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Changes of edogenous hormone levels during short-day inductive floral initiation and inflorescence differentiation of *Chrysanthemum morifolium* 'Jingyun'

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Abstract

Duration of various stages of inflorescence differentiation and hormone levels in apical buds eventually decide the harvest time, flower uniformity and quality of ornamental plants. The progress in inflorescence differentiation process in the chrysanthemum cultivar 'Jingyun' can be divided into nine stages. Following short day induction, it took 4d to reach the growing point hypertrophy stage, 8d to finish involucre primordia differentiation, 12d to finish floret primordia differentiation and 10d to finish crown formation. Under inducible conditions, the level of indole acetic acid (IAA) initially decreased, but maintained a relatively high level during the whole period of inflorescence differentiation. Both N6-(Δ2-isopentenyl)-adenosine (iPA) and abscisic acid (ABA) levels increased markedly during floral induction and floral initiation, peaking at the final involucre primordia differentiation stage. Thereafter, the iPA level remained high, while the ABA level decreased. A large decrease in the level of gibberellic acid (GA_3) occurred during floral induction, but this recovered by the final floret primordia differentiation stage. In conclusion, various stages of inflorescence differentiation take different time-span, the floret primordia differentiation takes the longest time-span, while time-span for involucre primordia differentiation is shortest. Moreover, an appropriate amount of IAA appears to be necessary for inflorescence differentiation, and a stable GA_3 and ABA level for crown formation. iPA plays a positive role both in floral induction and inflorescence differentiation.

Keywords: Chrysanthemum; Floral induction; Inflorescence differentiation; Plant hormones

Introduction

The transition from vegetative to reproductive growth is a critical stage during the plant development. The identity of mechanisms involved in this transition have been speculated by Bernier (1988), but as yet, the overall process remains unclear. Hormones, however, have long been known to play an essential role in floral induction and differentiation. The relationships between endogenous hormone levels and flower bud formation have been

widely investigated (Villacorta et al., 2008; Ding et al., 1999; Ulger et al., 2004). Auxin, as a key component, has been repeatedly implicated in floral induction (Smulders et al., 1988; Okada et al., 1991). A low concentration of auxin rather than a high concentration may be important for flower formation (Bernier, 1988). The level of the auxin indole acetic acid (IAA) increases during flower development in both *Coffea arabica* (Schuch et al., 1994) and *Polianthes tuberosa* (Ding et al., 1999). While, gibberellic acid (GA_3) and abscisic acid (ABA) levels vary during flower bud development rather differently. Inhanced level of ABA in *Citrus unshiu* increased the proportion of leafless inflorescences as well as the number of flower buds per node (Koshita et al., 1999), whereas it has little visible impact on flower induction in *Pharbitis nil* (Wijayanti et al., 1997). In *Olea europaea*, gibberellin-like substances accumulate progressively during the low temperature period, reaching a maximum shortly before floral initiation (Badr et al., 1970); however, others have reported that in this species, although a high GA_3 level is inhibitory, a high GA_4 level has a positive effect on flower formation (Ulger et al., 2004). A positive effect of high endogenous cytokinin levels on flower formation has been widely reported (Villacorta et al., 2008; Campos and Kerbauy, 2004).

Chrysanthemum (*Chrysanthemum morifolium*) is considered as one of the most popular cut flower and pot plant species. The process of inflorescence differentiation has been divided into nine stages in *D. occidentali-japonense* var. *ashizuriense* (Zhang et al., 1998), with the time required to complete each stage being highly cultivar-dependent. IAA levels decrease in the buds before the appearance of flower meristem (Harada and Nitsch, 1959), while the applied exogenous auxin just before flowering inhibits floral initiation (Tsukamoto et al., 1968). The effects of hormonal levels on inflorescence differentiation have not been explored, although it has been suggested that GA_3 is required for flowering of a short-day chrysanthemum plant (Sumitomo et al, 2009). The roles, if any, of ABA and cytokinin in floral transition and inflorescence differentiation and development have yet not to be defined. Thus, in the present study, we aimed to clarify time-span required for each stage of inflorescence differentiation and the relationship between inflorescence differentiation and hormone levels in the chrysanthemum cultivar 'Jingyun'. Here we firstly reported that chrysanthemum 'Jingyun' takes 8d to complete involucre primordia differentiation, 12d to complete floret primordia differentiation and 10d to complete crown formation. An appropriate amount of IAA appears to be necessary for inflorescence differentiation, and a stable GA_3 and ABA level for crown formation. iPA plays a positive role both in floral induction and inflorescence differentiation. These findings will do good to the manipulation of flowering process.

