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Thermoperiodic Control of Stem Elongation and Endogenous Gibberellins in *Campanula isophylla*

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Abstract. The effect of day/night temperature regimes on stem elongation and on the content of endogenous gibberellins (GAs) in vegetatively propagated plants of *Campanula isophylla* cv. Hvit have been studied. Compared with a constant temperature regime at 18°C (18/18°C), stem and internode elongation was enhanced significantly by a combination of high day/low night temperature (21/15°C) and inhibited by an opposite regime (15/21°C). Gibberellins A_1, A_{19}, A_{44}, A_{53}, and A_{97} were identified as endogenous components in *Campanula*. (GA_{97} was earlier referred to as 2β-OH-GA_{53}.) Quantitative analysis of the endogenous GAs indicates that temperature regimes that stimulate elongation growth are accompanied by an increase in the level of GA_1, GA_{19}, and GA_{44}. On the other hand, in plants grown under conditions that reduced stem elongation growth, there was an increased level of GA_{97}.

Key Words. *Campanula*—Stem elongation—Endogenous GAs—GA application—Internode elongation—Thermoperiodicity

Compared with growth at constant temperature, stem elongation in the long day plant *Campanula isophylla* has been shown to be enhanced when day temperature is higher than night temperature (positive DIF) and reduced when the situation is the opposite (Ihlebekk et al. 1994, Moe 1990). These studies showed that stem elongation in *Campanula* was influenced more by the difference between day and night temperature than by average daily temperature or absolute day or night temperature between 12 and 24°C. Similar responses have been reported for other species as well, *Fuchsia* × *hybridia* (Tangerås 1979), *Lilium longiflorum* (Erwin et al. 1989), and *Dianthus chinensis* (Moe 1983).

Inhibition of elongation growth in *Campanula* by negative DIF can be overcome by exogenous applied GA_3 or GA_{4+7}. Ancymidol, an inhibitor of GA biosynthesis (Coolbauch and Hamilton 1976), reduces stem elongation induced by positive DIF (Moe 1990, Tangerås 1979). These results suggest that GAs might be involved in thermoperiodic regulation of stem elongation. GA_1 has been suggested to be one of the effector GAs of elongation growth in higher plants (Graebe 1987, Phinney 1984, Reid and Ross 1991). A number of studies indicate that photoperiodic control of elongation growth could be related to photoperiodic regulation of biosynthesis of GA_1 (Gilmour et al. 1986, Junttila and Jensen 1988, Metzger and Zeevaart 1986, Olsen et al. 1994, Talon and Zeevaart 1990, Zeevaart et al. 1991). There are also results indicating the effects of temperature on endogenous GAs (Metzger 1990, Pinthus et al. 1989). However, there are, to our knowledge, no reports of the effects of diurnal temperature rhythms on endogenous GAs in relation to stem elongation.

The purpose of the present study was to investigate the role of GAs in thermoperiodic regulation of elongation growth in *C. isophylla*. In this paper we report the identification of various endogenous GAs in *C. isophylla* and the quantification of these GAs in plants growth at positive DIF, negative DIF, and at constant temperature, as well as results from application experiments with various GAs.

Abbreviations: DIF, difference between day temperature and night temperature; GA, gibberellin; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass chromatography; SPE, solid phase extraction; TMS, trimethylsilyl; MSTFA, *N*-methyl-*N*-TMS-trifluoroacetamide; KRI, Kovats retention index; SIM, selected ion monitoring; D2, deuterated.

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Materials and Methods

Plants

Pretreatment. C. isophylla Moretti cv. Hvit was propagated vegetatively by cuttings. After rooting they were planted in fertilized peat (Floralux) in 12-cm pots and grown for 2 weeks at 18°C in a 12-h Photoperiod in a greenhouse. Supplementary light was given with high pressure sodium lamps (50 a-T from Philips); photosensitive active radiation was 90 μmol m^{-2} s^{-1}. The plants were fertilized frequently during pretreatment and experimental period with a complete solution containing 153 ppm nitrogen, 32 ppm phosphorus, and 191 ppm potassium plus microelements.

