Anatomical Characterization of Flower-bud Blasting and Suppression Following Hormone Application in *Eustoma grandiflorum* (Raf.) Shinn.

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Flower-bud blasting is a constraint for producing *Eustoma grandiflorum* so a preventative strategy is needed. Flower-bud blasting occurs under low light intensity and high fertilizer input. To gain insight into the mechanisms of flower-bud blasting, we conducted a detailed characterization of flower development under normal and blast-inducing conditions. We found that floral buds under low light intensity ceased to grow at the stamen and gynoecium differentiation stages, although sepals and petals were initiated normally. Aborted flowers rarely had normal ovules. Moreover, an expanded apical meristem was observed. These results show that the differentiation and development of reproductive organs are critically suppressed by blast-inducing conditions; however, combined application of 300 ppm benzylaminopurine and 200 ppm gibberellic acid-3 to floral buds resulted in about five-fold greater frequency of flower opening compared to controls. Blasting inhibition also resulted from excising the inflorescent branch, suggesting the decrease in assimilates in flower buds would be attributed to flower-bud blasting. Moreover, hormone application combined with excision had an additive effect for preventing flower-bud blasting, suggesting that these treatments independently inhibit flower-bud blasting. These results suggest that flower-bud blasting in *Eustoma* is a break in floral development around the stamen and gynoecium initiation stages and is integrally induced by the factors related to hormone biosynthesis and the decrease in assimilates.

**Key Words:** assimilate, cytokinin, *Eustoma*, flower-bud blasting, gibberellin.

**Introduction**

*Eustoma grandiflorum* (Raf.) Shinn (lisianthus) originated from North America and is widely grown as an ornamental flower in temperate areas (Shimizu-Yumoto and Ichimura, 2010). When the external environmental conditions are inappropriate, damage such as rosette formation, tip burn, and flower-bud blasting is inevitable, resulting in unsuccessful flower opening. Flower-bud blasting is a type of abortion in flowers and commonly refers to the arrest of perfect flower development. The mechanism of flower-bud blasting remains uncertain, although low light intensity and the application of high concentrations of nitrogen promote blasting in *Eustoma* (Ushio and Fukuta, 2010).

Flower-bud blasting is observed in many popular flowering plants, such as lily, *Iris*, *Zinnia elegans*, and *Chrysanthemum*. Although experiments have been conducted to identify the cause of blasting, the mechanism remains poorly understood. Many studies have focused on two possible factors that may be involved in regulating flower-bud blasting, hormones and photoassimilation. Ethylene promotes flower-bud blasting in the tulip and lily (Demunk, 1973; Mason and Miller, 1991). Hormonal changes may also be associated with flower-bud blasting, such as smaller amounts of cytokinin and larger amount of abscisic acid in *Iris* (Vonk and Ribot, 1982; Vonk et al., 1986). Exogenous gibberellin and cytokinin treatment results in the decreased occurrence of flower-bud blasting in tulip and *Cymbidium* (Demunk and Gijzenberg, 1977; Ohno, 1991), suggesting that blasting is induced by the factors related to hormone biosynthesis in tulip and *Cymbidium*. Kernel abortion in maize, however, is apparently not initiated by a hormone signal, as a reduction in hormones is observed only after abortion occurs (Reed and Singletary, 1989). This suggests that a careful examina-
tion considering the timing of sampling is needed.

Another factor, photoassimilation, seems to be associated with flower-bud blasting among various species (Chrysanthemum, Cymbidium, Iris, Lathyrus odoratus; Fudano et al., 2009; Fukai et al., 1981; Ohno, 1991; Vonk and Ribot, 1982). In the pepper, flower-bud blasting appears to be caused by the low accumulation of assimilates in the floral bud (Aloni et al., 1997).

Information on the development of floral organs in Eustoma is essential to overcome flower-bud blasting; however, the developmental profile of the Eustoma flower is not fully understood, even under normal conditions, except for gametophytes (Yang et al., 2007). Here, we conducted a detailed investigation into the initial stages of flower development. Additionally, we investigated the effect of the plant hormones cytokinin and gibberellin on flower-bud blasting. We demonstrated that gibberellin and cytokinin treatments inhibit flower-bud blasting caused by blast-inducing conditions in Eustoma.

