Identification of Endogenous Gibberellins and Their Role in Rosetted Seedlings of *Eustoma grandiflorum*

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**Summary**

Endogenous gibberellins (GAs) were extracted from shoots with floral buds of *Eustoma grandiflorum* and analyzed by full scan gas chromatography/mass spectrometry (GC/MS). Consequently, five C31-hydroxylated GAs, GA1, GA19, GA20, GA44, and GA53, were identified. The presence of these GAs suggests that one major GA biosynthetic pathway, the early C31-hydroxylation pathway, is operating in these shoots. Stem elongation of rosetted plants was promoted by applications of GA1, GA4, GA9, and GA20. C31-hydroxylated GAs, GA1, and GA20, were more active in stem elongation than were the non-C31-hydroxylated GAs, GA4, and GA9. Inhibition of stem elongation on Uniconazol-P (UCZ) and Prohexadione-calcium (P.Ca)-treated plants was wholly reversed by application of GA3, where the inhibition by P.Ca was partially overcome by GA20. These results suggest that the activation steps of precursory GAs, such as 3β-hydroxylations, from GA20 to GA1 and from GA9 to GA3, are functioning in the rosetted plants and that the endogenous GA1 is physiologically important in regulating stem elongation of *E. grandiflorum*.

**Key Words:** gibberellin, *Eustoma grandiflorum*, Uniconazol-P, Prohexadione-calcium.

**Introduction**

*Eustoma grandiflorum* (Raf.) Shinn. (syn. *Lisanthus russellianus* Hook.) is the leading cut flower in Japan. The number of cut flowers has increased to 111.6 million stems in 1994. Stems of most *Eustoma* cultivars begin to elongate after they develop the third leaf-pair. However, rosetting occurs when the plants are grown at an average temperature of above 25 °C and a minimum temperature of 20 °C (Ohkawa et al., 1991; Harbaugh et al., 1992). Rosetting is a major problem in *Eustoma* cultivation. Seedlings of *E. grandiflorum* are most sensitive to high temperature for induction of rosetting during the first two weeks after seed imbibition. The sensitivity to high temperature gradually declines as leaves develop and is not apparent after the second leaf-pair stage (Ohkawa et al., 1991). These rosetted plants require an exposure to 10-15 °C over 4-6 weeks for stem elongation and flowering to occur (Ohkawa et al., 1994). Stem elongation of the rosetted plants was induced by application of GA3, but the plants formed an aerial rosette at a higher nodal position after several internodes elongated (Takeda, 1988). However, the relationship between endogenous GAs and rosetting in *E. grandiflorum* is not cleared. In this study, we investigated endogenous GAs in *E. grandiflorum* and their role in stem elongation.

**Materials and methods**

*Extraction and purification of GAs*

Seeds of *E. grandiflorum* cv. Azumano Yosooi were sown on October 20, 1994 and were grown in a greenhouse maintained above 15 °C. The shoots with floral buds (350 g, fresh weight) were harvested on March 27, 1995. Endogenous GAs were extracted from the fresh materials with 90% aqueous methanol (MeOH). After filtration, MeOH was removed in vacuo at 45 °C; the aqueous residue was adjusted to pH 2.5 with 1N HCl and partitioned against ethyl acetate (EtOAc, 40 ml×3 times). The combined EtOAc phase was then partitioned against 0.5 M phosphate buffer of pH 8.3 (40 ml×3 times). The combined aqueous phase was mixed with polyvinylpolypyrrolidone (5 g) and then filtered. The aqueous phase was adjusted to pH 2.5 with 5N HCl and partitioned against EtOAc (60 ml×3 times). The combined EtOAc phase was dehydrated over anhydrous sodium sulphate. After filtration, EtOAc fraction was evaporated in vacuo and then re-dissolved...
in a small amount of MeOH. The methanolic solution was loaded on a Bondesil DEA (5 g, Varian Associates, CA, USA) column (packed with methanol) and the column was washed with MeOH (100 ml); GAs were eluted with MeOH containing 0.75% acetic acid (100 ml), and the eluate reduced to dryness in vacuo. The residue was dissolved in a small amount of 45% aqueous MeOH containing 0.1% acetic acid (referred to as acidic MeOH). The GAs in the solution were separated by HPLC on a Develosil ODS column (10 mm i.d. × 150 mm, Nomura Chemical, Aichi, Japan) by eluting with a linear gradient of H2O-MeOH solution containing 0.1% acetic acid. The linear gradient elution conditions were as follows: MeOH increased from 45% to 50% for 25 min, from 50% to 80% for another 25 min, 10 min with 80% acetic methanol; and finally 20 min with acetic methanol. The total elution time was 80 min, with a flow rate of 2 ml min⁻¹; and 2 min fractions were collected. The fractions were dried in vacuo and bioassayed by a rice microdrop procedure (Nishijima, 1992). The biologically active fractions were further isolated by HPLC on Nucleosil N (CH₃)₂ columns (10 mm i.d. × 150 mm, Senshu Chemical, Tokyo, Japan) by eluting with acidic MeOH at a flow rate of 2 ml min⁻¹; 2 min fractions were collected, dried and bioassayed.