Materials and Methods

Plant material

'Jingyun', a short day plant (SD), is a commercial cut chrysanthemum cultivar. Plant material was obtained from the Germplasm Resource Preserving Center, Nanjing Agricultural University. Rooted cuttings at the six leaf stage were planted in 15 cm pots in a standard peat mixture on Sep. 9, 2008, and grown in a greenhouse (day/night temperature ~25/18 ºC, relative humidity ~70%). In order to prevent flower induction, the plants were

maintained under long day (LD) conditions for six weeks (from Sep. 9 to Oct. 20, 2008) supplemented by 6h natural light with 60W incandescent light bulbs. Thereafter, flowering was induced by applying short day conditions (from Oct. 21, 2008 to the end of experiment), achieved by covering the plants with a black satin cloth for 5h during the period of supplemental illumination. The control plants still were maintained under LD conditions.

Inflorescence differentiation

Twenty apical buds were collected every two days. The young leaves were detached from the bud, and inflorescence differentiation was observed under a dissecting microscope (İsfendiyaroğlu and Özeker, 2009). The definition of various differentiation stages follows the description by Zhang et al. (1998). The day when 80% of sampled apical buds had reached a given stage was considered as the initiation time for this stage. Apical buds of non-induced plants continuously grown under LD conditions were concurrently collected as control samples.

Measurement of endogenous hormone levels

Apical buds at the nine inflorescence stages were frozen in liquid nitrogen, and then crushed, lyophilized and stored at-20 ºC until required. The methods used for the extraction and analysis of IAA, N6-(Δ2-isopentenyl)-adenosine (iPA), ABA and GA_3 followed the protocol of Zhang et al. (2008), with minor modifications. Briefly, ~0.5 g lyophilized plant material was immersed in 5ml cold 80% (v/v) methanol containing 10 $mg.l^{-1}$ butylhydroxytoluene at 4 ºC for 4h. Particulates were removed by centrifugation at 10k x g for 15 min at 4 ºC, and the supernatant collected and passed through a C^{18} Sep-Pak cartridge (Waters Corp., Millford, MA, USA). A 200µl aliquot of the eluate was dried under a stream of nitrogen gas, and the residue dissolved in 200 µl phosphate buffered saline (1.3 mM NaH_2PO_4, 8.7 mM Na_2HPO_4, 0.14 M NaCl, pH 7.4) prior to analysis by ELISA. ELISA kits directed at IAA, iPA, ABA and GA_3 were obtained from Crop Chemical Control Institute, China Agriculture University. Absorbances of the ELISA reactions were measured in a microplate reader at 490 nm. Apical buds of non-induced plants were sampled concurrently as a control. Hormone contents (ng per gram fresh weight (fw)) were calculated from the mean of three replicates, where each replicate comprised six apical buds.

Data analysis

One-way analysis of variance (ANOVA) was used to test the significant difference among treatments, and multiple comparisons were performed with SPSS v11.5 (SPSS, Chicago, IL, USA) with the least significant difference set at P=0.05 (Ahmadikhah et al., 2008).

