J Plant Growth Regul (2010) 29:487–499
DOI 10.1007/s00344-010-9160-1

Dormancy and Flowering Are Regulated by the Reciprocal Interaction Between Cytokinin and Gibberellin in *Zantedeschia*

Arvind Kumar Subbaraj · Keith Allen Funnell · David John Woolley

Received: 24 January 2010 / Accepted: 7 May 2010 / Published online: 1 July 2010

Abstract Floral productivity of *Zantedeschia* is dependent on the conversion rate of buds to shoots, which is controlled by varying intensities of para- (apical dominance), endo- (dormancy), and ecodormancy. We present evidence of cross-talk between cytokinin and gibberellin in their complementary roles to alleviate bud dormancy and enhance flowering in a perennial geophyte. We assessed the impact of cytokinin and gibberellin, applied alone and in sequential combinations, on bud fate during three phases along the ontogeny of growth, which coincide with the progressive transition of buds from apical dominance to dormancy. Given that cytokinin can stimulate branching and gibberellin can induce flowering in *Zantedeschia*, we measured these phenotypic responses as parameters of bud commitment. The efficacy of cytokinin alone to stimulate branching declined with the transition to dormancy (phase $1 = 3.8 \pm 0.2$ shoots; phase $3 = 1.0 \pm 0.3$ shoots). To sustain branching during this transition, a sequential application of gibberellin was necessary. Gibberellin alone failed to stimulate branching. The efficacy of gibberellin alone to stimulate flowering diminished with the transition to dormancy. Any flowering during this transition occurred only after the sequential application of cytokinin. Cytokinin alone failed to stimulate flowering. Alleviating bud dormancy and enhancing flowering in *Zantedeschia*, achieved by the reciprocal cross-talk between cytokinin and gibberellin, contributes to the pool of evidence drawing common mechanisms between dormancy and flowering and may have commercial implications.

A. K. Subbaraj (✉) · K. A. Funnell · D. J. Woolley
Institute of Natural Resources, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand
e-mail: A.Subbaraj@massey.ac.nz

Keywords Geophyte · Dormancy · Flowering · Cytokinin · Gibberellin · Sequential

Introduction

Calla lilies (*Zantedeschia* sp. Family: Araceae) are gaining commercial importance worldwide as a cut flower and potted flowering plant (Kuehny 2000) and are New Zealand's second most exported cut-flower crop (Funnell and others 2002). A herbaceous perennial geophyte, the growth habit of *Zantedeschia* is sympodial (Funnell 1993), where floral transformation (spathe and spadix) of the apical bud triggers a flowering cascade in one or two axillary buds below it (Halligan and others 2004). This is, however, preceded by the development of buds to shoots, so that the floral productivity of *Zantedeschia* is a direct consequence of the conversion rate of buds to shoots.

The conversion rate of buds to shoots is controlled by an inherent developmental program (Naor and others 2005a), which in turn is executed via different degrees of para- (apical dominance) and endodormancy (dormancy) (Lang 1987; Faust and others 1997). Physical removal of the apical bud (Thimann and Skoog 1933) promotes axillary bud outgrowth (branching) in *Zantedeschia* (Clark and others 1987), thereby demonstrating the existence of apical dominance. Though the progressive establishment of bud dormancy is acknowledged (Corr and Widmer 1988; Naor and others 2005a), the transition of buds from apical dominance to dormancy is not visually represented. To predict the onset and duration of dormancy, a heat-unit accumulation model was developed (Carrillo Cornejo and others 2003).

The growth cycle of *Zantedeschia* is marked by a unique set of morphological attributes. There is a period of active

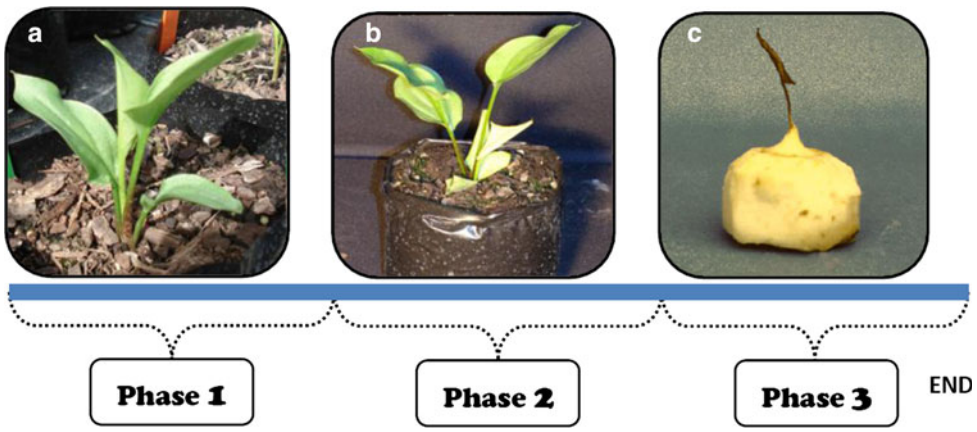

Fig. 1 Single-shoot system of *Zantedeschia* cv. 'Best Gold' showing the three developmental phases of the growth cycle, demarcated based on visual clues. Plant in (**a**) phase 1, with four leaves, during its active growth period, (**b**) phase 2, with seven leaves, after the cessation of new leaf production, and (**c**) phase 3, after the formation of an atrophied leaf, as revealed by removing the last emerged leaf

growth in which there is continuous production of new leaves, followed by the cessation of new leaf production, then the formation of an atrophied leaf (senescence before development) in the axil of the last emerged leaf (Carrillo Cornejo and others 2003; Halligan and others 2004), and subsequently the end of the growth cycle. Using these visual signposts, we divided the growth cycle of *Zantedeschia* into three phases: phase 1 comprises the active growth period prior to the cessation of new leaf production (Fig. 1a); phase 2, from the cessation of new leaf production until the formation of an atrophied leaf (Fig. 1b); and phase 3, from the formation of an atrophied leaf through to the end of the growth cycle (Fig. 1c). These phases hypothetically track the transition of buds from apical dominance to dormancy.

Cytokinins are thought to act antagonistically to terminally derived auxin (Sachs and Thimann 1967; Bangerth and others 2000) and promote branching in many plant species (Miller 1961), including monocots (Hussey 1976). In earlier studies involving *Zantedeschia* tubers (Naor and others 2005a) and plantlets in vitro (Ngamau 2001; Naor and others 2005a), the application of the cytokinin 6-benzyl aminopurine (BAP) was reported to stimulate branching. Although the role of cytokinin in apical dominance and shoot branching is widely acknowledged, the role of cytokinin in dormancy is still poorly understood (Horvath 2009). Gibberellins influence several plant processes (Stowe and Yamaki 1959; Hedden and Kamiya 1997), including the transition to flowering (Boss and others 2004). In *Zantedeschia*, irrespective of meristem size and age, the application of gibberellin dictates floral differentiation (Corr and Widmer 1987; Naor and others 2005b).

