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Hormonal Profile in Vegetative and Floral Buds of Azalea: Levels of Polyamines, Gibberellins, and Cytokinins

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Abstract The floral transition includes a complex system of factors that interact and involve various biochemical signals, including plant growth regulators. The physiological signals involved in the control of the floral transition have been sparsely studied and mainly in plant species whose genetics are poorly known. In this work, the role of polyamines, gibberellins, and cytokinins was investigated by analyzing their endogenous content in vegetative and floral buds of azalea. The results showed that there is a clear distinction between floral and vegetative buds with respect to the levels of these plant hormones, with floral buds containing higher amounts of conjugated polyamines, gibberellins (GAs) from the non-13-hydroxylation pathway (GA_9, GA_7, and GA_4), and cytokinins (particularly isopentenyl-type species), and vegetative buds containing higher amounts of free polyamines and gibberellins from the early 13-hydroxylation pathway and fewer cytokinins. In conclusion, there is a specific pattern of endogenous hormone profiles in both vegetative and floral bud development in azalea, which may be relevant for future research on the control of flowering by exogenous hormone applications.

Keywords *Rhododendron* sp. · Polyamines · Gibberellins · Cytokinins · Flowering

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Introduction

Azaleas comprise a small sector of the large genus *Rhododendron* L. (*Ericaceae*) that contains approximately 1000 described species and thousands of commercial hybrids. In the ornamental industry it is commercially important to produce plants with full blooms and high floral quality (Meijón and others 2009a). The transition to flowering is a major event in a plant's life that is marked by a switch in the shoot apical meristem from leaf production to the initiation of floral organs. Such a developmental transition takes place only when environmental and endogenous factors are most favorable for reproductive success. The initiation of flowering is synchronized to the changing seasons, with day length and temperature being the environmental cues used to achieve this (for a review see Corbesier and Coupland 2006; Kobayashi and Weigel 2007). Plant hormones are chemical messengers that connect environmental changes to plant responses such as floral transition.

The physiological study of the floral transition has led to the identification of several putative floral signals such as sucrose, cytokinins (CKs), gibberellins (GAs), and reduced N-compounds that are translocated in the phloem sap from leaves to the shoot apical meristems (Corbesier and Coupland 2006; Davis 2009). On the other hand, the genetic approach developed more recently in *Arabidopsis thaliana* has led to the discovery of numerous genes that

control flowering time. These genes operate in cascades within four promotive pathways: photoperiodic, autonomous, vernalization, and GA pathways. All of these pathways converge on a small number of integrator genes, *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, that lead to the activation of floral meristem identity genes *APETALA1* (*AP1*) and *LEAFY* (*LFY*) (Blázquez and others 2006; Wilkie and others 2008), which trigger the transition from vegetative to reproductive phase. GAs and other plant hormones (abscisic acid, salicylic acid, or bassinosteroids) are able to control flowering time through the regulation of the floral integrators *FT* and *SOC1* or through other secondary regulators of *LFY* and *AP1* such as *FLOWERING LOCUS C (FLC)* (Eriksson and others 2006; Davis 2009).

The transition to the flowering stage includes a complex system of factors that interact and involve various biochemical signals (Davis 2009), including the plant hormones polyamines (PAs), GAs, and CKs.

The PAs spermidine (Spd) and spermine (Spm) and their diamine precursor putrescine (Put) are endogenous regulators or intracellular messengers that are involved in many physiological processes such as morphogenesis, embryogenesis (Uribe and others 2008; Klimaszewska and others 2009), floral transition, fruit set (Tiburcio and others 1993; Sood and Nagar 2004), growth, and response to environmental stresses (Hu and others 2006; Baron and Stasolla 2008). In recent years, the PAs have also been implicated in cell cycle regulation (Wallace and others 2003; Palavan-Unsal and others 2006; Baron and Stasolla 2008), hence their close relationship with the proliferation and differentiation processes in both plants and animals.

The involvement of GAs in flowering has been demonstrated by both biochemical and molecular techniques (Blázquez and Leon 2006), with regulation not only at the synthesis, degradation, or translocation level, but also at the level of cellular sensitivity (Oka and others 2001). These phytohormones also seem to play an important role in the promotion of flowering induced by the short-day photoperiod through *LFY* regulation (Blázquez and others 2002), although it is not easy to elucidate the specific role of every GA due to their complex metabolism.

