Paclobutrazol Modulates Endogenous Level of Phytohormones in Inducing Early Flowering in *Camellia tamdaoensis* Hakoda et Ninh, a Golden *Camellia* Species

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Abstract. Golden camellia flowers are treasured for their unique yellow color and bioactive chemical compounds. Because of its high market demand, there is strong interest in inducing early flowering in golden camellias for earlier harvest. Previously, we have successfully induced flowering in *Camellia chrysantha* (Hu) Tuyama juvenile grafted plants and seedlings with paclobutrazol (PBZ). During this study, we investigated the efficacy of PBZ on *C. tamdaoensis* juvenile rooted cuttings. *C. tamdaoensis* is a yellow-flowering camellia species that is native to Vietnam and valued by the local population. It was found that applications of 100 and 200 ppm PBZ generated an average of 13 and 30 flowers per 5-year-old plant, respectively. None of the control plants flowered. The average flower diameter was 17.2 cm for 100-ppm-induced flowers and 26.0 cm for 200-ppm-induced flowers. The dynamics of various phytohormones (indoleacetic acid, abscisic acid, salicylic acid, and jasmonic acid) were altered by PBZ treatment. It is suggested that low indoleacetic acid, high abscisic acid, and jasmonic acid and a gradual increase in salicylic acid benefit floral initiation of golden camellias. The study provided the first insight regarding the action mechanism of PBZ for the initiation of camellia flowering.

Sexual reproduction is critical to survival and evolution of an organism because it results in genetic variations in offspring. In agriculture, plant reproduction is also fundamental to the success of the entire agricultural economy because most of the agricultural activities on a farm begin with seed and end with seed (Yadava et al., 2019), and flowers, fruits, and seeds are important food sources. The onset of reproduction is marked by flowering, thus signaling the transition from juvenility to maturity.

The juvenility length varies among species. For perennial trees, it may last several years, such as for hardwood species, to decades, such as for conifers (Albani and Coupland, 2010). A long juvenile phase not only hinders conventional breeding efforts but also adversely impacts the incomes of relevant businesses. Several environmental conditions as well as internal factors, including the photoperiod, temperature, hormones, and nutrients, have important roles in flowering initiation. Extensive genetic and molecular analyses of model species, such as *Arabidopsis*, have demonstrated that endogenous and environmental factors contribute to flowering through five main pathways: photoperiod; vernalization; autonomous; gibberellins [gibberellin acid (GA)]; and age (Teotia and Tang, 2015). These pathways are interwoven and channel the signals through several floral integrators, such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *AGAMOUS-LIKE 24* (*AGL24*), *LEAFY* (*LFY*), and *APETALA1* (*API*) (Mouradov et al., 2002). These flowering time genes are regulated by extensive DNA methylation, histone modifications,

small RNA-mediated chromatin silencing, and alternative splicing mechanisms (Fornara and Coupland, 2009; Zhou et al., 2013). In addition to GA, other hormone types can have a role in flowering. Auxin promotes the onset of flower formation in *Arabidopsis* through *AUXIN RESPONSE FACTORS/MONOPTEROS* (*ARF5/MP*) (Yamaguchi et al., 2016). Furthermore, sugars contribute to floral induction by serving as energy and acting as a signal. Seo et al. (2011) reported that the *Arabidopsis* INDETERMINATE DOMAIN transcription factor AtIDD8 regulated photoperiodic flowering by modulating sugar transport and metabolism. Wahl et al. (2013) demonstrated that the trehalose-6-phosphate (T6P) pathway regulated flowering at two sites in *Arabidopsis*: in leaves, *TREHALOSE-6PHOSPHATE SYNTHASE* (*TPS*) is required for the induction of *FT*; and at the shoot apical meristem (SAM), the T6P pathway acts via the age pathway directly, independently of the photoperiod pathway, thus affecting the expression of important flowering time and flower patterning genes.