Cross-talk between cytokinin and gibberellin has been implicated in diverse plant processes, including cell differentiation and meristem fate (Horvath and others 2003; Weiss and Ori 2007). At different developmental stages meristem fate is regulated by reciprocal interactions between both of them, where cytokinin inhibits gibberellin biosynthesis (Jasinski and others 2005) and gibberellin inhibits cytokinin synthesis (Greenboim-Wainberg and others 2005). A significant consequence of regulating the meristem fate by means of this cross-talk involves alleviating bud dormancy in a range of woody plant species and geophytes (Wareing and Saunders 1971; Suttle 2004).

Regulating the meristem fate depends on the mode of manipulating this hormonal cross-talk. Simultaneous application of cytokinin and gibberellin completely arrested axillary bud outgrowth in cuttings of *Solanum andigena*, whereas their individual application promoted the development of buds either as orthotropic shoots or diageotropic stolons, respectively (Woolley and Wareing 1972). In *Zantedeschia*, although the simultaneous application of cytokinin and gibberellin as Promalin (Valent BioSciences Corp., Walnut Creek, CA, USA) enhanced the total number of flowers per plant, the proportion of flowering buds was not significantly enhanced compared to the application of gibberellin alone (Funnell and others 1991), and branching was not significantly enhanced compared to the application of cytokinin alone (Funnell and MacKay 1988). A sequential application of cytokinin followed by gibberellin on suppressed buds of soy bean was found to be more successful in promoting branching compared to the simultaneous application of cytokinin and gibberellin or the application of cytokinin alone (Ali and Fletcher 1971).

Considering the existing evidence on the simultaneous application of cytokinin and gibberellin in *Zantedeschia* and evidence from other plant species that a greater response was obtained with sequential application, the sequential application of these two hormones to *Zantedeschia* was examined in the current experiment. Studies on the complementary roles of cytokinin and gibberellin and the effects of sequential applications of cytokinin

followed by gibberellin and vice versa on the meristem fate are limited. Although the reciprocal interaction between cytokinin and gibberellin on meristem fate is evident at the biosynthesis and signal transduction levels, phenotypic expression of this cross-talk has been scarcely reported. Given that in *Zantedeschia* these effects are phenotypically manifested as branching and flowering responses, the objective of the present study was to examine the roles of cytokinin and gibberellin, applied alone and in sequential combinations, in regulating bud dormancy and flowering in *Zantedeschia* as affected by (1) the three developmental phases of the growth cycle, (2) the sequential order of application, and (3) the concentration of each hormone.

Materials and Methods

Plant Material and Culture

Zantedeschia cv. 'Best Gold' is less prone to branching (D'Arth and others 2007), and the seedlings produce only one primary shoot (single-shoot system) during their first vegetative growth cycle (Funnell and Go 1993) (Fig. 1). Therefore, any axillary bud outgrowth can be easily associated with the apical bud. This advantage of the single-shoot system was employed in the current study.

Seeds of *Zantedeschia* cv. 'Best Gold' were germinated at 20 ± 2°C until plumule emergence. Seedlings were then transplanted at a uniform depth in 2.8 l polythene planter bags with a commercial growing medium (Daltons, New Zealand), containing 4.2 g of dolomite (Ravensdown Fertilisers, New Zealand), 0.9 N–0.2P–0.6 K g of 8–9-month Osmocote and 0.4 N–0.01P–0.3 K g of 3–4-month Osmocote (Scotts International B.V., Nijverheidsweg, The Netherlands) per bag. The plants were grown in a glasshouse at the Plant Growth Unit, Massey University, Palmerston North, New Zealand (40°20′S). The glasshouse was maintained at a minimum temperature of 15°C and vented at 19°C. The planter bags were placed on drained benches with capillary matting, and an irrigation frequency was maintained through drippers which supplied 50–60 ml of water per plant per day. Temperature within the glasshouse was monitored using multiple sensors and recorded every 10 min using a Squirrel 1200 data meter/logger (Grant Instruments Ltd., Cambridgeshire, UK). Accumulation of degree-days was calculated as described by Carrillo Cornejo and others (2003).

Three Developmental Phases and Treatment Application

Pilot studies were conducted to demarcate the three developmental phases based on an integral of the number of leaves present on the primary shoot, the presence of an atrophied leaf, calendar days, and degree-days. Seedlings normally produced 7 ± 2 leaves before the cessation of new leaf production. Therefore, in the current study, within each of the three phases (Fig. 1), seedlings with 4 ± 1 leaves (~85 days after sowing, ~1520 degree-days) were selected for phase 1; seedlings with 7 ± 1 leaves (~155 days after sowing, ~2700 degree-days) after the cessation of new leaf production and prior to the formation of an atrophied leaf, were selected for phase 2; and seedlings with 2 ± 1 leaves remaining (~210 days after sowing, ~3700 degree-days) after the formation of an atrophied leaf were selected for phase 3.

The effective range of concentrations of cytokinin and gibberellin to be applied was also determined from pilot studies. In the current study, cytokinin as BAP at 0, 2.2, 4.4, or 8.9 mM (0, 500, 1000, or 2000 mg l^{-1}, respectively) and gibberellin as GA_3 at 0, 0.3, 0.9, or 1.4 mM (0, 100, 300, or 500 mg l^{-1}, respectively) were applied as foliar sprays until runoff, at one of the four concentrations of each hormone. All concentrations of BAP and GA_3 (OlChemIm Ltd., Czech Republic) were dissolved in 15 ml of 1 N NaOH and 10 ml of 95% ethanol, respectively, and were then made up to the required volume with distilled water. Tween 20 (Sigma CAS No. 9005-64-5) was added as a surfactant. The control treatments comprised all components minus BAP and GA_3, respectively.

BAP and GA_3 were applied in two sequential orders which makes for two groups: group 1 (BAP → GA_3), where BAP was applied first (period 1) followed by the application of GA_3 (period 2) (Fig. 2a), and group 2 (GA_3 → BAP), where GA_3 was applied first (period 1) followed by the application of BAP (period 2) (Fig. 2b). Within any period, two sprays of each hormone were applied, with the intratime interval between two consecutive sprays of the same hormone and the intertime interval between the application of each hormone being 10 days (Fig. 2).

Experimental Design and Statistical Analysis

For each of the three developmental phases, the 16 combinations of the concentrations of BAP and GA_3 within each group were arranged in a 4 × 4 crossover design (Fletcher and John 1985). Eight individual plant replicates were used for each of the 16 combinations within a group (8 replicates × 16 combinations × 2 groups = 256 plants per phase).