Materials and Methods

Plant material and growth conditions

Eustoma grandiflorum ‘Piccorosa Snow’ seeds were sown in plug trays containing fertilized soil and maintained at 10°C in the dark for 5 weeks in a growth cabinet. Trays were then transferred to a growth chamber (Nihonika Co., Tokyo, Japan) maintained at 28°C under a 12 h light phase with a fluorescent lamps (50 μmol·m⁻²·s⁻¹ PPFD) and 18°C under a 12 h dark phase until the two-pair leaf stage. Each seedling was transplanted to a 10.5 cm plastic pot containing fertilized medium (Kureha Engeibaido, Kureha Chemical Industry Co., Ltd., Tokyo, Japan) and placed in a growth chamber at 28°C/18°C under a 14 h photoperiod supplemented with fluorescent lamps (400 μmol·m⁻²·s⁻¹ PPFD).

Induction of flower-bud blasting and evaluation of flower-bud blasting

At the transition stage from the vegetative to the reproductive phases (6 weeks after transplant), the plants were transferred to a growth chamber at 25°C in a 12 h light phase with fluorescent lamps (130 μmol·m⁻²·s⁻¹ PPFD) and 15°C in a 12 h dark phase. The solution of a compound fertilizer, OKF1 (N:P₂O₅ :K₂O, 15 : 8 : 17, Otsuka Chemical Co., Tokyo, Japan) including 75 ppm nitrogen was applied instead of water. Control plants were grown under the same conditions as in the vegetative phase. The first flower of each plant was evaluated for flower-bud blasting.

Measurement of flower buds

As sepals grew normally even if flower-bud blasting occurred, the length of flower buds was defined as the distance from the bottom end of the sepal to the top of the petal.

Histological analysis

Flower buds were collected 5 times at 5-day intervals from 3 days after transition to blast-inducing conditions. To obtain conclusive information, flower buds were collected 35 days after transition to blast-inducing conditions. For paraffin sectioning, we collected floral bud samples and fixed them overnight in formalin : glacial acetic acid : 70% ethanol; 1 : 1 : 18, followed by dehydration in a graded ethanol series. Following substitution with xylene, the samples were embedded in Paraplast Plus (Oxford Labware, St. Louis, USA) and sectioned to a thickness of 8 μm using a rotary microscope. Sections were stained with 0.05% toluidine blue and observed with a light microscope.

Branch removal

Inflorescence branches located next to the first flower were removed when benzylaminopurine (BA), dichlorofufenuron (CPPU), and gibberellic acid-3 (GA₃) and control liquid were applied to the first flower. To assess the effect of branch removal independent of hormone application, we removed an inflorescence branch and applied the below-mentioned control liquid instead of hormones to the first flower.

Hormone treatments

We assessed the effects of exogenously applied BA, CPPU, and GA₃ on flower-bud blasting. Three weeks after inducing flower-bud blasting, approximately 4 mm lengths of flower bud were inoculated with 300 ppm BA and 30 ppm CPPU in 20% (v/v) aqueous acetone containing 0.05% Tween20, respectively. Aqueous acetone (20%, v/v) containing 0.05% Tween20 was applied to flower buds of control plants. Five pots were used to administer each compound.

Seventeen days after inducing flower-bud blasting, approximately 4 mm lengths of flower bud were inoculated with various concentrations of BA (0, 30, 100, 300, and 3000 ppm) in 20% (v/v) aqueous acetone containing 0.05% Tween20. Six pots were used for each concentration with and without branch removal.

Two weeks after inducing flower-bud blasting, approximately 2.5 mm lengths of flower bud were inoculated with various concentrations of GA₃ (0, 40, 200, and 400 ppm) in 20% aqueous ethanol solution (v/v) containing 0.05% Tween20. Eight pots were used for each concentration with and without branch removal.

Two weeks after inducing flower-bud blasting, approximately 2.5 mm lengths of flower bud were inoculated with both 200 ppm GA₃ and 300 ppm BA. Eight pots were used.

Results

1. Developmental course of flower differentiation and flower-bud blasting in E. grandiflorum

Low light conditions and an increased level of nitrogen fertilizer significantly increased flower-bud blasting.
We compared sequential changes of flower development under blast-inducing conditions versus under control conditions. Under control conditions, floral buds become larger with age (Fig. 1C). In contrast, floral buds maintained under low light intensity ceased to grow (Fig. 1C).