Because of their longer juvenile phase and lengthy reproductive cycle, regulation of floral transition in perennial species is more complex but less studied. Although many homologs of *Arabidopsis* flowering-related genes, especially the floral integrator-encoding genes, are found to have similar functions in perennial woody species, mechanisms regulating flower induction and related pathways that promote flowering in perennial woody plants are not the same when compared with those of annual plants. For instance, exogenous GA₃ inhibited the formation of flower buds in many fruit tree species (e.g., mango, peach, and apple) (Southwick and Glozer, 2000), different from *Arabidopsis*, where GA₃ accelerated the transition from vegetative development to the first inflorescence stage of reproductive development (Yamaguchi et al., 2014). Muñoz-Fambuena et al. (2012) revealed that GA₃ inhibited flowering in citrus by repressing *FT* expression in leaves. Additionally, no clear *FLOWERING LOCUS C* (*FLC*) homologs have been identified in the poplar (*Populus trichocarpa* v3.1., *P. deltoides* v2.1, Phytozome 13) genome (the best match has an E value of 2E-23). Therefore, additional comprehensive studies are warranted to understand the similarities and differences in the transition of vegetative growth to flowering between annual and perennial species rather than relying entirely on inference from annual model systems.

To induce early flowering, physical wounding (such as girdling) and hormone treatments are commonly used. For example, Yin and Liang (1994) successfully induced female and male cone formation in 5-year-old *Metasequoia glyptostroboides* seedlings with indole butyric acid and zeatin ribonucleoside, resulting in approximately two cones per treated shoot. Without intervention, seeds are not produced until the trees are 25 to 30 years old (Kuser, 1982). Male strobili of *Cryptomeria japonica* were induced by GA₃ sprayed onto the shoots (Kurita et al., 2020).

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Therefore, the effects of hormones on sexual reproduction vary according to species and hormone types.

In this study, we reported the flowering induction of *C. tamdaoensis*, which is a rare camellia species with yellow flowers from Vietnam (Manh et al., 2019). Commonly known as golden camellias or yellow camellias, yellow-flowering camellias comprise 52 species. In addition to their aesthetic appeal, golden camellia flowers are valued for tea because they contain chemical compounds that may improve health (Lin et al., 2013; Wang et al., 2015). The active ingredients include polysaccharides, polyphenols, and saponins; furthermore, flavonoids are well-known characteristics of golden camellias. Currently, it costs US\$320 to US\$700 for 1 kg of dry flowers (Manh et al., 2019). Generally, it takes 6 to 8 years for golden camellias to start setting flower buds (Chai et al., 2009; Jiang and Zhao, 1997). Therefore, there is strong interest in inducing early flowering in golden camellias for earlier harvest. Previously, we have successfully produced flowers in juvenile *C. chrysantha* (Wei et al., 2017, 2018), another yellow-flowering camellia species, with PBZ. PBZ is a synthetic triazole-type cytochrome P₄₅₀ growth inhibitor that is used extensively in horticulture to improve the performance, yield, and quality of crops (Desta and Amare, 2021). Its ability to effectively induce and manipulate flowering or fruiting has been demonstrated in species such as mango, plum, and grapes (Desta and Amare, 2021). For *C. chrysantha*, the PBZ-induced flowers contain similar levels of active chemical components when compared with naturally occurring flowers (Wei et al., 2018). The current study focused on the effects of PBZ on *C. tamdaoensis* and provided the first insight regarding the action mechanism of PBZ underlying camellia flowering initiation in terms of the endogenous hormone response.

Materials and Methods

Plant materials and experimental treatment. Cuttings from a mature *C. tamdaoensis* plant in Vietnam were rooted in Aug. 2015 and grown in nonwoven fabric garden bags (height, 30 cm; diameter, 25 cm) containing yellow podzolic soil with a pH range of 4.5 to 6.0. Healthy plants with uniform growth (height, 103.4–120.6 cm; basal diameter, 12.3–15.1 mm) were selected for the study. Two levels of PBZ concentrations (100 and 200 ppm) were prepared with tap water; then, 1 L of solution was applied onto garden bags on the mornings of 9 Apr., 24 Apr., and 9 May 2020, respectively. The control plants were managed in the same way as the treated group except no PBZ was applied in the garden bags. The PBZ (Chemical Abstracts Service No. 76738-62-0) was purchased (Anyang Quanfeng Biological Technology Co., Ltd., He Nan Province, China) and contained 95% active components. There were four plants per treatment. The study was conducted in the Camellia Nursery of Guangxi