The plants were maintained in three rows of benches along the length of the glasshouse and were apportioned equally to each phase, across the three rows, as they progressed along the ontogeny of growth from phase 1 to phase 3. For each phase, all 32 combinations of the concentrations of BAP and GA_3 (16 combinations × 2 groups)

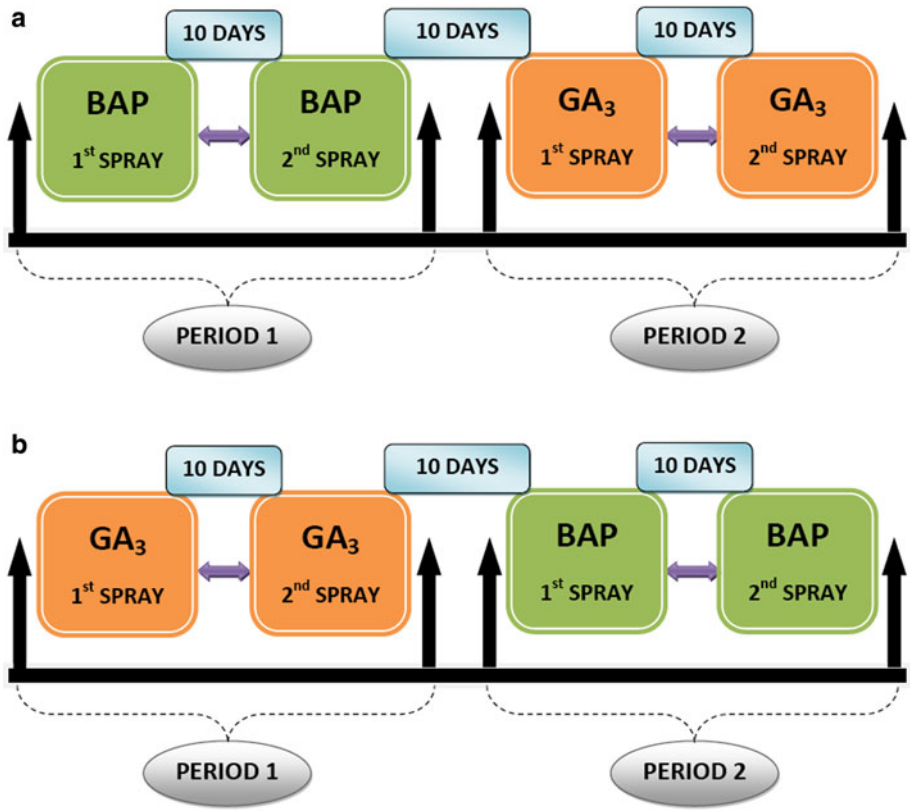

Fig. 2 Pictorial representation of the sequential order and timing of the application of each combination of BAP and GA_3 concentration used within each developmental phase of the study. **a** Group 1 (BAP → GA_3): two foliar sprays of BAP in period 1 were followed by two sprays of GA_3 in period 2. **b** Group 2 (GA_3 → BAP): two foliar sprays of GA_3 in period 1 were followed by two sprays of BAP in period 2. Two sprays of each hormone were applied within any period, with the intratime interval between two consecutive sprays being 10 days and the intertime interval between each hormonal application being 10 days

were assigned to the 256 plants in a completely randomized manner. Pilot studies and temperature sensors at multiple locations did not reveal any positional or temperature gradients across the glasshouse.

Analyses of variance for the branching and flowering data, tests of proportions for the percentage of flowering plants and primary and axillary flowering data, means ± standard errors, mean separation tests and contrasts between the two groups, and the three phases and four concentrations of each hormone were carried out using PROC GLM of SAS version 9.2 (SAS Institute, Cary, NC, USA) and MS Excel (Microsoft Corp., Redmond, WA, USA).

Observations

For each phase, the number of visible axillary shoots per plant (branching) at a minimum height of 1 cm above the growing medium were counted at the start of the experiment (time-zero), after the application of either BAP alone in group 1 or GA_3 alone in group 2 (10 days after period 1), and after the sequential application of GA_3 or BAP, respectively (10 days after period 2) (Fig. 2). The total number of visible flowers per plant (flowering) partitioned as those emerging from primary and axillary shoots was counted as and when they appeared. A floral stem was characterized by a peduncle bearing a spadix. A final count of the flowers, if present, was recorded 75 days after the last foliar spray.

Histological Examination

Floral differentiation at the apical and three axillary buds along the longest axis of the tuber, representing incremental phyllotactic distance and age, was determined using a stereomicroscope. For each phase, untreated plants (control), plants treated with the highest concentrations of BAP (8.9 mM) followed by GA_3 (1.4 mM) in group 1, and those treated vice versa for group 2 were destructively harvested for bud dissections at time-zero, 10 days after period 1, and 10 days after period 2. For each time of dissection, three individual plant replicates were used for control and treatments applied in groups 1 and 2.

Results

Overview

The plastochron of untreated (control) plants of *Zantedeschia* cv. 'Best Gold' grown under the conditions described above was 10 days. Continuous leaf production was observed until the formation of 7–8 leaves. Subsequently,

new leaf production ceased and withering of existing leaves commenced, starting with the older leaves. With two leaves remaining in the primary shoot, an atrophied leaf was noticed in the axil of the last emerged leaf, which was followed by the withering of all leaves and thus the end of the growth cycle.

Visible axillary bud outgrowth, marked by the initial emergence of one to two cataphylls, was normally observed 10 days after two consecutive applications of BAP at a 10-day interval. One to three ovate leaves unfurled subsequently. In group 2 ($GA_3 \rightarrow$ BAP), however, axillary shoots with longer petioles bearing lanceolate leaves were observed. During the flowering growth cycle, visible flowering is normally observed within 60 days after GA_3 application. Because the current study was undertaken during the vegetative growth cycle, flowering time was sporadic and large variability in the flowering data was observed (Fig. 5d–f). Flowers produced in this study were mostly malformed, denoted by the presence of a short peduncle and deformed spathes.

Branching

Time-Zero

At the beginning of each developmental phase, all plants comprised only the primary shoot, that is, axillary shoots were absent.

10 Days After Period 1

In phase 1 (Fig. 3a), increased branching occurred with increasing concentrations of BAP ($P < 0.0001$). This ability to stimulate branching declined gradually ($P < 0.0001$) for all concentrations of BAP in phases 2 and 3 (Fig. 3a). For example, branching stimulated by the highest concentration of BAP (8.9 mM), declined from 3.8 ± 0.2 shoots ($n = 32$) in phase 1 to 1.0 ± 0.3 shoots ($n = 32$) in phase 3.