Because environmental factors lead to floral transition, there should be full synchronization between the plant and its environment, controlled by phytohormones, specifically the GAs, which play a key role as transducers of environmental information. Another clear example involves temperature, with falling temperature associated with a reduction in stem length growth and a decrease in the endogenous GA_1 levels, which in turn is linked to higher synthesis of GA-2 oxidase, a GA_1-deactivating enzyme (Stavang and others 2007). Conversely, higher temperature is associated with increases in the levels of endogenous GA_1 and GA_9 (Fernández and others 1997). Also, the vernalization and autonomous pathways of floral induction are correlated with the blockade of *FLC* expression, a repressor of flowering that plays a key role during floral transition in *Arabidopsis* and other species (Wilkie and others 2008). In both pathways, *FLC* regulation is controlled by GAs (Wilkie and others 2008).

Another proposed model for the relationship between the levels of GAs and environmental factors suggests that an increase in GA biosynthesis contributes to the promotion of flowering by long-day photoperiods and the regulation of *FT* and *SOC1* (Eriksson and others 2006; Wilkie and others 2008). Thus, the active GA species GA_1 and GA_4 accumulated when short-day-grown *Arabidopsis* plants were induced to flower by transferring them to a long-day photoperiod; in addition, exogenous application of GAs to certain monocots or *Arabidopsis* mutants can be as efficient as single long-day treatments in the promotion of flowering (Eriksson and others 2006; King and others 2006). On the other hand, GA-deficient mutants are able to flower under long days, although the severity of the delay caused by the lack of GAs varies with the experimental conditions, indicating that there is no simple relationship between day length and the role of GAs in floral induction (Blázquez and others 2002). In *Rhododendron* sp. the interrelationship between photoperiod and GA biosynthesis has been described previously. Pemberton and Wilkins (1985) suggest that after treatment with GAs, long-day photoperiods are able to force flowering in winter in the Prize cultivar of azalea. In addition, Macmillan and others (2005) and Eriksson and others (2006) reported an increase in GAs and their functionality in the later stages of floral development in species exposed to a long-day photoperiod.

Another group of plant hormones that participate in the transfer of the floral stimulus from the leaves are the CKs. Although the involvement of the CKs during the floral transition has not yet been clearly defined, they likely play an essential role in the processes of cell division and differentiation of the floral meristem. The CK content increases significantly in the leaves and in the leaf phloem sap from 16 h after the start of floral stimulus (Corbesier and others 2003; Stern and others 2003). Subsequently, the CK content increases in the apical meristems at the time of early mitotic activation (Jacqmard and others 2003). In addition, exogenous CK application to vegetative plants grown in short-day conditions can induce various cellular and molecular changes in the shoot apical meristem that are normally associated with the floral transition (Chang and Chang 2003).

Advances in the techniques for hormone quantification have allowed a far more comprehensive analysis of the changes in plant hormone status in the different developmental processes in plants. Because the PAs, GAs, and CKs play a key role during floral induction in numerous

species, it is essential to study the endogenous levels of these plant hormones as physiological parameters that may be indicators of the developmental state during the floral transition in azalea. The main objective of this work was therefore to characterize the levels of endogenous PAs, GAs, and CKs in floral and vegetative buds during stages of vegetative growth and flowering in azalea, to establish each as a potential marker for floral development.

Material and Methods

Plant Material

Four-year-old plants from two cultivars of azalea with different growth capacities (Blaauw's Pink and Johanna with high and low vegetative growth, respectively) and with a known ability to bloom under long-day conditions were used for the analysis. Rooted cuttings of azalea were supplied by Fomento Vegetal, S.A. (Asturias, Spain) in May 2003. The plants were grown outside in experimental fields belonging to SERIDA (Servicio Regional de Investigación y Desarrollo Agroalimentario de Asturias) in Villaviciosa, Spain (5°25′ W, 43°28′ N) with a planting density of 3 plants/m^2. They were fertilized/irrigated three times a week with the following nutrient ratio (N-P-K): 1-0.5-0.5 from May to July, 1-5-0 in August, 1-2-2 in September, and 1-1.5-3 from October to April. Each spring the plants were transplanted into bigger pots containing *Pinus* bark as the substrate, supplemented with slow-release fertilizers (3.5 g/l, Basacote Plus 6 M, Compo).

Vegetative buds, in growth (Fig. 1), and floral buds, which were already producing floral organs (Fig. 1) (Meijón and others 2009b), were selected separately from 20 different plants per cultivar during spring outgrowth, 1 month before bloom (Meijón and others 2010). Samples were lyophilized and stored at –20°C until analysis.