Forestry Research Institute (lat. 22°56'N, long. 108°21'E, 95 m above sea level) in China. The area has a subtropical monsoon climate and annual precipitation more than 1300 mm. Detailed climate information was reported previously by Wei et al. (2017, 2018). All plants were maintained under a shade canopy that was ≈2.9 m aboveground and blocked 50% to 60% sunlight.

Sample and data collection. Plant height and stem basal diameter were measured before PBZ treatment on 8 Apr., as well as on 29 Oct. 2020, when seasonal growth largely stopped. Flower buds and leaf numbers were counted on 26 Jan. 2021. Four fully opened flowers per plant were randomly sampled, and flower diameter and fresh weight were recorded on the same day.

Leaf samples from four flowering plants and four nonflowering plants were collected separately on 8 Apr. 2020 (1 d before PBZ treatment), 14 Apr. 2020 (1 week after the first treatment), 1 May 2020 (1 week after the second treatment), and 14 May 2020 (1 week after the third treatment). They were kept frozen until they were used for endogenous hormones analyses.

Analysis of endogenous phytohormones. Leaf samples preserved at –80 °C were ground into fine powders. Approximately 1 g of each sample was weighed and dissolved in a 0.4 mL-extract solution containing methanol, water, and formic acid (v:v:v = 15:4:1). Indoleacetic acid (IAA), abscisic acid (AA), salicylic acid (SA), and jasmonic acid (JA) were analyzed with high-performance liquid chromatography and tandem mass spectrometry by Nanjing Webiolotech Biotechnology Co., Ltd., as reported by Ma et al. (2008).

The liquid phase conditions were as follows:

1. Chromatographic column: Waters ACQUITY UPLC HSS T3 C18 1.8 μm, 2.1 m × 100 mm;
2. Mobile phase: water phase was ultrapure water (0.04% formic acid added) in the water phase, acetonitrile (0.04% formic acid added) in the organic phase;
3. Elution gradient: 0 min water/acetonitrile (90:10 v/v), 5.0 min 40:60 v/v, 70 min 40:60 v/v, 7.01 min 90:10 v/v and 10.0 min 90:10 v/v;
4. The flow rate of 0.35 mL/min, column temperature of 40 °C, and injection volume of 2 μm.

The following mass spectrometry conditions were used: electrospray ionization (ESI) temperature of 500 °C; mass spectrometry voltage of 5500 V, curtain gas of 35 psi; the collision-activated dissociation parameter was set to medium in the dissociation; and each ion pair was scanned according to the optimized cluster voltage and collision energy.

The hormonal content obtained during the analysis was expressed as mg/g fresh weight.

Data analysis. A completely randomized design was used for the PBZ experiments. Dunnett's multiple comparison method was used to test whether the difference among treatment types was statistically significant at $P = 0.05$, as previously reported by Wei et al. (2017, 2018). These analyses were performed using SPSS version 17.0 (IBM Corp., Armonk, NY).