In phase 1, though not concentration dependent (Fig. 3b), the application of GA_3 alone was sufficient to stimulate branching, at least by an additional shoot ($P < 0.05$). However, in phases 2 and 3, GA_3 alone had no effect on branching. At 10 days after period 1, axillary shoots were not observed in the control plants.

10 Days After Period 2

At this time (Fig. 4a–c), the stimulating effect of BAP alone on branching was more prolific than that noted at 10 days after period 1 (Fig. 3a) in all three phases and across all concentrations of BAP. For example, 6.6 ± 0.5 shoots per plant ($n = 8$) were stimulated by 8.9 mM in phase 1 at 10 days after period 2 (Fig. 4a) compared with

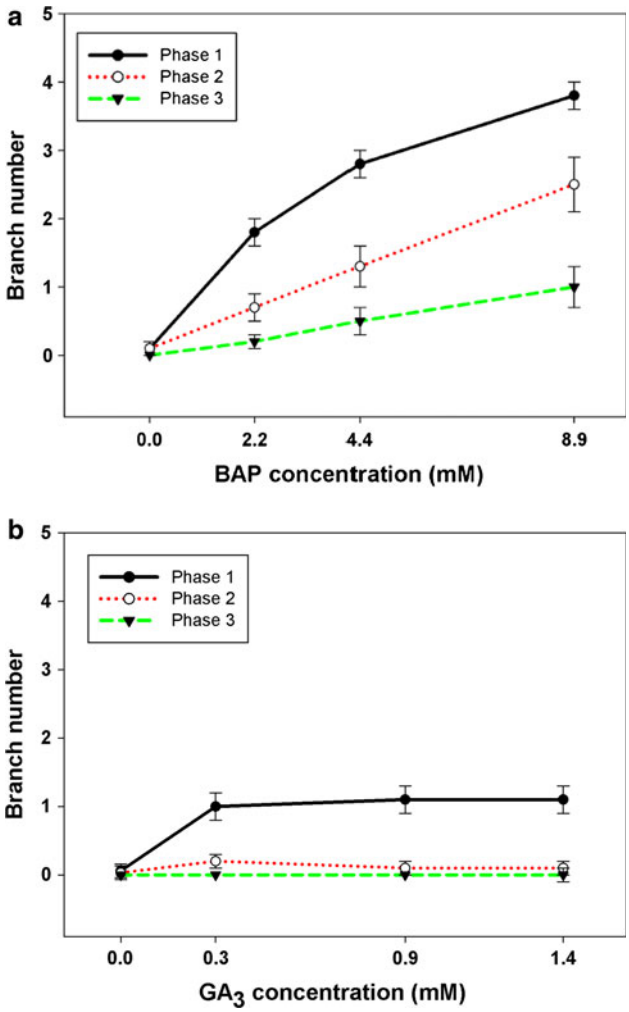

Fig. 3 Number of axillary shoots per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' at 10 days after period 1 after the application of **a** BAP alone in group 1 and **b** GA_3 alone in group 2 ($n = 32$)

3.8 ± 0.2 shoots ($n = 32$) observed at 10 days after period 1 (Fig. 3a). At 10 days after period 2, 1 ± 0.2 axillary shoots were observed in the control plants. In phase 1 (Fig. 4a), at 2.2 mM, a sequential application of GA_3 (BAP $\rightarrow$ GA_3) was effective in stimulating branching significantly compared to the application of BAP alone ($P < 0.05$). However, for the other concentrations of BAP (4.4 and 8.9 mM), a sequential application of GA_3 (BAP $\rightarrow$ GA_3) failed to enhance branching significantly.

GA_3 alone did not stimulate branching significantly in phase 1 (Fig. 4d). Any increase in branching ($P < 0.0001$) observed in group 2 occurred only after the sequential application of BAP ($GA_3 \rightarrow$ BAP). Compared to the branching stimulated by all concentrations of GA_3 alone (1.8 ± 0.17; $n = 32$), significant ($P = 0.05$) incremental branching was observed with increasing concentrations of a sequential application of BAP ($GA_3 \rightarrow$ BAP), with