Extraction, Purification, and Separation of PAs

The PAs Put, Spd, and Spm were extracted, separated, and quantified according to the method described by Fraga and others (2002). In brief, powdered material (25 mg DW) was extracted in 1 ml of cold 5% perchloric acid (PCA) in an ice bath for 15 min. After extraction, samples were centrifuged at 27,000 *g* for 20 min. The supernatant containing the free PAs (PAs-S) was stored until dansylation. Aliquots of 300 µl of supernatant were acid-hydrolyzed with 300 µl of 12 M HCl at 100°C for 20 h to release the PCA-soluble fraction conjugated to low-molecular-weight PAs (PAs-SH). The resulting mixture was filtered through a 45-µm filter (Millipore) and dried in a stream of N_2 at 80°C. After dansylation of both fractions, PAs were separated and quantified by reverse-phase high-performance liquid chromatography (HPLC) in a Waters 600 liquid chromatography device equipped with a fluorescence detector (Waters 474), following the method described by Fraga and others (2002). A Kromasil 100 reverse-phase column (Kromasil C18, 5 µm, 150 × 4.6 mm; 5 µm) was used. The mobile phase was acetonitrile and Milli-Q water. The PAs were separated with the following elution gradient: 68% acetonitrile for 4 min, then a 1-min linear gradient to reach 100% acetonitrile, which was kept constant for 4 min, and finally a 1-min linear gradient to 68% acetonitrile. The flow rate was kept constant at 1.5 ml/min. Eluted samples were excited at 350 nm and fluorescence emission was monitored at 500 nm. For quantitative purposes, 1,7-diamine heptane (HTD) was used as an internal standard. Three biological replicates were analyzed per sample.

Fig. 1 Vegetative and floral bud of Johanna cultivar. *Vb* vegetative bud; *Fb* floral bud

Losses during extraction and dansylation of samples were determined by the addition of 0.925 kBq of [C^{14}]-Spm (Amersham Ibérica®) to the initial extract of each sample. Results were corrected to account for the total losses determined for each sample.

Extraction, Purification, and Separation of GAs and CKs

GAs and CKs were extracted according to the methods described by Villacorta and others (2008) with several modifications. Samples (100 mg DW) were homogenized in

8 ml of extraction buffer (MeOH:HCOOH:H_2O + 0.001% BHT) (80:1:19 v/v) and extracted overnight at 4°C; prior to extraction, 50 ng of [$^2H_2$]GA_1, [$^2H_2$]GA_3, [$^2H_2$]GA_4, [$^2H_2$]GA_7, [$^2H_2$]GA_9, [$^2H_2$]GA_{20} (Olchemim®) and 0.015 TBq of 8-[^{14}C]-benziladenine (Amersham Ibérica®) were added to calculate GAs and CKs losses. Samples were centrifuged (1100 *g*, 20 min, 4°C), re-extracted for 1 h in 5 ml of extraction buffer, and recentrifuged. Both supernatant fractions were pooled and passed through a C18 SPE (BondElut® Varian, 500 mg, 6 ml, 30/PK) to remove pigments. Organic solvents were removed under reduced pressure at 40°C, the pH of the water phase was adjusted to 3, and H_2O at pH 3 was added to a final volume of 20 ml. The sample was applied onto a 10-ml column of monoammonium ionic-form cellulose phosphate (Sigma), equilibrated with acidified water (pH 3 with acetic acid), with a C18 SPE cartridge (BondElut® Varian, 500 mg, 6 ml, 30/PK) coupled underneath (Fig. 2). The cartridges were rinsed with 20 ml of distilled water at pH 3. The cellulose phosphate was eluted with 10 ml of 2 M NH_4OH, recovering the CK bases and nucleosides (basic fraction), whereas the C18 cartridge was eluted with 10 ml 80% MeOH + 0.1% HCOOH, recovering the GAs (acidic fraction). Both fractions were further purified separately.