Temperature Treatments. After a 2-week pretreatment the plants were moved to a daylight phytotron where three temperature treatments were used: 18/18°C (zero DIF), 21/15°C (positive DIF), and 15/21°C (negative DIF) day/night temperature regimes. The effect of various combinations of day-night temperatures on elongation growth of *Campanula* has been studied earlier (Moe 1990), and the described treatments provide adequate comparisons of the DIF effects. The temperatures were controlled to ±0.5°C. The day period was from 8 a.m. to 8 p.m., which comprised natural daylight plus supplementary light with high pressure sodium lamps; 95 μmol m^{-2} s^{-1} photosensitive active radiation. This 12-h high irradiance photosynthetic light period was defined as the day temperature period. The plants were given 12-h daylength extension with fluorescent lamps (SL lamps from Philips) at a fluence rate of 2–7 μmol m^{-2} s^{-1}. This 12-h low irradiance photoperiodic light period was defined as the night temperature period. Humidity was adjusted to give approximately 0.5-kPa water pressure deficit.

Stem length and all internodes were measured two to three times each week. The number of days from the start of temperature and long day treatments until visible flower bud and anthesis, total stem length, number of leaves per stem, and total number of flower buds per plant were recorded at anthesis. Results were analyzed using standard statistical procedures of Statistical Analysis Systems programs (PCSAS V604, SAS Institute Inc.).

Extraction and Purification

Identification of Endogenous GAs. Plant material, consisting of leaves and stems, was harvested 35 days after transfer to the zero DIF temperature treatment and long days. The plant material (418 g, fresh weight) was homogenized and extracted in 2 liters of 80% ethanol in 0.1 M sodium phosphate buffer,pH 8.0, and filtered. The residue was resuspended in 80% ethanol, extracted, and filtered. The pooled filtrates were evaporated to the aqueous phase in vacuo and slurried with insoluble polyvinylpolypyrrolidone (≈1 g/10 mL of extract), filtered, and adjusted to pH 2.5 by 1 N hydrochloric acid. The acidic aqueous fraction was evaporated to dryness in vacuo, and the sample was redissolved in 0.2 mL of methanol and 1.8 mL of water adjusted to pH 8.0 by 0.2 M NaOH. The sample was then applied to a QAE-Sephadex A-25 ion exchange column. The column (1 × 10 cm) was washed with 30 mL of water at pH 8.0, and the GAs were eluted with 40 mL of 1 N acetic acid. The GA-containing fraction was taken to dryness by a Speed-Vac system, redissolved in methanol:water:acetic acid (10:89: 1), and applied to a 500-mg solid phase extraction (SPE) column (Waters Sep-Vac 3CC). The column was washed with 4 mL of methanol-:water:acetic acid (10:89:1), and the GAs were eluted by 5 mL of methanol:water:acetic acid (80:19:1). The fraction containing the GAs was taken to dryness in a Speed-Vac system.

Quantitative Analysis. Plant material for quantitative analysis of GAs was harvested in a separate experiment. In this experiment, stem elongation rates were approximately 0.3, 0.45, and 0.62 cm/day for 15/21°C, 18/18°C, and 21/15°C, respectively. The method of extraction and purification for quantitative analysis of GAs was essentially the same as described above for qualitative analysis. But prior to extraction, 500 ng of $[^2H_2]GA_1$ and $[^2H_2GA_8$ and 1,000 ng of $[^2H_2]GA_{19}$, $[^2H_1]GA_{44}$, $[^2H_2]GA_{20}$, and $[^2H_2]GA_{53}$ were added to each sample as internal standards (Professor L. Mander, Australian National University, Canberra, Australia). The amount of plant material in each sample was 234 g, fresh weight (15/21°C treatment); 218 g, fresh weight (18/18°C treatment); and 275 g, fresh weight (21/15°C treatment). After extraction, each sample was divided into two equal volumes.

