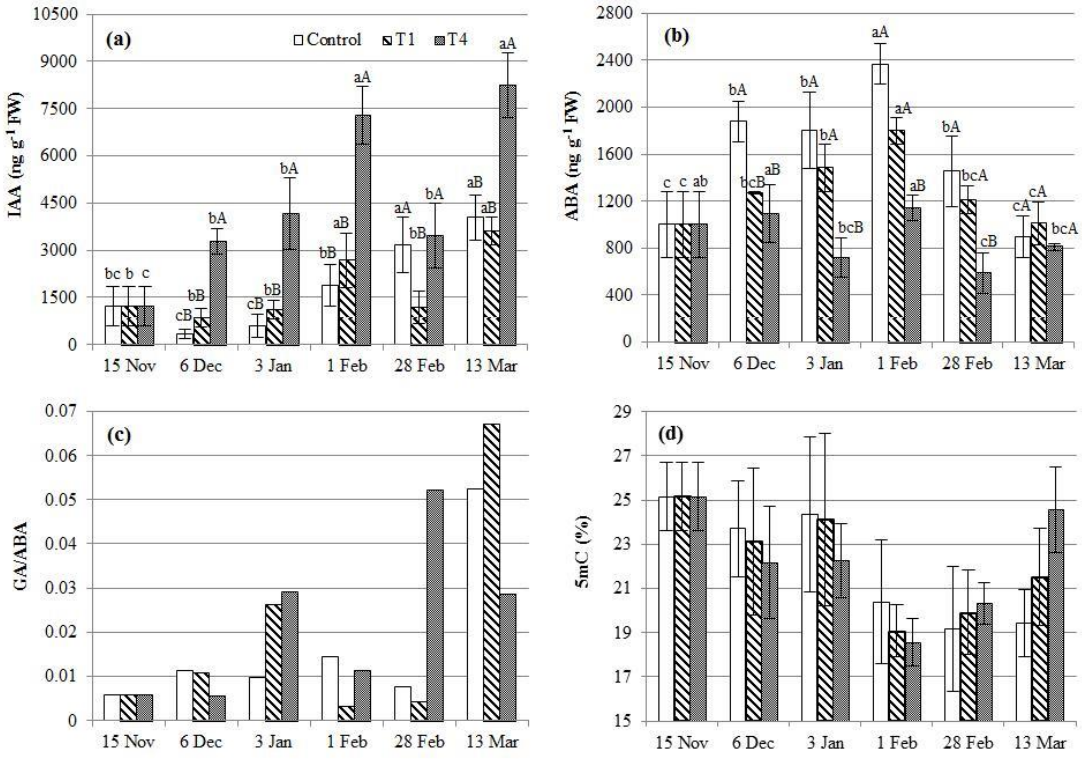

Figure 1. Changes of endogenous hormones and global DNA methylation in different treatments during the whole experimental period. (a) IAA content; (b) ABA content; (c) the ratio of GA content to ABA content; (d) global DNA methylation. The different lowercase letters indicate significant differences between dates within each treatment ($P \leq 0.05$, n = 3), whereas, the different capital letters show significant differences between treatments within each date ($P \leq 0.05$, n=3) (for abbreviation of treatments refer to Table 1).

DISCUSSION

Bud dormancy is a complex biological problem. For the past century, much research has been published based on different aspects. The effects of chemicals on the initiation and development of flower buds could be totally opposite when treated at different stages of growth. Bodson (1986) found out that GA (4+7) could lead to a bud abortion when treatment applied at the time of floral transition. However, when the treatment was carried out on the inflorescence buds, it substituted for chilling in overcoming dormancy. It is speculated from the change of bud volume that buds were probably under dormant stage between December and January, so we sprayed the plants in the middle of November just when the dormancy was about to begin. Plants treated only with 300 mg L^{-1} GA_3 (T1) showed color of buds 16 days earlier and bloomed 14 days earlier than controls. Likewise, in Chang (2000) research, 500 mg L^{-1} GA_3 was used in the middle of dormancy (the end of December) and got a 10 or 9 days advancement on the blooming of two azalea species. The differences in blooming might be due to different concentrations of GA_3, plant material and the start time of treatments. In our study, when treated with both GA_3 and 5-azaC, the effects were incredibly good with a promotion of 25 and 17 days (of bud showing color and blooming, respectively) earlier than controls. We found it interesting that it took longer than controls for the treated plants to bloom since the buds started showing color. It may because the controls accumulated more GDD units than treatments (Meijon, 2011).

Abscisic acid (ABA) plays a crucial role in the establishment and maintenance of dormancy (Gotz, 2014). It was found in Camelia a clear downward trend of flower buds ABA levels as the cold treatment continued (Berruti, 2012). In our experiment, GA_3 and 5-azaC treatments both induced a lower level of ABA than controls. When the dormancy of flower buds was inhibited (mainly during February), the ABA content decreased. The correlation of endogenous hormones, DNA methylation and flower bud volume was also tested (Table 4). Both ABA content and ratio of GA to ABA were significantly related with flower bud volume. IAA and GA content seemed to be not so relevant with the bud growth.

Table 4. Correlation coefficients matrix for flower bud volume, endogenous hormones and DNA methylation.

	ABA	IAA	5mC	Flower bud volume	GA/ABA	GA
ABA	1.000					
IAA	-0.525*	1.000				
5mC	-0.028	-0.273	1.000			
Flower bud volume	-0.549*	0.462	-0.149	1.000		
GA/ABA	-0.509*	0.314	-0.085	0.593*	1.000	
GA	-0.114	0.114	-0.014	0.410	0.885**	1.000

* Significance at the 0.05 probability level; ** Significance at the 0.01 probability level.

Treating plants with the demethylating agent, 5-azaC, brought early flowering by demethylation of global DNA (Finnegan, 1998; Lizal, 2001; Brown, 2008). Meijon (2010) observed that on the floral transition of azalea, there is a sharp decrease of DNA methylation. When floral differentiation is completed, the degree of global DNA methylation keeps increasing till flowering (Meijon, 2011). The dynamics of DNA methylation during flower development in our study was a little different with Meijon's (2011) results. However, azalea cultivars used in Meijon' (2011) studies don't need to be vernalized during floral transition and anthesis. DNA methylated after flower buds formed. Whereas, the cultivar in our study has a clear requirement of cold storage to overcome dormancy. In the dormant stage, DNA demethylated in flower buds with different speed. The faster DNA methylated, the earlier the plant bloomed. All these indicated that the chilling requirement could partially be substituted by the applying 5-azaC, but the effect might not be that obvious compared with GA_3 treatment.

***Conclusion*:** The use of both GA_3 and 5-azaC with different combination has shown to break the early bud dormancy in azalea. The foliar application of these chemicals was more effective in the demethylation of DNA and decrease of ABA levels, and could partly substitute with chilling requirement. This encourages us to improve the time and concentration of chemical uses on flowering forcing, and may provide a well strategy for early breaking of flower bud dormancy of azalea plants for commercial production at the time of New Year in the country.

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