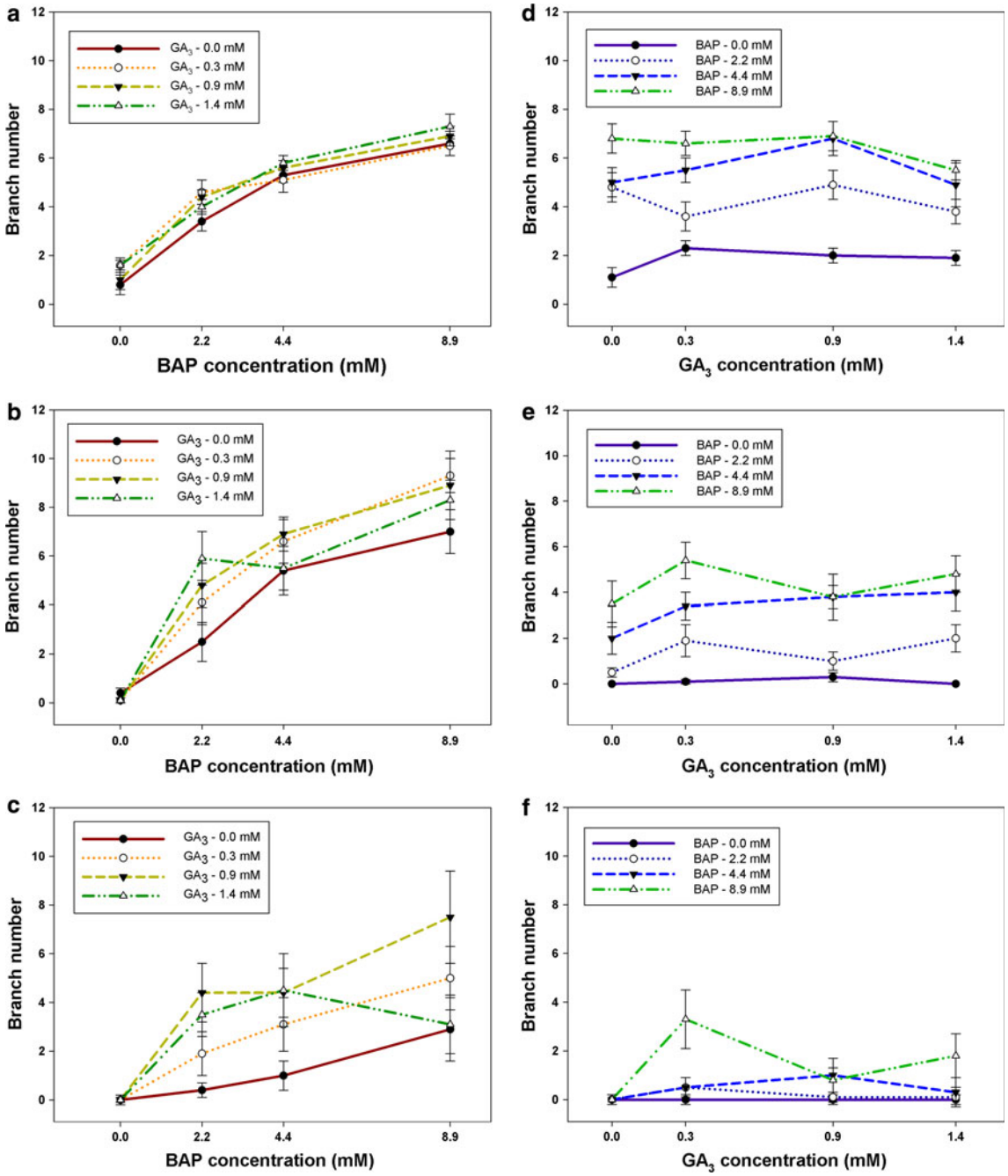

Fig. 4 Number of axillary shoots per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' at 10 days after period 2 after the sequential application of BAP followed by GA_3 in group 1 (BAP → GA_3) in **a** phase 1, **b** phase 2, **c** phase 3, and after the sequential application of GA_3 followed by BAP in group 2 (GA_3 → BAP) in **d** phase 1, **e** phase 2, and **f** phase 3 ($n = 8$)

4.25 ± 0.28, 5.5 ± 0.35, and 6.4 ± 0.29 shoots ($n = 32$ each) stimulated by 2.2, 4.4, and 8.9 mM concentrations of BAP, respectively. A comparison of branching between the two sequential orders of application (group 1 versus group 2), showed that the order of application was insignificant in eliciting a branching response in phase 1 (Table 1A).

In phase 2, with the progressive decline in the branching effect of BAP alone (Fig. 3a), a sequential application of GA_3 (BAP → GA_3), compared to the application of BAP alone, significantly ($P < 0.02$) and marginally ($P < 0.1$) enhanced branching following the application of BAP at 2.2 and 8.9 mM concentrations, respectively (Fig. 4b), but it failed to enhance branching significantly ($P > 0.1$) following the application of BAP at 4.4 mM. However, a comparison between branching stimulated by each concentration of BAP, combining all concentrations of GA_3

Table 1 Effects of the sequential orders of application of BAP and GA_3 on (A) the number of axillary shoots (branching) and (B) flowers (flowering) in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1	Phase 2	Phase 3
(A) Branching (number of axillary shoots per plant)			
Group 1 (BAP → GA_3)	4.40 a	4.73 a	2.54 a
Group 2 (GA_3 → BAP)	4.51 a	2.27 b	0.41 b
(B) Flowering (total number of flowers per plant)			
Group 1 (BAP → GA_3)	0.63 a	0.05 a	0.00 a
Group 2 (GA_3 → BAP)	1.41 b	0.30 b	0.15 b

Different *small letters* indicate significant differences ($\alpha = 0.05$) between the two sequential orders of application. Mean separation by DMRT ($n = 128$)

($n = 32$), revealed significant ($P = 0.05$) incremental branching with increasing concentrations of BAP, where 4.3 ± 0.48, 6.1 ± 0.44, and 8.3 ± 0.50 shoots were stimulated by 2.2, 4.4, and 8.9 mM concentrations of BAP, respectively (Fig. 4b).

In phase 3, the stimulating effect of BAP alone declined further (Fig. 3a). Compared to BAP applied alone, the sequential application of GA_3 in group 1 (Fig. 4c) significantly increased branching for 2.2 mM ($P < 0.01$) and 4.4 mM ($P < 0.03$) concentrations of BAP, and marginally increased ($P < 0.1$) branching for BAP at 8.9 mM concentration. However, a significant ($P = 0.05$) increase in branching was caused by the sequential application of 8.9 mM BAP followed by 0.9 mM GA_3 (Fig. 4c).

As in phase 1 (Fig. 4d), GA_3 alone failed to provoke a significant branching response in phases 2 and 3 (Fig. 4e, f), and branching in group 2 occurred only after the sequential application of BAP. This ability of BAP to stimulate branching when applied sequentially after GA_3 (GA_3 → BAP) also diminished gradually ($P < 0.05$) from phase 1 to phase 3. BAP at 8.9 mM, which stimulated 6.5 ± 0.3 shoots in phase 1 across all concentrations of GA_3 (Fig. 4d), stimulated only 1.5 ± 0.7 shoots in phase 3 (Fig. 4f). Overall, a comparison of branching between the two sequential orders of application (group 1 versus group 2) in phases 2 and 3 (Table 1A) showed that group 1 (BAP → GA_3) stimulated more branching than group 2 (GA_3 → BAP).

Flowering

Visible Flowering

In phase 1, the application of GA_3 alone was sufficient to induce a linear flowering response ($P < 0.05$) (Fig. 5d). However, compared to the application of GA_3 alone, the sequential application of BAP in group 2 (GA_3 → BAP) (Fig. 5d) enhanced the flowering response ($P < 0.05$). For example, the application of GA_3 at 1.4 mM followed by BAP at 8.9 mM in phase 1 (Fig. 5d) produced 3.5 ± 0.8 flowers ($n = 8$) per plant compared to 1.4 ± 0.4 flowers ($n = 8$) obtained with the application of GA_3 alone at 1.4 mM. In phases 2 and 3 (Fig. 5e, f), the efficacy of GA_3 alone to provoke flowering was depleted. Any flowering observed in these phases was only after the sequential application of BAP in group 2. For example, no flowering occurred in phases 2 (Fig. 5e) and 3 (Fig. 5f), even with the highest concentration of GA_3 (1.4 mM) applied alone, whereas 1.9 ± 0.9 and 0.9 ± 0.4 flowers per plant were induced, respectively ($n = 8$), after the sequential application of BAP at 8.9 mM.

BAP alone, regardless of the concentration, had no effect on flowering during all three phases of growth (Fig. 5a-c). Flowering in group 1 (BAP → GA_3), therefore, occurred only after the sequential application of GA_3. In phase 1, increased flowering occurred with increasing concentrations of GA_3 ($P < 0.001$) (Fig. 5a), and the ability of GA_3 to provoke flowering in group 1 diminished as the buds progressed toward phase 2 (Fig. 5b) and was completely eliminated in phase 3 (Fig. 5c). Overall, a comparison of the total number of flowers per plant produced by the two sequential orders of application (group 1 versus group 2) in the three phases of growth (Table 1B) showed that group 2 (GA_3 → BAP) always produced significantly ($P = 0.05$) higher numbers of flowers than group 1 (BAP → GA_3).