HPLC

The HPLC systems used were standard commercially available instruments from Waters (Milford, MA, U.S.A.). Samples were injected onto a radially compressed reversed phase (Waters Novapak C-18 4 μm) column (10 cm × 8 mm inner diameter) with a flow rate of 2 mL min^{-1} using a linear gradient of 20–80% methanol in 1% acetic acid over 25 min, and then with 80% methanol for an additional 20 min. The column temperature was 25°C. Two-mL fractions were collected for 30 min, and each fraction was taken to dryness in a Speed-Vac system.

Combined GC-MS

Combined HPLC fractions were methylated using etheral diazomethane, and the samples were purified further using 100-mg aminopropyl SPE columns. the samples were dissolved in 500 μL of methanol, applied to the column, and eluted with 2.5 mL of methanol and reduced to dryness by gentle blowing with nitrogen. The samples were analyzed by GC-MS as the methyl ester trimethylsiyl (TMS) ether derivatives by derivatizing the samples further by the use of *N*-Methyl-*N*-TMS-trifluoroacetamide (MSTFA). The samples were injected in the splitless mode into a HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a 25-m × 0.22-mm inner diameter nonpolar (HP-1 Ultra) fused silica capillary column. The film thickness was 0.11 μm, and the phase ratio was 450. The injector temperature was 250°C. The column temperature was maintained at 50°C for 2 min, then increased by 10°C/min to 170°C, followed by 2°C/min to 240°C and then by 30°C/min to 280°C. the column was introduced via a splitless interface to the electron impact ion source of a VG Trio 1000 Quadropole mass spectrometer (Fisons Instruments, Manchester, UK). The temperature of the interface was 250°C and of the ion source 200°C. Ions were generated with 70-eV electrons at an emission current of 300 μA. Qualitative analysis were performed by scanning from 80 to 700 atomic mass units at a scan rate of 1 s/scan. Quantitative analyses were performed using the selected ion monitoring (SIM) technique. For each GA and their deuterated analogs, two characteristic ions were recorded. All data were processed by a VG Lab Base data system.

Results

Stem and Internode Elongation

Both the rate of stem elongation and the rate of internode elongation were affected significantly by temperature treatments, enhanced by positive DIF, and inhibited by negative DIF compared with constant temperature (Table 1). Mean internode lengths were 5.7, 7.6, and 11.2 mm at 15/21, 18/18, and 21/15°C, respectively. The measured

Table 1. Effect of difference between day and night temperature on duration and rate of internode elongation in *C. isophylla* grown under a long photoperiod. Day temperature was given during the 12-h period of high irradiance, night temperature during the 12-h period of day-length extension. Results are means ± S.E.

Temperature (°C)	Duration of elongation (days)	Rate of elongation (mm/day)
15/21	18.9 ± 0.35	0.30 ± 0.012
18/18	24.1 ± 0.39	0.31 ± 0.010
21/15	25.8 ± 0.24	0.44 ± 0.010

differences in mean internode length were statistically significant ($p < 0.01$). These differences were due both to an increased rate of elongation and longer elongation period at positive DIF. At 21/15°C the elongation rate was 0.44 mm/day and at 15/21°C 0.33 mm/day; the length of the elongation period for an internode was 25.3 and 18.9 days ($p < 0.05$), respectively. In plants harvested for the qualitative analysis, differences in elongation rate were even more obvious, 0.6 mm/day at 21/15°C and 0.3 mm/day at 15/21°C.

Identification of Endogenous GAs

Full scan analysis of the HPLC-purified fractions from extracts of *C. isophylla* revealed the presence of GA_1, GA_{19}, GA_{44}, GA_{53}, and GA_{97}. All except GA_{97} were identified by comparing mass spectra and Kovats retention index (KRI) with those of reference substances. The results are presented in Table 2, supported by visual inspection of the spectra and by HPLC retention time. Identification of GA_{97} was based on comparing the obtained spectra with spectra and KRI values given in the literature (Gaskin and MacMillan 1991). Also, the putative GA_{97} was found in the same 6-mL HPLC fraction as GA_1, and it is reported in the literature that GA_{97} elutes close to GA_1 in reversed phase HPLC (Talon and Zeevaart 1990).