Results

Inflorescence differentiation

In order to monitor the inflorescence differentiation process and to determine the time-span for each differentiation stage of 'Jingyun', we tracked the whole process by using a dissecting microscope. The inflorescence differentiation 'Jingyun' can be divided into nine stages, namely, vegetative (S1, a flat shoot apex with leaf primordia), growing point hypertrophy (S2, the shoot apex formed a hemispherical shape), initial involucre primordia differentiation (S3, involucre primordia differentiated at the periphery of the dome), final involucre primordia differentiation (S4, the dome became flat and many involucre differentiation completed), initial floret primordia differentiation (S5, floret primordia differentiated at the periphery of the dome), final floret primordia differentiation (S6, more than 50% of dome was covered with floret primordia), initial crown formation (S7, the petal primordia differentiated in the floret primordia at the base of dome), metaphase crown formation (S8, the floret primordia covered the dome and further development of each floret progressed acropetally) and final crown formation (S9, the florets with closed petals filled the dome) (Figure 1). Inflorescence differentiation began within 4d of exposure to SD (Figure 2). The time required to pass from S1 to S2, from S2 to S3, from S3 to S4, from S6 to S7 and from S8 to S9 was 4d; from S4 to S5 and S5 to S6 was 6d; and from S7 to S8 was 2d (Figure 2). It took 8d to complete involucre primordia differentiation, 12d to complete floret primordia differentiation and 10d to complete crown formation. Namely, 30d from floral initiation (S2) to complement of inflorescence differentiation (S9) for cut chrysanthemum 'Jingun'. No inflorescence differentiation occurred in plants grown under non-inductive LD conditions. Inflorescence differentiation underwent 9 stages, and time-span for various stages varied for one another, the floret primordia differentiation took the longest time-span.

Hormone levels in the apical buds

To clarify the possible roles of plant hormones during inflorescence differentiation of chrysanthemum, levels of IAA, iPA, ABA and GA_3 were detected during the whole process. Endogenous IAA content was highest (4088 $ng.g^{-1}$ fw) during S1 (Figure 3). Thereafter, it declined sharply to 1701 $ng.g^{-1}$ fw at S2, recovered to 2476 $ng.g^{-1}$ fw at S3, and then remained rather stable throughout the subsequent stages. The IAA level in all SD treated apical buds was uniformly less (0.40 to 0.68 fold) than the control non-induced buds. The endogenous iPA content of the apical buds increased markedly after floral induction, reaching a peak at S4, and remained high through to S9 (Figure 3). iPA level at S4 (159 $ng.g^{-1}$ fw) was 1.97 fold to that presented during S1 (81 $ng.g^{-1}$ fw). From S2 onwards, the level of iPA in the induced apical buds was 1.56 to 2.08 fold higher than that in non-induced ones. Consequently, high levels of cytokinins were required for the formation of the involucre, the floret primordia and the crown. Endogenous ABA level in the apical buds increased from 2848 $ng.g^{-1}$ fw at S1 to 6063 $ng.g^{-1}$ fw by S4, fell to 4020 $ng.g^{-1}$ fw by S7, and thereafter remained relatively constant (Figure 3). Between S2 and S9, the ABA level in the induced buds was uniformly higher (by 1.19 to 2.11 fold) compared with that in the

non-induced ones. As a result, endogenous ABA promotes floral transition, while ABA level differed at various stages of inflorescence differentiation. Finally, the endogenous GA_3 content in SD treated apical buds decreased steeply after induction, falling from 988 $ng.g^{-1}$ fw at S1 to 459 $ng.g^{-1}$ fw by S4. It was in minimum 449 $ng.g^{-1}$ fw at S5. By S6, the level had risen to 669 $ng.g^{-1}$ fw, where it remained throughout the subsequent stages (Figure 3). The GA_3 level in the induced buds was uniformly lower (between 1.12 and 2.18 fold) compared with that in the non-induced ones. Therefore, a stable GA_3 level appears to be necessary for crown formation.