Results and Discussion

PBZ effectively induces flowering in golden camellias. Without intervention, none of the control plants flowered (Fig. 1A). In contrast, three of the four *C. tamdaoensis* plants treated with 100 ppm PBZ flowered (75%), whereas 50% of the plants treated with 200 ppm PBZ produced flowers (Fig. 1B–F). The lower concentration induced an average of 12.7 flowers per plant, and the higher concentration treatment produced 33.0 flowers per plant. Therefore, 200 ppm PBZ produced significantly more flowers than the lower concentration in *C. tamdaoensis*. Furthermore, flowers induced by 200 ppm were larger in diameter than those induced by 100 ppm (26.0 ± 0.8 cm for 200 ppm vs. 17.2 ± 0.6 cm for 100 ppm). Regarding fresh weight, a flower induced by 200 ppm had an average weight of 1.2 ± 0.3 g, and a flower induced by 100 ppm had an average weight of 0.8 ± 0.4 g. For 4-year-old juvenile *C. chrysantha* seedlings, both 100 ppm and 200 ppm concentrations generated a 75% flowering rate, whereas 300 ppm resulted in a 100% success rate (Wei et al., 2018). For 4-year-old *C. chrysantha* grafted plants, a PBZ concentration range of 150 to 750 ppm in combination with urea resulted in a 100% flowering rate (Wei et al., 2017). Therefore, PBZ effectively induces flowering in both *C. tamdaoensis* and *C. chrysantha* juvenile plants, and 200 ppm induces comparable numbers of flowers for these two yellow flowering species (33 for *C. tamdaoensis* vs. 20 for *C. chrysantha*). Furthermore, PBZ can increase the flower bud number of red-flowering *Camellia* hybrid rooted cuttings (*C. saluenensis* × *C. japonica*) (Wilkinson and Richards, 1988). Induced flowers of juvenile *C. chrysantha* seedlings were smaller than natural flowers of mature trees (Wei et al., 2018). No references regarding flower size were found for naturally occurring *C. tamdaoensis* flowers.

PBZ inhibits vegetative growth. As a growth restrictor, evidence of the growth inhibition of PBZ has been well-documented, especially in high concentrations. For instance, plant height was reduced by ≈30% in camellia cuttings with 500 ppm (Wilkinson and Richards, 1988). *C. chrysantha* grafted plants treated with 750 ppm PBZ were significantly shorter and experienced severe defoliation, and some of these treated plants did not survive after flowering (Wei et al., 2017). In the current study, with *C. tamdaoensis* rooted cuttings, 100 ppm significantly reduced plant height and 200 ppm significantly

Fig. 1. *Camellia tamdaoensis* plants and their paclobutrazol (PBZ)-induced reproductive buds and flowers. (A) Untreated shoots without flowers on 2 Mar. 2021. Reproductive buds and flowers induced by PBZ on 7 Feb. 2021 (B–E) and 2 Mar. 2021 (F). (G) An apical shoot stunted by treatment with 200 ppm PBZ (7 Feb. 2021). The scale bars represent 20 mm in each panel.

decreased the stem diameter and leaf number (Fig. 2). For some of the treated plants, apical growth was stunted (Fig. 1G).

In the study by Wei et al. (2018) involving 100 to 300 ppm PBZ, the growth reductions in the height and stem basal diameter of *C. chrysanth* seedlings were not statistically significant ($P > 0.05$) when compared with those of the control; however, defoliation occurred and a strong correlation between the severity of defoliation and PBZ concentration was observed. Therefore, the degree of reduction in vegetative growth may depend on how the plants are generated (seedlings, rooted cuttings, or grafted plants). Wei et al. (2018) found no negative effects of PBZ on photosynthesis activity and levels of water-soluble sugars and nutrients (phosphorus, nitrogen, potassium, and carbon) in *C. chrysanth* seedlings. However, there were significant increases in the activities of ascorbate peroxidase (APX) and catalase (CAT) with 200 ppm and 300 ppm PBZ treatments, as well as in superoxide dismutase (SOD) with 100 ppm to 300 ppm PBZ treatments. Considering the significance of the steady-state level of cellular reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), in many biological and physiological processes of plants, the enhanced activities of these enzymes suggest that PBZ treatment leads to an increased level of superoxide ($O_2^{\cdot -}$) radicals and peroxides (such as H_2O_2), thus causing premature defoliation.

PBZ alters endogenous phytohormones dynamics during flowering initiation. Plant hormones have important roles in regulating developmental processes, including flowering. Yin and Liang (1994) found that lower levels of IAA and ABA occurred during *Metasequoia* male cone initiation, whereas the opposite was observed for female cones. Furthermore, transition from vegetative buds to cone buds in *Metasequoia* required a higher level of GA_{1+3} (Yin and Liang, 1994), indicating the importance of GA in the transition. In comparison, high GA_3 levels had an inhibitory effect on floral formation of olive during the induction and initiation periods, and the high concentrations of GA_4 , ABA, and certain cytokinin levels were beneficial (Ulger et al., 2004). Similar GA_3 inhibitory effects have been reported for other fruit trees such as mango, peach, and apple (Southwick and Glozer, 2000).