The basic fraction (CKs) was purified again through a C18 SPE cartridge (BondElut® Varian, 500 mg, 6 ml, 30/PK). It was then eluted with 10 ml of 80% MeOH, recovering the CKs. Organic solvents were removed under reduced pressure at 40°C, the pH of the water phase was adjusted to 7, and 25 mM phosphate buffer saline (PBS) at pH 7 was added to a final volume of 10 ml. The fraction was further purified using an immunoaffinity column for isoprenoid CKs (Olchemim®). CKs were eluted with methanol and subsequently the solvent was evaporated. Finally, the fractions were resuspended in triethylamine acetate buffer (TEAA, 40 mM, pH 7). Separation of the isoprenoid-type CKs zeatin (Z), zeatin riboside ([9R]Z), dihydrozeatin ((diH)Z), dihydrozeatin riboside ([9R](diH)Z), isopentenyladenine (iP), and isopentenyladenine riboside ([9R]iP) was accomplished by HPLC in a Waters 600 liquid chromatography device equipped with a diode array detector (Waters 996) according to the method of Moncaleán and others (2005). The column used was a Kromasil C18 (150 × 4.6 mm; 5 µm) and the flow rate was 1.5 ml/min. The mobile phase was acetonitrile in a linear gradient from 5 to 20% acetonitrile (v/v) over 20 min. The eluted CKs were monitored at 265 and 275 nm and fractions of 2 ml were collected at 1.5-min intervals and reduced to dryness by speed-vac concentration. Quantitative determination of isoprenoid-type CKs was accomplished by enzyme-linked immunosorbent assay (ELISA). The dried HPLC fractions were resuspended in Tris-saline buffer (TSB, 25 mM, pH 7.5) and quantified with polyclonal rabbit antibodies raised in our laboratory (Fernández and others 1995). We used anti-[9R]Z to measure [9R]Z and Z, anti-[9R](diH)Z to measure [9R](diH)Z and (diH)Z, and anti-[9R]iP to measure [9R]iP and iP. Three biological replicates were analyzed per sample.

The acidic fraction (GAs) was purified through a C18 SPE cartridge (BondElut® Varian, 500 mg, 6 ml, 30/PK) and adsorbed onto 0.5 g of celite 545 (Prolabo®, Paris, France), dried under a stream of hot air, purified throughout a column of silicic acid (ICN Silica 32-100, active 60 Å, ICN Biochemicals GmbH) (5 g of SiO_2 in 50 ml of ethyl acetate:n-hexane 95:5 saturated with 0.5 M aqueous formic acid), and brought to a dry state. All GAs were analyzed by HPLC coupled to a triple-quadrupole mass spectrometer (1200L Varian LC/MS/MS) at the Research Support Service, University of Córdoba. Two biological replicates were analyzed per sample.

Statistical Analysis

The data were analyzed using SigmaStat 3.1 software. To analyze the results for the PAs and CKs, two one-way ANOVAs (analyses of variance) per hormone followed by the Holm–Sidak test were used (overall significance level was $p \leq 0.05$), the type of bud and hormone being the factors analyzed. For specific PA ratios, a one-way ANOVA and the Holm-Sidak test were used, the type of bud being the factor analyzed.

Results

Endogenous Levels of PAs in Floral and Vegetative Buds

The endogenous content of PAs-S in the two types of buds and in both cultivars, Blaauw's Pink and Johanna, is shown in Fig. 3a and b. In both cultivars, the levels of PAs-S (Put, Spd, and Spm) were always significantly higher in the vegetative bud, the greatest differences being in the levels of Put and Spm, with Spd being the major PA in both types of buds and cultivars.

In contrast, PAs-SH (Fig. 3c and d) were significantly higher in the floral buds, except for Put, which showed no significant differences between floral and vegetative buds in either cultivar. Again, the long-chain PAs predominated, although in this fraction Spm was the main PA, with the values of Spd and Spm three or four times higher in the floral buds.

The values of all ratios between the free and conjugated forms of PAs were higher in floral buds (Table 1) in both cultivars.