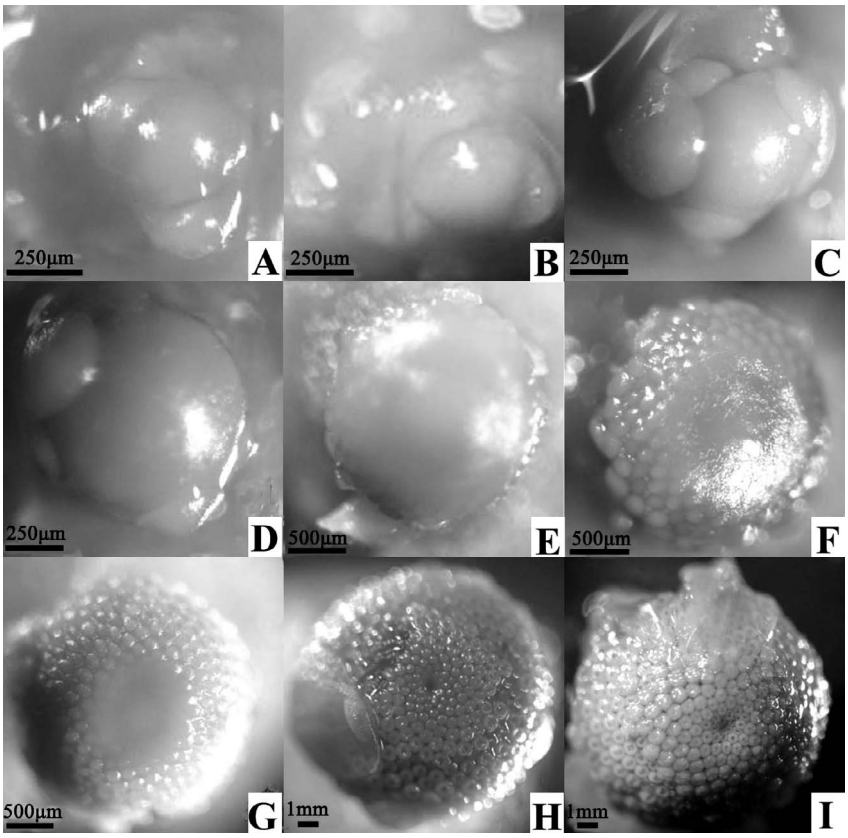

Figure 1. Stages of inflorescence differentiation in the chrysanthemum cultivar 'Jingyun'. (A): vegetative (S1，a flat shoot apex with leaf primordia); (B): growing point hypertrophy (S2, a hemispherical shape shoot apex); (C): initial involucre primordia differentiation (S3, involucre primordia differentiated at the periphery of the dome); (D): final involucre primordia differentiation (S4, flat dome and involucres differentiated); (E): initial floret primordia differentiation (S5, floret primordia differentiated at the periphery of the dome); (F): final floret primordia differentiation (S6, floret primordial covered >50% of dome); (G): initial crown formation (S7, the petal primordia differentiated); (H): metaphase crown formation (S8, floret developed and progressed acropetally); (I): final crown formation (S9, the florets with closed petals over the dome).