To understand the effects of PBZ on endogenous phytohormones, levels of IAA, ABA, SA, and JA in leaf were determined before and after PBZ treatment and compared between the PBZ-treated *C. tamdaoensis* flowering plants and the control (nontreated non-flowering plants). As shown in Fig. 3, a significant difference was found at least once during floral bud initiation for the phytohormones examined, thereby indicating the importance of hormone regulation. The spikes of IAA and SA on 1 May in the control group were suppressed by PBZ. In contrast, PBZ significantly enhanced the level of ABA on 14

Apr. and 14 May, of SA on 14 May, and of JA on 14 Apr. and 1 May (Fig. 3). These results support the findings of Fletcher and Hofstra (1988) and Rademacher (2000), who reported that PBZ treatment led to decreased abscisic acid catabolism. The GA level was not analyzed during the present study because it is well-known that PBZ inhibits GA biosynthesis by blocking the oxidative conversion of entkaurene to kaurenoic acid in plants, thus leading to decreased endogenous GA levels (Rademacher, 2000). Because both GA and ABA are synthesized via the terpenoid pathway, blocking gibberellins synthesis by PBZ may lead to the accumulation of precursors in the terpenoid pathway, thereby promoting ABA genesis (Rademacher, 2000).

Overall, our data suggest that low GA and IAA, high ABA and JA, and a gradual increase of SA benefit *C. tamdaoensis* floral initiation. The hormone dynamics in flowered *C. tamdaoensis* was largely the opposite of the observations for tree peony (*Paeonia suffruticosa*), which was successfully induced to flower with exogenous GA_3 ; high levels of endogenous GAs were accompanied by a reduced ABA level and increased IAA levels (Guan et al., 2019). Phytohormones, including GA, IAA, ABA, JA, and SA, have been reported to have a role in regulating the flowering network (Conti, 2017). Among them, GA is probably the most studied, as well as the most dominant, hormone in flowering. In *Arabidopsis*, different hormones appear to coordinately converge on the transcriptional activation of a small number of floral integrator genes, and the DELLAs signaling proteins of GA act as hubs for hormonal cross-regulation upstream of individual floral integrators (Conti, 2017). In addition, phytohormones have important effects on chromatin compaction mediated by DNA methylation and histone posttranslational modifications, suggesting the role of epigenetic regulation in flowering through hormone action.

In summary, the present study demonstrated the efficacy of PBZ in flowering induction in juvenile *C. tamdaoensis* rooted cuttings. For grafted plants (Wei et al., 2017), seedlings (Wei et al., 2018), and rooted cuttings of a golden camellia species, PBZ has been shown to be effective. Furthermore, this study provides the first insight regarding endogenous phytohormone dynamics during

Fig. 2. Effects of paclobutrazol (PBZ) on *Camellia tamdaoensis* height (A), stem basal diameter (B), and leaf number (C). Measurements were obtained on 8 Apr. 2020, before PBZ treatment, and on 29 Oct. 2020, when the active growing season ended. The leaf number was counted during blossom on 26 Jan. 2021. Different letters in each panel indicate a significant difference when $P < 0.05$. The bars indicate SD. N = 4.

Fig. 3. Dynamics of endogenous phytohormones during *Camellia tamdaoensis* bud initiation and differentiation. Samples were collected before (8 Apr. 2020) and after each paclobutrazol (PBZ) treatment (14 Apr., 1 May, and 14 May 2020). Endogenous phytohormones were analyzed using high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). Each treatment at each timepoint included at least three biological replicates. *Significant difference at $P < 0.05$.

golden camellia floral initiation. The PBZ treatment led to quantitative changes in endogenous IAA, ABA, SA, GA, and JA. These hormones may have synergistic or antagonistic pivotal regulatory roles in golden camellia floral bud induction.

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