Identification of GAs

Endogenous GAs in the shoots including flower buds of E. grandiflorum were identified using a JEOL Auto- mass 20 equipped with HP 5890 GC equipment (GC/MS). After purification by Nucleosil N (CH₃)₂ HPLC, the fractions showing GA-like activity were dried in vacuo and then dissolved in MeOH (20 μl) and methylated with ethereal diazomethane (100 μl) at room temperature. They were then dried and trimethylsilylated with bis-(trimethylsilyl)-trifluoroacetamide (20 μl, BSTFA, Tokyo Kasei Kogyo, Japan) in glass tubes at 75 °C. The samples (1 μl) were injected into a fused silica cross-linked 5% phenylmethylsilicone capillary column (0.25 mm i.d. × 15 m, 0.33 μm film thickness, J & W DB-1). The oven temperature program started at 120 °C and after 2 min was increased at 16 °C min⁻¹ to 216 °C and after 5 min was increased at 8 °C min⁻¹ to 280 °C. The electron energy was 70 eV and the ion source was kept at 200 °C. GAs were identified from mass spectra and Kovats retention indices (KRI, Kovats, 1958) in comparison with authentic GAs or published data (Gaskin and MacMillan, 1991).

Effect of applied GAs on rosetted plant

Experiment 1.

Seeds of E. grandiflorum cv. Azumano Yosooi were sown on March 9, 1996, and grown in a plug tray (cell size was 2.0 cm (L) × 2.0 cm (W) × 4.0 cm (D)), containing Metro Mix 350 (Scotts, OH, USA) in a chamber maintained at 30 °C under a 12-hr photoperiod until the second leaf-pair stage. The plants were transplanted to plastic pots (12 cm i.d., one seedling per pot) containing fertilized medium (Kureha Engeibai, Kureha Chemical Industry Co., Ltd., Tokyo, Japan) on April 20, 1996. The plants were grown in a natural-light phytotron maintained at 35/30 °C (06:00-1800 h/1800-0600 h) under a 16-hr photoperiod with supplemental fluorescent lamps (FL-40S-PG, National Co. Ltd., Osaka, Japan) until the fifth leaf-pair stage. These plants were then transferred to a greenhouse maintained under a 12-hr photoperiod (0600-1800 h) and grown until the end of experiment. During the experiment, the average, maximum and minimum temperatures were 28 °C, 35 °C, and 20 °C, respectively.

GA treatments were started when the plants reached to the sixth leaf-pair stage. Each of GA₁, GA₃, GA₅, and GA₂₀ was dissolved in 10% aqueous acetone at a concentration of 0.1 μg/μl. Ten μl of each GA solution or 10% aqueous acetone (control) was applied to a shoot tip. Applications were made 2 times per week for 5 weeks. Each treatment consisted of 12 plants and cultivated for 8 weeks after the first application.

Experiment 2.