Quantification of Endogenous GAs

The data used to estimate the amounts of GA_1, GA_{19}, GA_{44}, GA_{53}, and GA_{97} were obtained by using the GC-MS instrument in the SIM mode. Because of limited access to nondeuterated GA standards, the amounts given are not calculated by using standard curves, but by assuming that the response in the GC-MS of nondeuterated GA_n is the same as the response of the corresponding deuterated (D2) GA_n. With respect to GA_{97} we had no access to a deuterated standard, but as mentioned above, GA_{97} eluted in the same fraction as GA_1. By comparing the area below the peak $m/z = 536$ (GA_{97}) with the area below the peak $m/z = 508$ (D2-GA_1) we could obtain relative quantitative data also with respect to GA_{97}. The GC-MS analysis of one of these samples is illustrated in Fig. 1. These data contain, of course, a systematic error, but the amounts of GA_{97} in extracts from different temperature treatments can be compared with each other. The results are presented in Table 3.

Discussion

According to the present results, *C. isophylla,* like most angiosperms studied so far (Graebe 1987), contains GAs that are synthesized via the early 13-hydroxylation pathway. Consequently, GA_1 is the probable effector GA in *Campanula* with respect to elongation growth. Quantitative analyses showed increased amounts of the bioactive GA_1 correlated positively with stem and internode elongation growth (Table 3). The observed differences were rather small; the GA_1 content varied from 1.0 ng/g fresh weight, in plants grown under a negative DIF treatment to 1.9 ng/g, fresh weight, in plants grown under a positive DIF treatment. It can be argued that such small differences in the content of GA_1 can not explain the increase in the rate of elongation of 47% and the increase in average internode length of 97%. But there are reports indicating that differences in GA_1 content in plants given different treatments or in plants of different genotypes are small. The GA_1 content in a tall (Nihonbare) and a dwarf (Waito-C) rice cultivar was 0.17 and 0.10 ng/g, fresh weight, respectively (Takahashi and Kobayashi 1991). In wheat the level of GA_1 increased 1.6-fold when the growth temperature was increased from 10 to 25°C (Pinthus et al. 1989). It should also be emphasized that our samples consisted of both leaf and stem tissue and that the quantitative analysis does not necessarily reflect the differences that might be present in elongating stem tissue. In *Silene* the photoperiodic effects on endogenous GAs were more pronounced in shoot tips than in mature leaves (Talon and Zeevart 1992), and differences in endogenous GAs between a tall (Le) and a dwarf (le) genotype of *Pisum* were clearly evident in apical parts but not in lower leaflets and stem (Smith et al. 1992). It can further be noted that the ratio of GA_{19} to GA_1 is smaller in plants grown under positive DIF than the GA_{19}:GA_1 ratio in plants grown under negative DIF treatment. The ratio of GA_{19} to GA_{53} is the same in all treatments (Table 4), and this may reflect the fact that metabolism of both GA_{53} and GA_{19} seems to be equally affected by environmental conditions (Gilmore et al. 1986). On the basis of what is mentioned above, we conclude that the observed increase in elongation growth in plants grown under positive DIF can be explained partially by an increase in the biosynthesis of GAs and simultaneously increased rate of conversion of GA_{19} to GA_1. GA_{20} could not be detected in the samples by full scan MS, but the

Table 2. Four principal ions and KRI obtained from full scan GC-MS analysis of methyl ester (ME) TMS derivatives of authentic and endogenous GAs purified from extracts of *C. isophylla*.