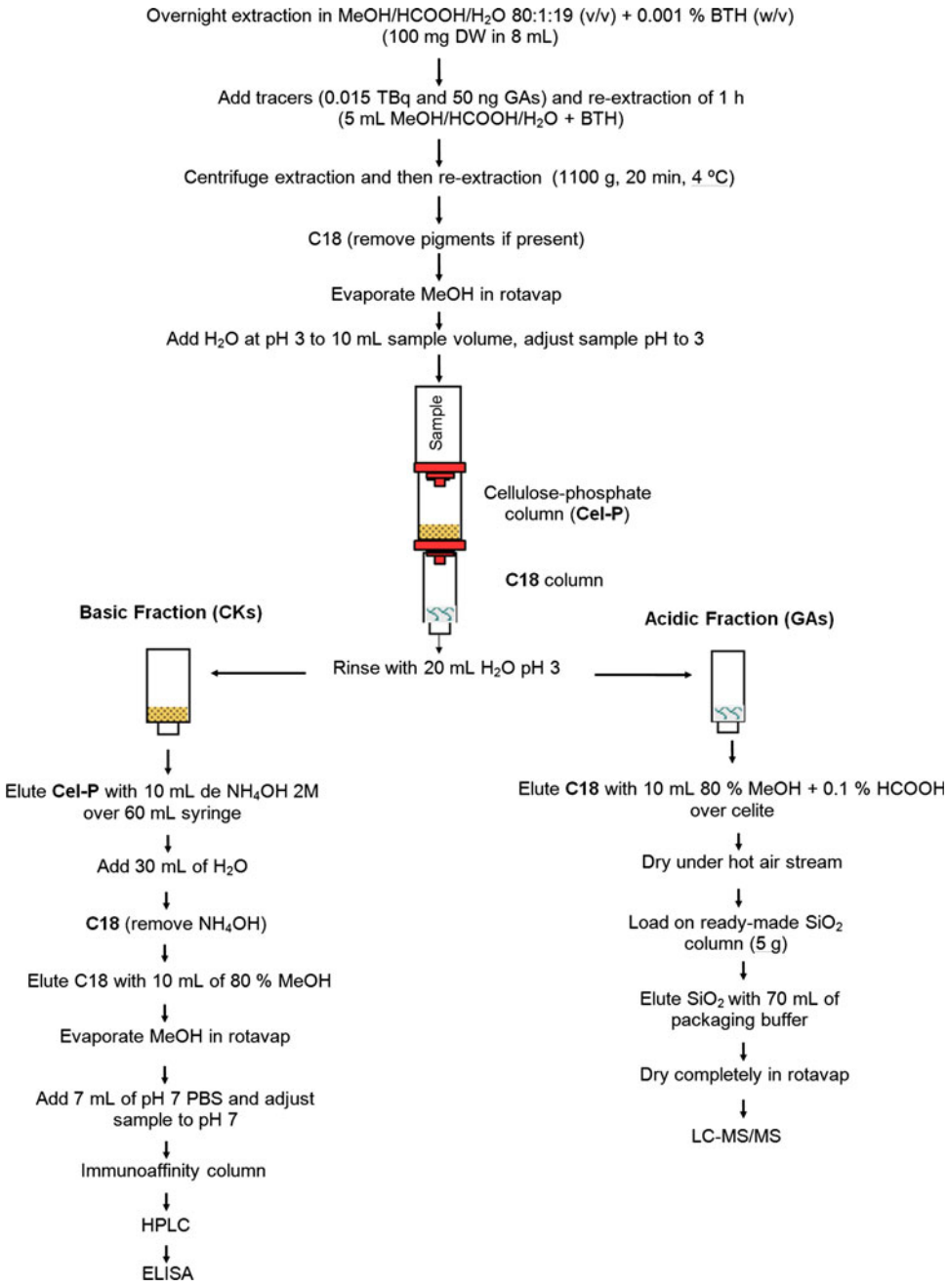

Fig. 2 Scheme of the protocol used for the extraction of endogenous GAs and CKs. For details see text

Endogenous Levels of GAs in Vegetative and Floral Buds

The distribution pattern of the endogenous levels of GAs was similar in both cultivars, although the Blaauw's Pink, which has a longer stem length than Johanna, had higher GA levels in the two types of buds (Fig. 4).

In both cultivars, GAs from the early nonhydroxylation pathway (GA_4, GA_7, and GA_9) were present at higher levels in floral buds, in contrast to the GAs from the early 13-hydroxylation pathway (GA_1, GA_3, and GA_{20}), which were higher in the vegetative buds. Nevertheless, GA_9 was the major GA in the two types of buds in both cultivars. All other tested GAs had values below 30 pmol/g FW except GA_7, which gave values around 76 pmol/g FW in floral buds of Blaauw's Pink. It is important to note that GA_9 reached higher values than its metabolites GA_4 and GA_7, whereas the levels of GA_1 and GA_3 were similar to their precursor GA_{20} in both bud types and cultivars.