Seeds of E. grandiflorum cv. Asukano Nami were sown on May 29, 1996, and grown in plug trays (cell size was 2.0 cm (L) × 2.0 cm (W) × 4.0 cm (D)), containing Metro Mix 350 in a chamber maintained at 30 °C under 12 hr-photoperiod until the second leaf-pair stage. Then, the plants were transplanted to plastic pots (9 cm i.d., one seedling per pot) containing fertilized medium (Kureha Engeibai) on July 10, 1996, and grown in a natural-light phytotron as in Exp. 1. The plants were separated into three groups and were administered the following treatment. Ten ml of aqueous Uniconazol-P (UCZ, Sumitomo Chemical Co. Ltd., Osaka, Japan, 50 mg/l) was applied to the soil of each pot in the first group when the plants reached to the fifth leaf-pair stage. To the second group, 10 μl of Prohexadione-calcium (PCA, Kumiai Chemical Industry Co. Ltd., Tokyo, Japan, 1 μg/μl in 10% aqueous acetone) was applied to shoot tips of plants on the same day of the UCZ treatment, and repeated 4, 8 and 11 days after the first treatment. Shoot tips of plants in the third group received 10 μl of 10% aqueous acetone. Subplots of plants in each group were received 10 μl of GA₁ and GA₂₀ (0.1 μg/μl in 10% aqueous acetone). The treatment was repeated 1, 5 and 8 days after the chemical treatments were started. Each plot and subplots consisting of 6 plants were cultivated for 45 days after the treatments were started.

Results

Identification of endogenous GAs

The fractions from Develosil ODS HPLC were com-
bined to give six groups according to their biological activity by a rice microdrop assay. These groups were subsequently purified by Nucleosil N (CH$_3$)$_2$ HPLC before GC/MS analyses. Consequently, five C13-hydroxylated GAs, GA$_1$, GA$_{19}$, GA$_{20}$, GA$_{44}$, and GA$_{53}$, were identified by comparison of mass spectra and KRI with those of authentic GAs (Table 1).

**Effect of applied GAs on rosetted plants**

**Experiment 1.**

Stem scarcely elongated in the control plants as they were rosetted (Fig. 1). However, the stem elongation of the rosetted plants was promoted by application of GA$_1$, GA$_4$, GA$_9$, and GA$_{20}$, GA$_{1}$ being the most active, followed by GA$_{20}$, GA$_4$, and GA$_9$ in order of the activity.

Flowering of the rosetted plants was not induced by GAs application. The control plants remained rosetted until the end of experiment, whereas the GA-treated plants reformed an aerial rosette at a higher nodal position after the internode elongated and remained thus until the end of the experiment.

**Experiment 2.**

Stems of the control plants grew slightly but still retained the rosette form (Fig. 2). The stems of UCZ-treated plants were suppressed compared to that of the control plants, whereas those of PCA-treated plants were obviously enhanced while still remaining rosetted. Stem elongation of the control and UCZ- and PCA-treated plants was promoted by application of GA$_1$ and GA$_{20}$. GA$_1$ was more active than GA$_{20}$ in causing the stem elongation. Stem elongation response of UCZ-treated plants to GA$_1$ and GA$_{20}$ was similar to that of plants administered the respective GAs alone. Growth inhibition by the PCA-treatment was partially reversed by GA$_{20}$ but wholly by the GA$_1$ treatment, compared to the control. Application of GA$_1$ and GA$_{20}$ to the control plants altered leaf shape by increasing the length and narrowing the width compared to the control (Fig. 3). Leaf expansion of the UCZ-treated plants was markedly inhibited compared to that of the control plants but the inhibition of leaf length was overcome by the application of GA$_1$ and GA$_{20}$, but not that of leaf width.

**Table 1.** Gibberellins identified by GC/MS as MeTMSi derivatives in shoots with floral buds of *E. grandiflorum* cv. Azuma no Yossoi.