To clarify the stage at which flower-bud blasting occurred, we observed longitudinal sections of flower buds. Under control conditions, flower meristems initiated sepal primordia and subsequently several layers of petals protruded (Fig. 1D, stage 1). Floral meristems produced stamen primordia when the first petal primordia were about 2 mm in length (Fig. 1E, stage 2). Carpals and placenta were produced when the first petal was approximately 3–5 mm in length (Fig. 1F and G, stages 3 and 4, respectively). It took about 2 weeks from stages 1 to 4. Subsequently, numerous ovules differentiated (Fig. 1H, stage 5) and megasporocyte differentiation occurred (Fig. 1I, stage 6).

Under low light intensity, all floral meristems initiated the development of normal sepal and petal primordia (Fig. 1B, C, J), whereas development of stamens and gynoecium was severely inhibited. Some plants initiated stamen primordia 2 weeks after treatment (Fig. 1K) and several floral buds achieved stages 3 and 4 (Fig. 1L, M). Low light intensity was associated with a low frequency of normal ovule differentiation. In addition to the pistil, petal development was also inhibited (Fig. 1B, N). We confirmed that the petal size at stage 4 under control condition was 4.2 mm and the cell division of petals continued from stage 4 onward (data not shown). These results suggested that blast-inducing conditions which caused smaller petals influenced cell proliferation as well as organ differentiation.

To obtain conclusive information regarding flower-bud blasting, we observed the floral buds 35 days after the plants had been transferred to blast-inducing conditions. Under control conditions, all flower buds

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**Fig. 1.** Developmental profiles of flower-bud blasting in *Eustoma grandiflorum*. A, Appearance of the plants under control (left) and blast-inducing conditions (right) 47 days after induction. All floral buds aborted under low light (arrows). Scale bar = 5 cm. B, Higher magnification of flower-bud blasting shown in A. Scale bar = 1 cm. C, Changing patterns in the length of floral buds during development. Vertical bars indicate SE (n = 7). D–M, Developmental profiles of flowers under normal conditions (D–I) and blast-inducing conditions (J–M). D, J, Petal initiation stage (stage 1). E, K, Stamen initiation stage (stage 2). F, L, Carpel initiation stage (stage 3). G, M, Placental differentiation stage (stage 4). H, Ovule differentiation stage (stage 5). I, Megasporegenesis stage (stage 6). Abbreviations: se, sepal; p, petal; p1, petal in the first layer; st, stamen; ca, carpel. Scale bars = 400 μm in D–M. N, Correlation of petal length with pistil length under normal and inducing conditions.
went through stage 6 (Fig. 2A). In contrast, up to 50% of floral buds ceased to produce stamens and gynoecium under blast-inducing conditions (Fig. 2A), and none of the flower buds enclosed ovules. Furthermore, aberrations in floral meristems were occasionally found. For example, just after initiating stamen primordial development, normal floral meristems were about 50 μm in width (Fig. 1E). In contrast, floral meristems in which flower-bud blasting occurred were up to 200 μm in width (Fig. 2B). Cells in the rib zone of blasting flowers expanded compared to those in normal flowers (Fig. 2B). These results demonstrated that blast-inducing conditions arrested two phenomena, organ differentiation and supply of undifferentiated cells, although they were not coincidental.

2. Effect of excising the inflorescence branch on flower-bud blasting

The inflorescence branch next to the flower bud continues to grow in Eustoma even if flower-bud blasting occurs (Fig. 1A). The most common symptom of flower-bud blasting is vigorous growth of the inflorescence branch. As flowers and inflorescence branches grow simultaneously, some competition may occur. To investigate the effect of accumulation and partitioning on floral organization, we removed inflorescent branches when flower buds next to them were approximately 2.5–4 mm long. Branch removal resulted in the decreased frequency of flower-bud blasting (Figs. 3A, C, D and 4A). The rate of flower-bud blasting appeared to be slightly but significantly ($P<0.05$, t-test) lower than in
plants in which the inflorescent branches were not removed. These results indicate that the inflorescent branch might arrest the development of the neighboring flower.