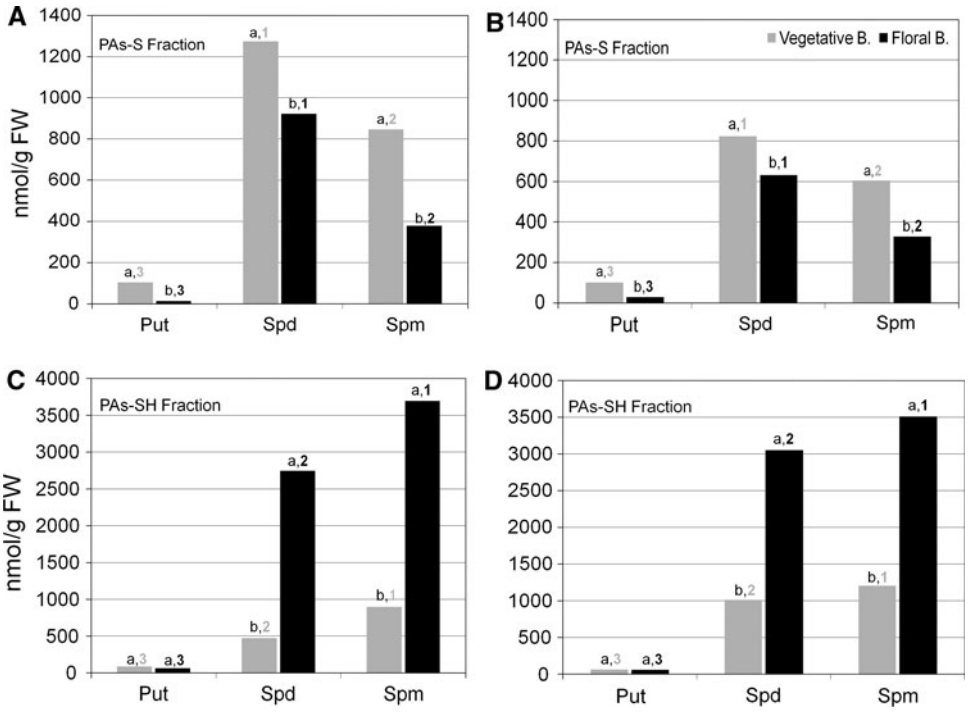

Fig. 3 Levels of free and conjugated PAs in the floral and vegetative buds from two cultivars of azalea (nmol/g FW). **a** Free PAs in Blaauw's Pink. **b** Free PAs in Johanna. **c** Conjugated PAs in Blaauw's Pink. **d** Conjugated polyamines in Johanna. Different letters indicate significant differences between types of buds within each hormone and cultivar ($p \leq 0.05$, $n = 3$). Different numbers indicate significant differences between hormone within each type of bud and cultivar ($p \leq 0.05$, $n = 3$). *PAs-S* free polyamines, *PAs-SH* low-molecular-weight polyamine conjugates, *Put* putrescine, *Spd* spermidine, *Spm* spermine

Table 1 Specific PA ratios in vegetative and floral buds of Blaauw's Pink and Johanna cultivars

Cultivar	Ratios	Vegetative buds	Floral buds
Blaauw's Pink	PAs-S/PAs-SH	1.69 a	0.20 b
	Put-S/Put-SH	1.22 a	0.21 b
	Spd-S/Spd-SH	2.50 a	0.30 b
	Spm-S/Spm-SH	1.01 a	0.10 b
Johanna	PAs-S/PAs-SH	0.67 a	0.15 b
	Put-S/Put-SH	1.56 a	0.51 b
	Spd-S/Spd-SH	1.01 a	0.20 b
	Spm-S/Spd-SH	0.51 a	0.09 b

PAs-S free polyamines, *PAs-SH* low-molecular-weight polyamine conjugates, *Put* putrescine, *Spd* spermidine, and *Spm* spermine

Within each ratio and cultivar, different letters indicate significant differences ($p \leq 0.05$, $n = 3$)

Endogenous Levels of CKs in Vegetative and Floral Buds

The endogenous content of CKs is shown in Fig. 5. The CK profile was also similar in both cultivars, although the levels of all the CKs were always higher in the Johanna cultivar, in both vegetative and floral buds.

All tested CKs presented significantly higher levels in the floral bud with the exception of [9R]Z, which showed the lowest content in both cultivars and bud types. Among the tested CKs, [9R]iP was the highest in the floral buds. In general, the free forms of the CKs (Z, (diH)Z, and iP) showed greater differences between the two types of buds than their derivative ribosides ([9R]Z, [9R] (diH) Z, [9R] iP) in both Blaauw's Pink and Johanna.

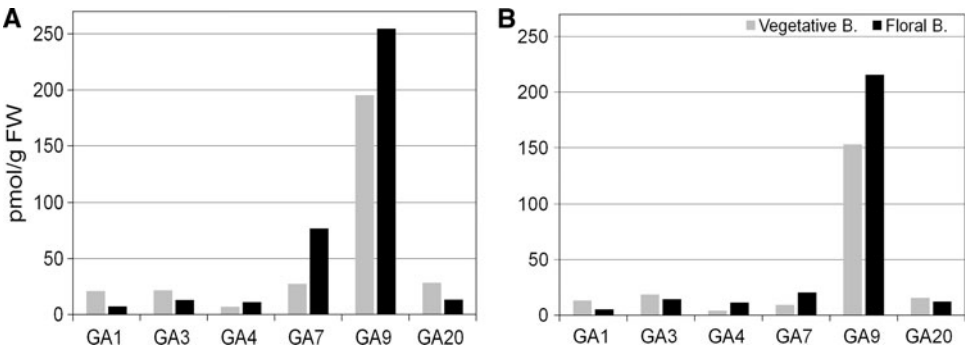

Fig. 4 Levels of endogenous GAs in the floral and vegetative buds from two cultivars of azalea (pmol/g FW). **a** Blaauw's Pink. **b** Johanna. The data represent the average of two analytical replicates with similar results (not statistically analyzed)