<table>
<thead>
<tr>
<th>C$_4$HPLC</th>
<th>N (CH$_3$)$_2$ HPLC</th>
<th>Relative intensities of major ions : m/z (%)</th>
<th>KRI$^c$</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 - 5$^7$</td>
<td>11 - 12$^7$</td>
<td>506 (100) 491 (12.4) 448 (30.8) 376 (26.9) 207 (85.1)</td>
<td>2661</td>
<td>GA$_1$</td>
</tr>
<tr>
<td>12 - 14</td>
<td>15 - 17</td>
<td>418 (100) 403 (17.4) 375 (85.4) 301 (23.9) 207 (23.8)</td>
<td>2491</td>
<td>GA$_{20}$</td>
</tr>
<tr>
<td>15 - 17</td>
<td>11 - 13</td>
<td>432 (19.5) 417 (6.1) 401 (5.4) 373 (11.2) 207 (100)</td>
<td>2800</td>
<td>GA$_{44}$</td>
</tr>
<tr>
<td>18 - 19</td>
<td>19 - 21</td>
<td>462 (17.6) 434 (100) 402 (47.1) 374 (97.1) 345 (69.7)</td>
<td>2588</td>
<td>GA$_{19}$</td>
</tr>
<tr>
<td>24 - 27</td>
<td>4 - 8</td>
<td>448 (63.9) 416 (19.6) 389 (45.4) 373 (13.4) 207 (100)</td>
<td>2493</td>
<td>GA$_{20}$</td>
</tr>
</tbody>
</table>

$^c$ Kovats' retention index.
$^7$ Number of fractions.

![Fig. 1. Effect of GA$_1$, GA$_4$, GA$_9$ and GA$_{20}$ on stem elongation in the rosetted plants of *E. grandiflorum* cv. Azuma no Yossoi at 31 days after the first application. Values are Mean ± SE (n = 12). Stem elongation was not observed in control.](image1.png)

![Fig. 2. Inhibition of Uniconazole (UCZ) and Prohexadione calcium (PCA) on stem elongation and their reversibility by GA$_1$ and GA$_{20}$ in rosetted plants of *E. grandiflorum* cv. Asuka no Nami. Data taken 45 days after the first application. Values are Mean ± SE (n = 6).](image2.png)
Flowering of the rosetted plants was not induced by any application of these chemicals and GAs in this experiment. The control plants remained rosetted until the end of experiment; GA-treated plants with their aerial rosettes, likewise, stayed unaltered until the end of the experiment.

**Discussion**

The presence of five C13-hydroxylated GAs, GA1, GA18, GA20, GA44, and GA33 in *E. grandiflorum* suggests that one major GA biosynthetic pathway, the early C13-hydroxylation pathway (→GA53→GA44→GA19→GA20→GA1→), is operating in the shoots and flower buds of *E. grandiflorum*. The current knowledge of GA biosynthesis suggests that the endogenously active GAs may be GA1 on the early C13-hydroxylation pathway and GA4 on the non-C13-hydroxylation pathway (Phinney and Spray, 1982; Nakayama et al., 1991). Although GA4 seems to be more biologically important than GA1 in some ornamental plants such as *Matthiola incana* (Hisamatsu et al., 1997), *Lilium elegans* (Takeyama et al., 1993) and tulip (Rebers et al., 1995), the absence of non C13-hydroxylated GAs suggests in *E. grandiflorum* that C13-hydroxylated GAs might be important for stem elongation. This hypothesis is supported by the result that C13-hydroxylated GAs (GA1 and its immediate precursor GA, GA20) were more biologically active for stem elongation than were the non-C13-hydroxylated GAs (GA4 and its immediate precursor, GA20) (Fig. 1).