The percentage of plants induced to flowering (minimum of 1 flower), though progressively diminishing from phase 1 to phase 3 (Table 2A), was also significantly ($P = 0.01$) higher in group 2 than in group 1 ($n = 128$). In group 2, the flowers emerging from the primary shoot contributed significantly more ($P = 0.01$) to the total flower count than in group 1 for phases 1 and 3 (Table 2B). Consequently, the percentage of flowers emerging from axillary shoots in phase 1 (72.8%) was significantly ($P = 0.01$) higher than that from the primary shoot (27.2%) in group 1 and that from axillary shoots in group 2 (52.2%). However, in phase 2, though axillary flowering was the main contributor for the total flower count, the differences between axillary and primary flowering within each group and axillary flowering between the two groups were not statistically significant ($P > 0.1$). Among the flowering plants, the total number of flowers (primary and axillary) per plant (Table 2C) was also higher in group 2 along the three phases, but was significantly ($P = 0.05$) higher than that in group 1 only in phases 1 and 3.

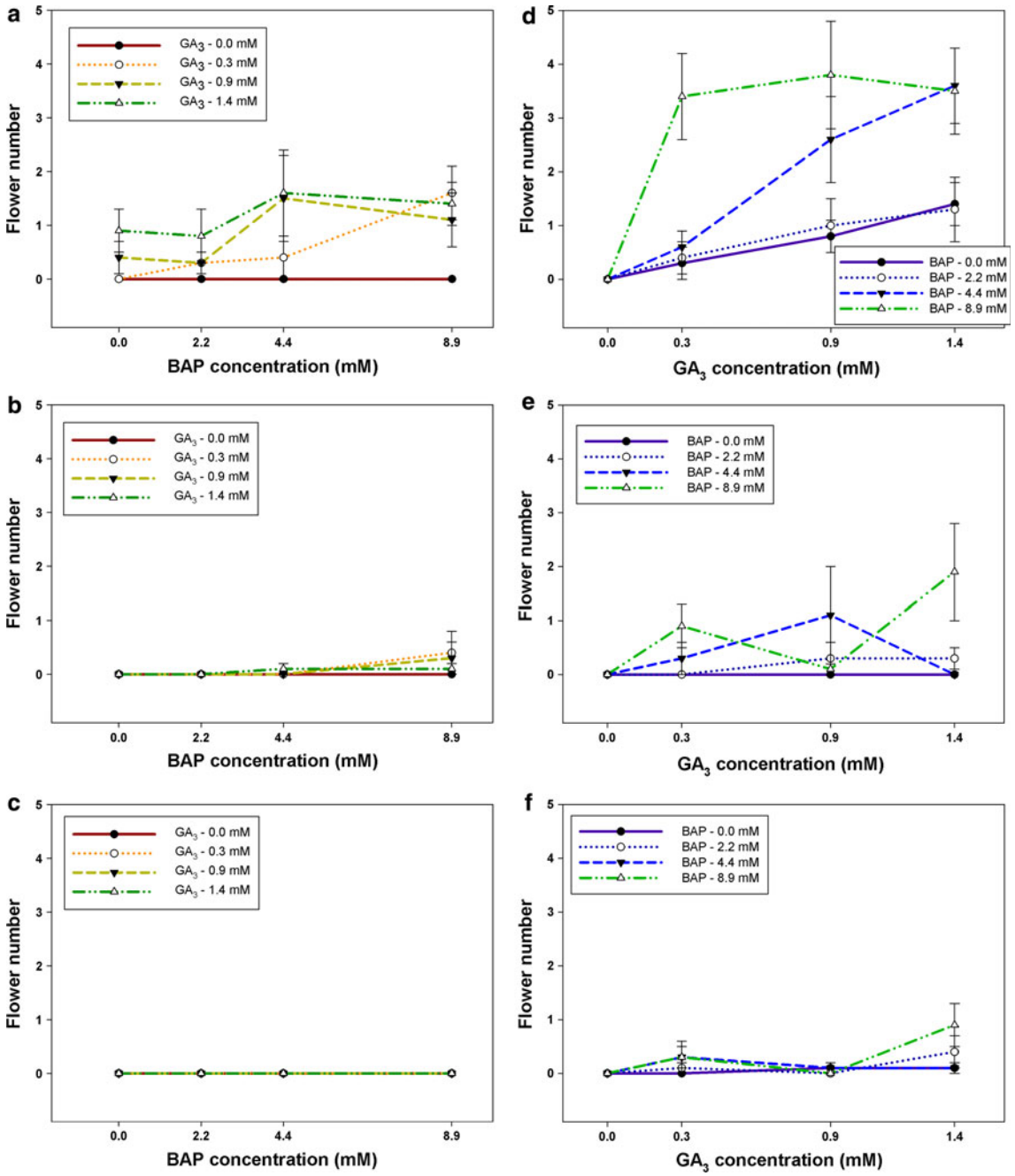

Fig. 5 Number of flowers per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' after the sequential application of BAP followed by GA_3 in group 1 (BAP → GA_3), in **a** phase 1, **b** phase 2, **c** phase 3, and after the sequential application of GA_3 followed by BAP in group 2 (GA_3 → BAP) in **d** phase 1, **e** phase 2, and **f** phase 3 ($n = 8$)

Histological Examination

Floral differentiation was identified by the formation of a smooth elongated apex and sometimes by the presence of a primordial spadix. Floral differentiation at the apical and three axillary buds, which represents increasing phyllotactic distance and age, was not noticed in untreated (control) plants ($n = 3$) at any of the three observation times (time-zero, 10 days after period 1, and 10 days after period 2), during all three phases of growth (Table 3). At 10 days after period 1, a floral primordium was noticed in the apex of the apical bud during the three phases only in plants treated with the highest concentration of GA_3 (1.4 mM) ($n = 3$) in group 2. At 10 days after period 2,

Table 2 Effects of the sequential orders of application of BAP and GA_3 on (A) the percentage of flowering plants, (B) percentage of flowers emerging from the primary and axillary shoots, and (C) the total number of flowers per flowering plant in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1	Phase 2	Phase 3
(A) Percentage of flowering plants ($\alpha = 0.01$)			
Group 1 (BAP → GA_3)	29.7 a	3.1 a	0.0 a
Group 2 (GA_3 → BAP)	47.7 b	13.3 b	10.2 b
(B) Percentage of primary (and axillary) flowers ($\alpha = 0.01$)			
Group 1 (BAP → GA_3)	27.2 a (72.8 a)	42.9 a (57.1 a)	0.0 a (0.0 a)
Group 2 (GA_3 → BAP)	47.8 b (52.2 b)	44.7 a (55.3 a)	68.4 b (31.6 b)
(C) Number of flowers per flowering plant ($\alpha = 0.05$)			
Group 1 (BAP → GA_3)	2.1 a	1.8 a	0.0 a
Group 2 (GA_3 → BAP)	3.0 b	2.2 a	1.5 b