Sample	KRI	Diagnostic ions *m/z* (% relative intensities)			
GA_1-MeTMS	2671	376 (12)	448 (22)	491 (12)	506 (100)
HPLC fractions 10–12	2675	376 (14)	448 (27)	491 (11)	506 (100)
GA_{10}-MeTMS	2595	374 (68)	402 (39)	434 (100)	462 (5)
HPLC fractions 19–21	2593	374 (71)	402 (42)	434 (100)	462 (5)
GA_{44}-MeTMS[a]	2784	373 (13)	401 (4)	417 (7)	432 (42)
HPLC fractions 19–21[a]	2779	373 (18)	401 (3)	417 (7)	432 (38)
GA_{53}-MeTMS[a]	2483	373 (10)	389 (32)	416 (21)	448 (59)
HPLC fraction 25–26[a]	2478	373 (12)	389 (38)	416 (24)	448 (54)
GA_{97}-MeTMS[a]	2695	477 (11)	504 (10)	521 (6)	536 (27)[b]
HPLC fractions 10–12[a]	2694	477 (9)	504 (7)	521 (7)	536 (21)

[a] Intensities calculated by using $m/z = 207$ as base peak.
[b] Taken from data published by Gaskin and MacMillan (1991).

Fig. 1. Quantitative analyses of GA_{97} were performed by comparing ions characteristic of GA_{97} (**A** and **B**) with the M^+ ion of D_2-GA_1 (**C**).

presence of GA_{20}, at low quantities, was indicated by SIM technique. Previous application experiments have shown that the growth-enhancing effect of GA_{20} was relatively less at negative than at positive DIF, and this could indicate that conversion of GA_{20} to GA_1 is reduced by negative DIF (Ihlebekk et al. 1994).

From Table 3 it can be noted that the content of GA_{97} is higher in plants grown under negative DIF than in plants grown under positive DIF. Hydroxylatin in the 2β-position is a common inactivation step of GAs (Reeve and Crozier 1975), and production of GA_{97} could act as a side shunt of GA_{53} when production of GA_1 is reduced. Similar observations are made in spinach (Zeevaart et al. 1991). Based on the observations mentioned above, we believe that there is an increased rate of conversion of GA_{53} to GA_{97} in plants grown under nega-

Table 3. Quantification of endogenous gibberellins in *C. isophylla* grown at different day/night temperature rhythms. Values are given as ng/g, fresh weight, of plant material.

	Day/night temperatures (°C)		
Gibberellin	15/21	18/18	21/15
GA_1	1.0 (±0.1)	1.5 (±0.1)	1.9 (±0.1)
GA_{19}	4.4 (±0.2)	5.8 (±0.1)	6.2 (±0.2)
GA_{44}	0.8 (±0.2)	1.8 (±0.1)	1.5 (±0.2)
GA_{97}	2.1 (±0.1)[a]	1.7 (±0.1)[a]	1.3 (±0.1)[a]
GA_{53}	1.6 (±0.2)	2.1 (±0.1)	2.3 (±0.1)

[a] Since these values are obtained by comparing the area below the peak $m/z = 536$ (corresponds to GA_{97}) with the area below the peak $m/z = 508$ (corresponds to D_2-GA_1) they do contain a systematic error. The systematic error can be calculated by comparing the response of GA_{97} with the response of D_2-GA_1 in the mass spectrometer.

Table 4. Ratio of content of various GAs in plants given different temperature treatments.

	Positive DIF	Zero DIF	Negative DIF
GA_{19}/GA_1	4.40	3.86	3.26
CA_{19}/GA_{53}	2.75	2.76	2.70

tive DIF compared with the rate of conversion of GA_{53} to GA_{97} in plants grown under positive DIF.

Both the present studies and earlier results with *Campanula* (Moe 1990) and some other species as well (Erwin et al. 1989) suggest an involvement of GA in the thermoperiodic regulation of elongation growth. In addition to effects on the levels of bioactive GAs, changes in tissue sensitivity to GA can be influenced by environmental factors (Nick and Furuya 1993, Pinthus et al. 1989, Reid and Ross 1991). The mode of action of GA is not known, but both cell division and cell elongation can be affected. Erwin et al. (1994) have shown that stem elongation responses to DIF in *L. longiflorum* are elicited primarily through effects on cell elongation.

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