Both UCZ and PCa are mainly used as dwarfing chemicals in paddy fields but the modes of action of the two chemicals are different. UCZ is a triazol type compound which inhibits the earlier stages of GA biosynthesis such as the conversion of ent-kaurene to ent-kaure-noic acid (Izumi et al., 1984), resulting in reduction of total endogenous GA levels. Thus, stem elongation which was suppressed by UCZ treatment (Fig. 2), was reversed by the application of GA1 and GA20. The stem elongation response to GA1 and GA20 in UCZ-treated plants was similar to that in the control plants. These results indicate that metabolic pathway from GA20 to GA1 through 3β-hydroxylation is functioning in the rosetted stems. It also implies that the GA biosynthetic pathway to the precursor, GA20, is not fully functioning in rosetted stems. On the other hand, PCa is an acyclcyclohexanedione compound which inhibits the late stages of GA biosynthesis. Hydroxylation, particularly, at positions 2β and 3β of gibberellane skeleton appears to be the primary targets of this compound (Nakayama et al., 1990a, 1990b). The hydroxylation at position 3β leads to a formation of biologically active GAs, such as GA1 from GA20 and GA2 from GA0, respectively, and at position 2β leads to a formation of biologically inactive GAs such as GA8 from GA1 and GA34 from GA4, respectively (Graebe, 1987). PCa treatment markedly reduced the stem elongation response to the applied GA20, compared to the control (Fig. 2) which is attributed to an inhibition of 3β hydroxylation step from GA20 to GA1, resulting in reduction of GA1 level. Thus, GA1 is a physiologically important GA and could regulate the stem elongation in *E. grandiflorum* similar to that in maize (Phinney and Spray, 1982), pea (Ingram et al., 1984), rice (Takahashi and Kobayashi, 1990) and spinach (Zeevaart et al., 1993). The promotion of stem growth by PCa-treated plants compared to that of the control plants (Fig. 2) supposedly resulted from the inhibition of 2β-hydroxylation step of endogenous GA1 and consequently its accumulation. Stem elongation by GA1 application to PCa-treated plants was slightly promoted over than that in the control plants (Fig. 2) which also suggest that 2β-hydroxylation of applied GA1 was inhibited.

In *E. grandiflorum*, stems of rosetted plants do not elongate, but their leaves expand. That active GAs level play a regulatory role on leaf growth has been demonstrated with GA-biosynthesis mutants of *Brassica rapa* (Zanewich et al., 1990) and *Lathyrus odoratus* (Ross et al., 1993). They showed that a certain level of active GA is necessary for the production of normal, wild-type leaf, whereas its deficiency results in substantially shorter and narrower leaves. On the other hand, mutants with excessive GA or a wild-type treated with a large dose of active GA results in a slightly longer, markedly narrower, smaller leaves than the untreated wild types. We observed the same response with GAs applied to leaves of rosetted plants (Fig. 3). Leaf expansion of UCZ-treated plants was markedly inhibited compared to that of the control plants, whereas the inhibition was reversed to the control level by GA applications. These results indicate that enough GA1 for leaf expansion is endogenously produced in the rosetted
plants. However, the distribution of endogenous GA_{1} in the stems and leaves of *E. grandiflorum* is still unknown. If GA_{1} remains primarily in the leaves, it may indicate that there is a tissue-specific GA synthesized in rosetted plants. On the other hand, if GA_{1} is partitioned to the stems and leaves, it indicates that there is some difference in responsiveness to GA_{1} among the different tissues. Further quantitative studies are required to explain these speculations.

**Acknowledgment**

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トルコギキョウの内生ジペレリンの同定とロゼット化実生におけるそれらの役割

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摘要

トルコギキョウのロゼット化とジペレリンとのかかわりを明らかにするために、GC/MSを用いてトルコギキョウの内生ジペレリンを同定し、さらにロゼット化実生に対するジペレリン合成阻害剤と類似ジペレリンの影響を調査した。その結果、内生ジペレリンとしてGA₃、GA₁₉、GA₂₆、GA₄₄およびGA₅₃を同定し、初期13位水酸化ジペレリンが機能していることを推定した。処理実験からはトルコギキョウの茎の伸長生長には13位水酸化ジペレリンであるGA₃およびGA₂₆の活性が非13位水酸化ジペレリンであるGA₄およびGA₉の活性よりも高いことが明らかになった。また、ロゼット化実生においてもジペレリン合成経路の重要な活性化機構である3β水酸化は機能していることを示した。さらにジペレリン合成阻害剤とGA₃またはGA₂₆の複合処理からはGA₃がトルコギキョウの茎の伸長生長の生理的制御に重要であることを示した。