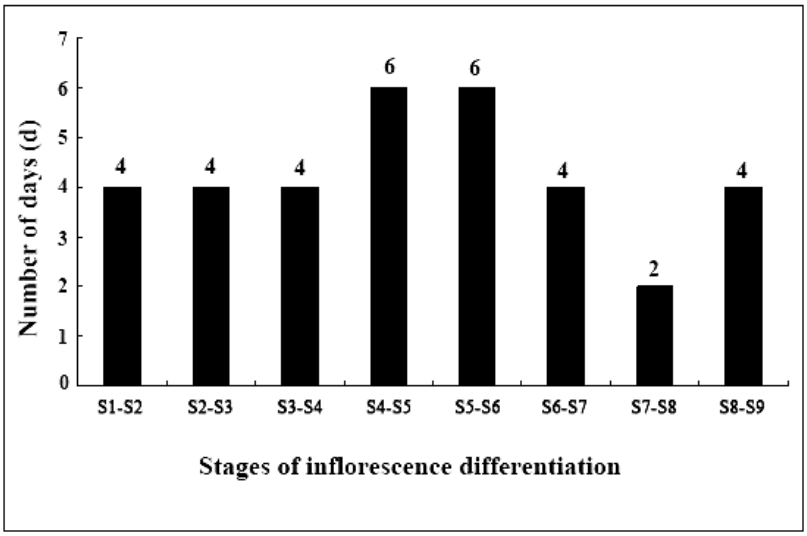

Figure 2. Days required for each stage of inflorescence differentiation in chrysanthemum 'Jingyun'. Numbers on the top of each pillar represent the time-span for each stage. S1 to S9 represent nine different stages of inflorescence differentiation of 'Jingyun'.

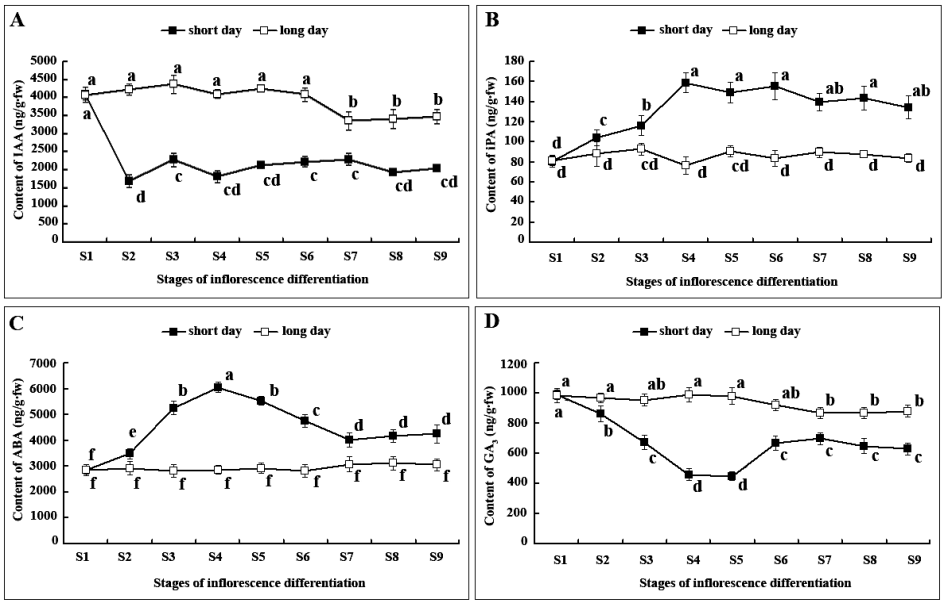

Figure 3. Different endogenous hormone levels during the inductive inflorescence differentiation process in chrysanthemum 'Jingyun'. (A): IAA levels; (B): iPA levels; (C): ABA levels; (D): GA_3 levels. S1 to S9 represent nine different stages of inflorescence differentiation of 'Jingyun'. Small letters along the lines represent the significant difference at 0.05% level.

Discussion

The majority of plants switch from vegetative to reproductive growth in response to a change in their environment, commonly temperature, photoperiod or a combination of these. For short day plants, photoperiod is the over-riding signal, so flower growers can manipulate flowering time by artificial extension or foreshortening of the day length. Here, we show that the chrysanthemum cultivar 'Jingyun' responds to photoperiodic induction within 4d of exposure to short days, which compares well with the behaviour of 'White Horim' that became committed to flowering after a 3-7d exposure (Van et al., 1984).