3. Effect of cytokinin treatment on flower-bud blasting.

Our anatomical study indicated that the blast-inducing condition likely influenced cell proliferation of floral organs in *Eustoma*. Cytokinins are plant hormones that regulate plant cell division (Riou-Khamlichi et al., 1999). To estimate the effect of cytokinins on flower-bud blasting, we applied the pure cytokinin \textit{N}6-benzylaminopurine (BA) and the phenylurea cytokinin forchlorfenuron (CPPU), a synthetic compound with cytokinin-like activity. Both BA and CPPU resulted in increased length of flower 42 days after hormone inoculation (Fig. 3A). The appearances of flowers with cytokinin treatment and branch removal under blast-inducing condition were comparable to those under normal conditions (Figs. 1A and 3B). These results demonstrated that cytokinins act to suppress flower-bud blasting.

Next, we applied various concentrations of BA to further investigate the effect of BA on the suppression of blasting. The applications of 30 ppm, 100 ppm, and 300 ppm BA increased the frequency of flower opening, respectively (Fig. 3C). Flower-bud blasting was significantly prevented by applying 30 ppm BA, regardless of whether the inflorescent branch was removed (Fig. 3D).

To investigate the relationship between excising the inflorescent branch and BA treatment, we applied a combined treatment of excising the inflorescent branch and treating with BA and CPPU. Combined treatment was more effective than the individual treatments (Fig. 3C, D), suggesting that excising the inflorescent branch and treating with cytokinins acted independently to affect flower-bud blasting.

4. Effect of gibberellic acid-3 (GA$_3$) treatment on flower-bud blasting.

Gibberellins (GAs) play an important role in the development of flowers in *Eustoma* (Hisamatsu et al., 1999, 2004); thus, we applied GA$_3$ at various concentrations to investigate the effect of GA$_3$ on flower-bud blasting. Treatment significantly inhibited flower-bud blasting (Fig. 4A, B). In particular, the flower opening rate was restored with the application of 200 ppm GA$_3$ (Fig. 4A). Moreover, the effect was enhanced by branch removal (Fig. 4A, C), suggesting that GA$_3$ and branch removal act independently to prevent flower-bud blasting. Even low levels of GA$_3$ prevented flowers from blasting in case of branch removal (Fig. 4A, C). The application of 200 ppm GA$_3$ and excising the inflorescent branch reproducibly resulted in complete flower development (data not shown).

Combined application of 300 ppm BA and 200 ppm GA$_3$ to floral buds resulted in about five-fold greater frequency of flower opening than in controls (Fig. 4A) and brought about flowers which were similar in size to those under control condition (Figs. 1C and 4B). The exogenous, simultaneous application of GA$_3$ and BA caused a higher frequency of flower opening than single applications (Figs. 3C and 4A), suggesting that GA$_3$ and BA act to resolve flower-bud blasting independently.

Discussion

We conducted a detailed investigation of flower-bud blasting to develop an application to suppress flower-bud blasting. Our anatomical approach showed that flower-bud blasting is a spontaneous end to floral development in the stamen and gynoecium initiation stages. An incomplete gynoecium was observed even in petals up to 10 mm long, demonstrating that floral-bud blasting represents a break in floral development rather than necrosis of the flower bud. It is unlikely that low light intensity results in a shortage of energy sources...
because the inflorescent branches next to aborted floral buds grew normally (Fig. 1). Inadequate conditions did not result in improper anabolism but rather in damage associated with flower development.

To gain insight into this damage, we investigated the effects of hormones by which floral organ development is almost certainly regulated. When we inoculated hormones to flower buds under blast-inducing condition, the flower buds were 2.5–4 mm in length. Our anatomical analysis demonstrated that the stages in which flower buds were about 2.5–4 mm in length were stage 2 to stage 3. GA$_3$ and cytokinin applications resulted in increase of the frequency of flower opening. Thus, GA$_3$ and cytokinins affected flower buds producing the stamen and gynoecium.

The highest frequency of blasting suppression by a single treatment was observed in experiments in which 200 ppm GA$_3$ was applied (Fig. 4A). GA$_3$ acts as a functional inhibitor of DELLA proteins, which are repressors of gibberellin signaling in Arabidopsis (Cheng et al., 2004; Yu et al., 2004). In Arabidopsis, GAs play a role in upregulating the expression of floral homeotic genes that control stamen and carpel identity (Yu et al., 2004). They also promote the expression of the flower meristem identity gene LEAFY, which directly activates genes including stamen and carpel identity genes (Blazquez and Weigel, 2000; Busch et al., 1999; Parcy et al., 1998). In addition, exogenous GA modulates flower development in various plants (Demunk and Gijzenberg, 1977; Ohno, 1991; Zhang et al., 2008; Zieslin et al., 1977). In light of these findings and our analysis, GA-mediated promotion of flower development appears to be conserved in Eustoma.