Different *small letters* indicate significant differences ($\alpha = 0.01$ or 0.05) between the two sequential orders of application. Mean separation by DMRT and test of proportions by two-tailed *t* test

Table 3 Effect of the sequential orders of application of BAP and GA_3 on floral differentiation at the apical and three axillary buds representing incremental age and phyllotactic distance observed upon histological examination at time-zero, 10 days after period 1, and 10 days after period 2 in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1				Phase 2				Phase 3			
	Axillary buds			Apical bud	Axillary buds			Apical bud	Axillary buds			Apical bud
	1	2	3		1	2	3		1	2	3	
Time-zero												
Control												
10 days after period 1												
Control												
Group 1 (BAP)												
Group 2 (GA_3)				+				+				+
10 days after period 2												
Control												
Group 1 (BAP → GA_3)	+	+	+	+	+	+	+	+	+	+	+	+
Group 2 (GA_3 → BAP)	+	+	+	+	+	+	+	+	+	+	+	+

+ denotes the presence of a floral primordium at the apex of the bud observed on histological examination ($n = 3$)

floral differentiation was noticed in the apical and all three axillary buds of the three phases with the application of BAP at 8.9 mM and GA_3 at 1.4 mM concentrations ($n = 3$), irrespective of the sequential order of application (groups 1 and 2) (Table 3).

Discussion

The Three Developmental Phases and the Progressive Establishment of Dormancy

The developmental program in autonomous geophytes such as *Zantedeschia* is controlled primarily by innate mechanisms (Naor and Kigel 2002). Physiological and/or morphological representation of these underlying mechanisms is lacking and, therefore, a chronological analysis of the developmental changes would provide a better understanding of these processes (Naor and others 2008). These developmental changes are different episodes in the life of the shoot apical meristem (SAM), which passes through distinct phases during its post-embryonic growth (Poethig 1990). Though the cessation of new leaf production and the formation of an atrophied leaf cannot be regarded as definitive indicators of the onset of dormancy (Halligan and others 2004), to our understanding, the progression from an active growth period characterized by continuous production of new leaves to the cessation of new leaf production and the formation of an atrophied leaf reflects a series of developmental changes in the SAM. These steps also act as useful landmarks in the ontogeny of *Zantedeschia*. These changes in the SAM, manifested as visual

clues, formed the basis of our partition of the growth cycle of *Zantedeschia* into three developmental phases.

A simple diagnostic tool to test the onset of dormancy is decapitation, which would fail to trigger branching during bud dormancy. Likewise, the establishment of bud dormancy can also be diagnosed by the deteriorating sensitivity of the buds to cytokinin, indicated by the branching response. A change in sensitivity is regarded as a change in the magnitude of an induced response (branching in this study) by an organ (bud) to a known amount of exogenous hormone (BAP) (Firn 1986). We showed that in phase 1 (Fig. 3a), where buds are predominantly controlled by apical dominance, a linear branching response can be stimulated with increasing concentrations of BAP. However, as the buds progressed toward phases 2 and 3 (Fig. 3a), there was a concomitant decline in the magnitude of the branching response with the same concentrations of BAP. Therefore, the transition of buds from apical dominance to dormancy could in part result in the loss in sensitivity of the buds to cytokinin (effect), or a loss in the sensitivity of the buds to cytokinin could signal the establishment of dormancy (cause). This study, therefore, highlights the significance of the changes in sensitivity of the buds to cytokinin as a key determinant in the progressive establishment of dormancy. This study also corroborates the parallel inception of dormancy during the three developmental phases of *Zantedeschia*, which vindicates our classification of the growth cycle based on the visual landmarks (Fig. 1).

Alleviating Bud Dormancy in *Zantedeschia*

Hormonal signals are vital for altering the developmental pathway of the SAM and, therefore, maintaining the flexibility of the bud meristems (Traas and Vernoux 2002). The differential response of buds to exogenous cytokinin at different developmental stages was noted in soy bean when Ali and Fletcher (1970) showed that the application of cytokinin alone was sufficient to stimulate branching in 8-day-old seedlings. However, in 16-day-old seedlings, cytokinin alone was not sufficient to stimulate branching after mitosis had ceased. A sequential application of gibberellin was required for sustained axillary bud outgrowth. A role for cytokinin in promoting mitosis and a role for gibberellin in enhancing shoot elongation was emphasized.

In the current study, as noted in soy bean, BAP alone was sufficient to alleviate bud dormancy, predominantly governed by apical dominance in phase 1 (Fig. 3a). In phase 2, after the cessation of new leaf production, presumably mitosis had ceased and, therefore, the developmental pathway of the SAM was altered. The efficacy of BAP alone to alleviate bud dormancy had diminished, and a sequential application of GA_3 in group 1 was required to sustain branching (Fig. 4b). In phase 3, after the formation of an atrophied leaf, which marked another cornerstone in the transition toward stronger bud dormancy, the efficacy of BAP alone to stimulate branching declined further, and the dependence on a sequential application of GA_3 to alleviate bud dormancy intensified further (Fig. 4c). Therefore, along the three developmental phases, with continuing modifications in the developmental pathway of the buds, the progressive establishment of dormancy synchronized with the gradual decline in the efficacy of BAP to alleviate bud dormancy and a concomitant increase in the need for a sequential application of GA_3.

In two different studies in *Zantedeschia*, endogenous cytokinin (D'Arth and others 2007) and gibberellin (Naor and others 2008) levels were found to be higher in sprouting buds than in dormant buds, which in the present study correspond to buds in phases 1 and 3, respectively. In phase 1, the application of BAP alone, interacting with endogenous gibberellins, may have been sufficient to elicit a branching response, which deemed the order of application of BAP immaterial in phase 1 (Table 1A). This also explains why the sequential application of GA_3 in group 1 failed to enhance the branching response significantly in phase 1 (Fig. 4a). This theory would also help to explain the increased branching, by at least one additional shoot, measured with the application of GA_3 alone in phase 1 (Fig. 3b). The application of GA_3, interacting with endogenous cytokinin, may have been sufficient to provoke a mild branching response in phase 1, which eventually declined with the establishment of dormancy in phases 2 and 3 (Fig. 3b). In dormant buds (phase 3), after the decrease in endogenous cytokinin and gibberellin levels (D'Arth and others 2007; Naor and others 2008), the application of BAP, by promoting mitosis, may have catalyzed the transformation of the dormant bud to its responsive state, and the sequential application of GA_3, by promoting shoot elongation, may have catalyzed the subsequent transition to a growing shoot (Ferguson and Beveridge 2009). The key to alleviating bud dormancy in *Zantedeschia*, therefore, involves cross-talk between BAP and GA_3, where GA_3 enhanced branching only when applied sequentially after BAP (BAP $\rightarrow$ GA_3) and had no effect on branching when applied alone (Table 4).