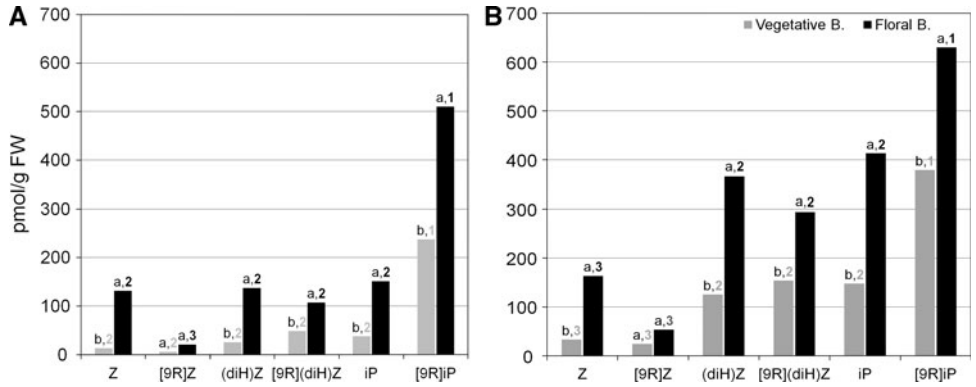

Fig. 5 Endogenous levels of CKs in floral and vegetative buds from two cultivars of azalea (pmol/g FW). **a** Blaauw's Pink. **b** Johanna. Different letters indicate significant differences between types of buds within each hormone and cultivar ($p \leq 0.05$, $n = 3$). Different numbers indicate significant differences between hormone within each type of bud and cultivar ($p \leq 0.05$, $n = 3$). *Z* zeatin, *[9R]Z* zeatin riboside, *(diH)Z* dihydrozeatin, *[9R](diH)Z* dihydrozeatin riboside, *iP* isopentenyladenine, *[9R]iP* isopentenyladenine riboside

Discussion

The hormonal regulation of floral induction has been assessed in numerous studies (Blázquez and León 2006; Wilkie and others 2008) and, based on those results, the application of exogenous plant growth regulators (PGRs) is the usual practice for improving flowering (Marosz and Matysiak 2005; Meijón and others 2009a). Most of the theoretical models for floral stimulus are based on the existence of multiple, nonhormonal and hormonal factors that interact and act in coordinated fashion during floral bud development (Corbesier and Coupland 2006). PAs (Sood and Nagar 2004), GAs (Eriksson and others 2006; King and others 2006), and CKs (Chang and Chang 2003; Corbesier and others 2003) are the plant hormones most closely associated with the success of the floral transition in numerous species.

In azalea, our results showed differences in the levels of plant hormones during bud differentiation. With respect to PAs, vegetative buds were characterized by high levels of the free forms, whereas PAs conjugated to molecules of low molecular weight (mainly Spm) predominated in floral buds. During vegetative growth and the early stages of flower development, high levels of PAs-S are related to active cell division (Sood and Nagar 2004). In contrast, PAs-SH play a key role in differentiation and maturation processes and correlate with advanced stages of flowering (Fraga and others 2002, 2003, 2004), as in the floral buds of azalea.

Free Put is the most abundant PA in tissues of *Pinus pinaster* during the phases of proliferation (Klimaszewska and others 2009), its increase being associated with growth and cell division, which could explain the high levels observed in the vegetative buds of azalea. Conversely, a decrease in Put is related preferentially to maturation and flowering (Fraga and others 2002, 2003, 2004), in agreement with our current findings in floral buds. On the other hand, the high levels of Spd and Spm found in the floral buds of azalea are associated with cell differentiation and advanced stages of flowering in different species, indicating the success of the floral transition (Sood and Nagar 2004; Pritsa and Voyiatzis 2005).

The relative proportions of free and conjugated PAs shown in azalea (Table 1) support the different roles of these regulators in flowering and vegetative growth, as previously reported by Sood and Nagar (2004), who also observed an increase in conjugated PAs and a decrease in free PAs during flower development in the rose.