In flower meristems of Arabidopsis, the gibberellin 3-oxidase (GA$_{3ox}$) gene, which catalyzes the final step in the synthesis of bioactive GAs, is only expressed in stamens and the gynoecium (Hu et al., 2008), and the flower homeotic gene AGAMOUS, which facilitates organ identity of stamens and the gynoecium, binds to the GA$_{3ox}$ promoter (Gomez-Mena et al., 2005). Thus, once floral organ differentiation proceeds through stamen and gynoecium stages, flower buds themselves would become capable of supplying sufficient GA. In Eustoma, almost all aborted-flowers were less than 10 mm long. Floral meristems under control conditions, in which flower buds are about 10 mm long, differentiated ovule primordia. This suggested that flower-bud blasting tend not to occur once the stamen and gynoecium initiation stages had passed; therefore, GA$_{3ox}$ induction at stamen and gynoecium primordia might be conserved in Eustoma.

In addition to GA, applying BA and CPPU reversed blasting in Eustoma. Cytokinins regulate a variety of developmental processes, including the development of floral organs (Barrina et al., 2011; Nishijima and Shima, 2006). Blast induction arrested the development of floral organs which had been differentiated (Fig. 1C), suggesting that cell division was suppressed. As cytokinin stimulates cell division by regulating the G1/S phase transition in Arabidopsis (Barrina et al., 2007; Riou-Khamlichi et al., 1999; Soni et al., 1995), cytokinins in inoculation experiments would act as cell-division activators in Eustoma.

Not only cell division, but also organ differentiation was suppressed by blast-inducing conditions (Fig. 2A). It has been reported that cytokinins facilitate cell differentiation as well as cell division in Arabidopsis (Werner et al., 2001, 2003). Similarly, organ differentiation is suppressed in cytokinin-deficient potato tubers (Hartmann et al., 2011). Our results demonstrated that the effects of blast-inducing conditions on organ differentiation and the supply of undifferentiated cells were not coincidental. Hence, it is likely that exogenous cytokinins promoted floral organ differentiation as well as cell division in Eustoma. As cytokinins are exported through the xylem and accumulate at high transpiration sites (Aloni et al., 2005; Boonman et al., 2007), a reduction in active cytokinins in meristems may be induced by low light intensity.

In contrast to cytokinins, it has not been reported that low light intensity decreased endogenous GA in flower buds; therefore, GAs may act as negative blasting regulators by decreasing sensitivity to other factor(s) that are induced by blast-inducing conditions and that promote blasting.

It has been reported that removing branches changes assimilate partitioning (Fudano et al., 2001) and the correlation between low tolerance to light stress and levels of assimilate in flower buds (Aloni et al., 1996; Turner and Wien, 1994). Our results demonstrate that the effect of branch removal was independent of hormone treatments; therefore, it is likely that branch removal leads to the suppression of blasting by increasing the assimilate in flower buds. Branch removal, however, unexpectedly did not lead to extreme inhibition of blasting. This result indicated that the increased levels of assimilation in floral buds might not be sufficient for perfective flower development. Decapitation together with hormone treatment led to marked inhibition of blasting compared to each single hormone treatment and single branch removal. These additive effects suggest that low amounts of assimilates in the floral bud were rate-limiting for overcoming blasting in the single hormone application experiment and vice versa.

Flower-bud blasting studies in diverse plant species have predicted that flower-bud blasting is caused by low levels of assimilate as an energy source (Aloni et al., 1997; Fudano et al., 2009; Fukai et al., 1981; Ohno, 1991; Vonk and Ribot, 1982). A possible explanation for the slight effect of branch removal in this study is that not only absolute amounts of assimilates in flower buds but also those in the whole plants which have not been exploited in vegetative organs may act as a signal that controls flower development. This is consistent with
the previous result that the shortage of acquired assimilates in the whole plant is not necessarily for blasting (Ushio and Fukuta, 2010). Sugars play pivotal roles regulating the expression of genes controlling cell division, cell proliferation, and death (Gibson, 2005; Leon and Sheen, 2003). To assess whether a decrease in assimilates acts as a signal or not, further studies are needed.

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