Enhancing Flowering in *Zantedeschia*

Gibberellin evokes different flowering responses in different plant species (Levy and Dean 1998) and collaborates with other signals such as photoperiod, vernalization, and autonomous control to culminate in floral initiation (Boss and others 2004). Gibberellin mediates the transition of activated buds, regardless of size and age, from a vegetative to a floral state in *Zantedeschia* (Naor and others

Table 4 Summary of results highlighting the cross-talk between BAP and GA_3 in their complementary roles on branching and flowering of *Zantedeschia* cv. 'Best Gold'

Hormone application	Branching	Flowering
BAP	Stimulated alone; efficacy declined with onset of dormancy	No effect
GA_3	No effect	Stimulated alone; efficacy declined with onset of dormancy
Group 1 (BAP → GA_3)	GA_3 complementary to BAP when applied sequentially	Attributed solely to GA_3 component; efficacy declined with onset of dormancy
Group 2 (GA_3 → BAP)	Attributed solely to BAP component; efficacy declined with onset of dormancy	BAP complementary to GA_3 when applied sequentially

2005b). In this study we have shown that in phase 1, where the buds are considered to be in an active state, the application of GA_3 alone induced a linear flowering response in group 2 (Fig. 5d). However, with the establishment of dormancy in phases 2 and 3, GA_3 alone failed to evoke any visible flowering response (Fig. 5e, f). Histological examination after the application of the highest concentration of GA_3 (1.4 mM) alone at 10 days after period 1, however, revealed a floral primordium in the apex of the apical bud in phases 2 and 3 (Table 3). In *Zantedeschia* plantlets grown in vitro and induced to floral transition with the application of gibberellin, inflorescence development ceased at later stages of growth, resulting in premature abortion (Naor and others 2004). Therefore, the floral differentiation in *Zantedeschia*, dictated by the application of gibberellin, does not guarantee visible manifestation of flowering. Apart from the size and age of the bud, flowering competence may involve a series of events exclusive of gibberellins (Mutasa-Gottgens and Hedden 2009). In *Zantedeschia*, day-neutral to flowering (Funnell 1993), this could involve a change in sensitivity of the buds to gibberellins implemented by the same innate mechanisms that govern the developmental program.

Though cytokinins are not frequently mentioned in flowering discussions, evidence supporting the definitive role of cytokinins in promoting mitosis and subsequent differentiation of floral organs is available for *Sinapis alba* (Lejeune and others 1994) and *Chenopodium* sp. (Machácková and others 1993). In *Zantedeschia* plantlets in vitro, cytokinin alone failed to induce inflorescence development, but it interacted with gibberellin, resulting in floral development (Naor and others 2004). In this study, the application of BAP alone, irrespective of concentration, had no effect on visible flowering (Fig. 5a–c) or floral differentiation in the apex (Table 3) during all three developmental phases.

However, when BAP was applied after GA_3 in group 2, the total number of flowers per plant (Table 1B) and the percentage of flowering plants (Table 2A) were significantly enhanced in all three phases, even in phases 2 and 3 where the efficacy of GA_3 alone to initiate flowering was entirely depleted (Fig. 5e, f). The percentage of flowering plants (Table 2A) and the number of flowers per plant (Table 1B) produced in group 1 (BAP → GA_3), can be attributed solely to the gibberellin factor, which progressively diminished with the establishment of dormancy in phases 2 and 3 (Fig. 5b, c), as observed in group 2 (Fig. 5e, f). Histological examination of apical and axillary buds at 10 days after period 2 (Table 3) revealed that floral differentiation had occurred in all the buds, irrespective of the order of application of BAP and GA_3 (group 1 or 2) and the phyllotactic distance and the age of the axillary buds. Visible manifestation of the differentiated primordia was, however, more predominant in group 2 (Fig. 5e, f).

The primary shoot possessed the largest number of flowering buds (Naor and others 2005b). In the current study, the percentage of the flowers emerging from the primary shoot was significantly higher in group 2 than in group 1 (Table 2B). Histological examination at 10 days after period 1 (Table 3) also revealed floral differentiation only in the apical bud of plants treated with GA_3 (group 2). Presumably, after the floral "switch" was turned on by the initial application of GA_3, the sequential application of BAP may have rekindled mitosis at the SAM, resulting in differentiation of floral organs, which triggered an increase in percentage of flowers emerging from the primary shoot and, therefore, the total flower number (Tables 1B, 2C) and percentage of flowering plants in group 2. The key to enhanced flowering in *Zantedeschia*, therefore, involves cross-talk between BAP and GA_3, where BAP enhanced floral productivity only when applied sequentially after GA_3 (GA_3 → BAP), and had no effect on flowering when applied alone (Table 4).

Hormonal Cross-talk Regulates Dormancy and Flowering in *Zantedeschia*

Cross-talk in plant physiology refers to signal integration from multiple hormone inputs, which could be either direct, indirect, or coregulatory, resulting in a common biological output (Chandler 2009). In this study we have shown that the application of BAP followed by GA_3 (BAP → GA_3) was the key to alleviating bud dormancy

(branching), and the application of GA_3 followed by BAP ($GA_3 \rightarrow$ BAP) was the key to enhanced flowering in *Zantedeschia*. The application of GA_3 alone had no effect on branching and the application of BAP alone had no effect on flowering. We assume that GA_3 complements the branching process initiated by BAP through its well-known role in shoot elongation (Hedden and Kamiya 1997), and BAP complements the flowering process initiated by GA_3 through its well-known role in mitosis (Campbell and others 1996). The respective biological outputs, branching and flowering, are more likely to be the result of coregulation between the independent pathways of cytokinin and gibberellin. Cross-talk between cytokinin and gibberellin and their reciprocal interaction in dormancy release or enhanced flowering have not been previously reported in a single plant species. The current findings, where alleviating bud dormancy (branching) and enhanced flowering were depicted as two facets of regulating meristem fate in day-neutral *Zantedeschia*, would contribute to the model, drawing common mechanisms between dormancy and flowering in plants (Horvath 2009).

Acknowledgments We thank the New Zealand Calla Council for providing plant material, James Slater and Dr. Panimalar Vijayan for technical assistance, Dr. Alasdair Noble for statistical assistance, and the Government of New Zealand for the financial assistance of Arvind Kumar Subbaraj.

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