With respect to GAs, the levels of all the forms analyzed varied between the two types of buds in both cultivars, with higher levels of all GAs in Blaauw's Pink. The results from this study suggest that GA_4, GA_7, and GA_9 (GAs from the non-13-hydroxylation pathway), which were found at higher levels in the floral bud, could have a role in floral differentiation, whereas GA_1, GA_3, and GA_{20} (GAs derived from the early 13-hydroxylation pathway), which were highest in the vegetative phase, could be more important for vegetative development. Moreover, these data are consistent with the observations of other authors in rice, *Matthiola incana*, *Pinus radiata*, and *Lolium temulentum* (Kobayashi and others 1990; Hisamatsu and others 2000; Fernández and others 2003; King and others 2006). They have suggested that there is an essential role of GAs from the non-13-hydroxylation pathway in the regulation of reproductive development, whereas the GAs from the early 13-hydroxylation pathway seem to play a more decisive role during vegetative growth and stem elongation.

The high levels of GA_9, a precursor of GA_4 and GA_7 (Hedden and Phillips 2000), as well as of GA_7 in both cultivars, suggest a high activity of the non-13-hydroxylation pathway in these species. High levels of GA_9 could act as a reservoir for the rapid synthesis of GA_7, which together with GA_4 seem to be the GAs that play the most

essential role in azalea anthesis. On the other hand, the early 13-hydroxylation pathway seems to be less efficient in azalea because the levels of the active GAs, GA_3 and GA_1, and their inactive precursor GA_{20} (Davis and others 1998; Hedden and Phillips 2000) were much lower.

The higher levels of CKs found in floral buds are consistent with results of previous studies of other species that indicated a general increase in endogenous CKs during flower development (Corbesier and others 2003; Sim and others 2008) and the promotion of flowering by exogenous application of these PGRs (Chang and Chang 2003; Blanchard and Runkle 2008).

[9R]iP is the first form in the de novo biosynthetic route of the CKs, which could perhaps explain the high levels of this hormone in both vegetative and floral buds. iP-type species reached the highest levels of all CKs in floral buds in both cultivars, whereas Z-type species showed the lowest values in the vegetative bud and greater differences between the two types of buds. Therefore, although the evolution of CKs during the floral transition has not been analyzed, an increase in the biosynthesis of these hormones could be assumed during the floral transition in azalea. According to Corbesier and others (2003), this increase in the iP forms of CKs is correlated with early events in floral transition after the implementation of photoperiods exceeding 16 h of light.

On the other hand, the large increase in free bases in floral buds (especially of the Z-type species) could be related to the activity of CKs. Experiments have shown that the free form of *trans*-zeatin, but not its derivative riboside or ribotide, binds directly to the receptor CYTOKININ RESPONSE 1 (CRE1) (Yamada and others 2001), indicating that the free base is the only active form of this CK.

Finally, although the CK profile was similar in both cultivars, the concentration of endogenous CKs was always higher in the cultivar Johanna (unlike the PAs and GAs). This is probably related to the physiological development of this cultivar, which has a higher density of branches and less stem elongation compared to Blaauw's Pink (Meijón and others 2009a).

The CKs are essential for stimulating cell division in meristems. The elevated number of cell divisions that take place during bud development led Chang and others (1999) to suggest the possibility that these phytohormones play an essential role in floral induction, a process that involves reorganization and cell reprogramming prior to floral differentiation. Cell reprogramming should begin with an increase in the rates of cell division, which is essential for triggering the new morphogenic pathway, and CKs may play a role in this process.

There is much evidence about the interrelationships between these phytohormones at both the metabolic and the physiological levels. Thus, plants with high levels of total Put (the amounts of free and conjugated Put) show a decrease in the endogenous levels of GAs as well as dwarfism and late blooms (Alcázar and others 2005). Alcázar and others (2005) were able to demonstrate that Put accumulation in *Arabidopsis* affects the metabolism of GAs through the repression of the biosynthetic steps catalyzed by GA 20-oxidase and GA 3-oxidase, and that this inhibition affects plant growth and floral development. Our results seem to confirm this because in the vegetative buds high levels of Put were associated with a decrease in the GAs from the non-13-hydroxylation pathway, whereas in floral buds the increase in GA from the early 13-hydroxylation pathway was associated with low levels of Put. On the other hand, Villacorta and others (2008) also interrelated changes in the levels of GAs with variations in the levels of CKs during the floral transition in hops, finding that increases in GA_3 and GA_4 were accompanied by high levels of [9R] iP. Again, our results seem to confirm theses observations because in floral buds elevated levels of GAs were associated with increases in all of the CKs, especially the iP-type species.

In conclusion, our results showed for the first time in azalea a specific pattern in endogenous hormonal profiles of PAs, GAs, and CKs in both vegetative and reproductive bud development, which may be relevant for future research on the control of flowering by exogenous hormone applications.

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