After floral induction, the four endogenous hormone levels significantly changed. Harada and Nitsch (1959) have shown that level of IAA decreases in chrysanthemum buds just prior to the appearance of the floral meristem, and a similar pattern was observed in *Pharbitis nil* (Wijayanti et al., 1997). However, in other species e.g., *Coffea arabica* (Schuch et al., 1994), IAA levels increased during flower development. In *Polianthes tuberosa*, the early floral initiation stage is correlated with a reduction in the content of unbound IAA, but the level rises during the floral differentiation stage (Ding et al., 1999). Here, we observed that the IAA content in the chrysanthemum apical bud decreased significantly after floral induction, which suggests that IAA acts as a negative regulator of floral induction. However, the stable IAA content during the inflorescence differentiation process implies that a certain amount of IAA is required to maintain the growth of the inflorescence.

In *Arabidopsis thaliana*, cytokinins isopentenyladenine and zeatin contents in the LD induced shoot apical meristems were higher than those in non-induced vegetative meristems (Corbesier and Coupland, 2005; Jacqmard et al., 2003). Soon after induction, *Sinapis alba* shoot apical meristems accumulate substantial levels of iPA (Jacqmard et al., 2002). Here, we have shown that in chrysanthemum also, the iPA content increased markedly after floral induction, which implies that a transient increase in cytokinin is associated with the early events of the floral transition. The iPA level reached its peak at the final stage of involucre primordia differentiation and remained high thereafter, a period characterized by rapid cell division. Since cytokinins are primarily associated with the acceleration of cell division, it is reasonable to suppose that the observed high levels of cytokinins at these stages is required for the formation of the involucre, the floret primordia and the crown.

The role of ABA in flowering is not settled. In *Olea europaea*, high ABA concentrations during the induction and initiation periods have been associated with a positive effect on flower formation (Ulger et al., 2004), as well as on flower morphology in *Citrus unshiu* (Koshita et al., 1999). However, in *Polianthes tuberose* corm, endogenous free ABA levels significantly decreased at the floral initiation stage (Su et al., 2002), as also occurred in *Dendrobium* buds after floral induction (Campos and Kerbauy, 2004). Endogenous ABA inhibited/promoted flowering in *Pharbitis nil* under LD/SD conditions, respectively (Wilmowicz et al., 2008). In present study, we observed significant fluctuations in endogenous ABA content as a response to floral induction. Endogenous ABA level during inflorescence differentiation was higher than that in vegetative growth control all throughout, indicating that endogenous ABA promote floral transition, while different stages need different ABA content during the inflorescence differentiation of chrysanthemum.

Gibberellin is a general regulator of floral development (Pharis and King, 1985). In *Arabidopsis*, gibberellin promotes petals and stamens development (Cheng et al., 2004). GA_3, an active form of gibberellin, plays many roles in regulation of plant growth, flowering, cell division and elongation. GA_3 promotes the flowering of *Eustoma grandiflorum* (Kawabata et al., 2009). Here, the gradual decrease in endogenous GA_3 from S1 to S4 in 'Jingyun' grown under induced conditions indicates that lower content of GA_3 appears to be necessary for floral induction and floral initiation in chrysanthemum. Stages S6 to S9 during inflorescence differentiation are characterized by quick formation and growth of florets, especially the growth of petals and formation of stamens. Higher GA_3 level during these stages may be responsible for accelerating the cell division and elongation of florets.

Inflorescence differentiation is an elaborate process at both morphological and physiological levels. Endogenous hormone levels responded to floral induction, and fluctuated throughout the process of floral induction and inflorescence differentiation. An inference can therefore be drawn that IAA, iPA, ABA and GA_3 all participate in the process of floral induction and inflorescence differentiation, although they probably play different roles at specific stages. Both IAA and GA_3 levels fell down in response to floral induction, while those of iPA and ABA rose. The steady level of IAA after its initial decrease suggests that there is a minimal requirement for inflorescence differentiation, and similarly both GA_3 and ABA seem likely to be necessary for crown formation.

Taken together, inflorescence differentiation of 'Jingyun' underwent nine stages, and time-span for different stages varied for one another, five kinds of plant hormones all played roles in the differentiation. Our findings will be helpful for the manipulation of flowering process such as by applying exogenous plant growth regulators.

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