J Plant Growth Regul (1993) 12:85–90

Journal of
Plant
Growth
Regulation

© 1993 Springer-Verlag New York Inc.

Light Effects on Endogenous Levels of Gibberellins in Photoblastic Lettuce Seeds

Tomonobu Toyomasu,[1] Hiroko Tsuji,[1] Hisakazu Yamane,[1] Masayoshi Nakayama,[1] Isomaro Yamaguchi,[1] Noboru Murofushi,[1] Nobutaka Takahashi,[1,*] and Yasunori Inoue[2]

[1]Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; and [2]Department of Applied Biological Science, Science University of Tokyo, Noda-shi, Chiba 278, Japan

March 2, 1992; accepted May 27, 1993

Abstract. Gibberellin A_1 (GA_1), 3-*epi*-GA_1, GA_{17}, GA_{19}, GA_{20}, and GA_{77} were identified by Kovats retention indices and full-scan mass spectra from gas chromatography-mass spectrometry analysis of a purified extract of mature seeds of photoblastic lettuce (*Lactuca sativa* L. cv. Grand Rapids). Non-13-hydroxylated GAs such as GA_4 and GA_9 were not detected even by highly sensitive radioimmunoassay. These results show that the major biosynthetic pathway of GAs in lettuce seeds is the early-13-hydroxylation pathway leading to GA_1, which is suggested to be physiologically active in lettuce seed germination. Quantification of endogenous GAs in the lettuce seeds by gas chromatography-selected ion monitoring using deuterated GAs as internal standards indicated that the endogenous level of GA_1 increased to a level about three times that of dark control 6 h after a brief red light irradiation, and that far-red light given after red light suppressed the effect of red light. The contents of GA_{20} and GA_{19} were not affected by the red light irradiation. Evidence is also presented that 3-*epi*-GA_1 is a native GA in the lettuce seeds.

It is well known that the germination of photoblastic lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds is under phytochrome-mediated photocontrol (Bewley and Black 1982). Red light induces seed germination and far-red light, given after red light, completely suppresses the effect of red light (Borthwick et al. 1952). Inoue (1990) reported that decoated Grand Rapids seeds germinated in the dark without red light irradiation, and suggested that the fruit wall has inhibitory effects on the seed germination. From the fruit wall, abscisic acid (ABA) was identified as an inhibitor of germination, its content being about 0.33 μg/g (Inoue 1990). On the other hand, gibberellin A_3 (GA_3) is known to mimic the effect of red light on the germination of lettuce seeds (De Greef and Fredericq 1983). This suggests that red light increased endogenous GA levels to cancel the suppression of seed germination by the fruit wall. However, very little information on endogenous GAs in the lettuce seeds has been obtained so far, although the metabolism of exogenously applied GAs has been studied (Durley et al. 1976).

We wish to report here the identification of endogenous GAs from mature lettuce seeds and the quantification of endogenous GAs in the seeds incubated in different light conditions.

* Present Address: The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

Materials and Methods

Qualitative Analysis

Plant Materials. Mature lettuce seeds (*Lactuca sativa* L. cv. Grand Rapids) were purchased from Sakatashubyo Co. (Japan). The seeds were 2 years old and had been stored at 4°C until used. One hundred grams of the seeds were used for qualitative analysis.

Extraction and Purification. Mature seeds were extracted three times with methanol (MeOH, 0.3 L) using a mortar and a pestle. The MeOH extract was evaporated in vacuo and the resultant aqueous (aq.) residue was fractionated by the usual method (Endo et al. 1989) to give an acidic ethyl acetate (EtOAc) fraction (AE fraction). The AE fraction was subjected to Sep-Pak (ODS) treatment and reversed-phase high-performance liquid chroma-

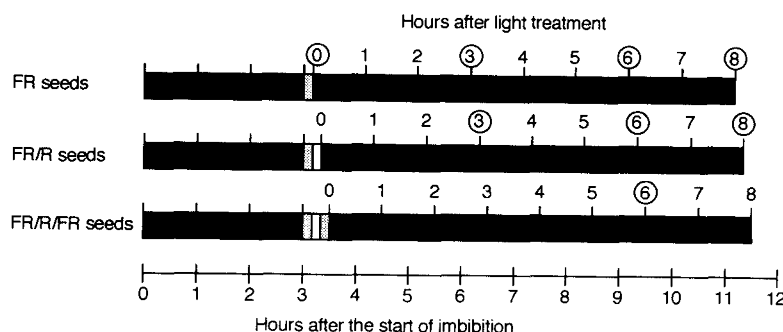

Fig. 1. Preparation of plant materials for quantitative analysis of endogenous GAs in lettuce seeds. Light treatments were carried out 3 h after the start of imbibition. The first far-red light irradiation was done to suppress the small percent of germination in the complete dark. Seeds were harvested at the chosen time (circled). ■, in the dark at 25°C; ▨, far-red light irradiation (4.6 Wm^{-2}, 10 min); □, a red light irradiation (5.0 Wm^{-2}, 10 min).

tography (RP-HPLC) on a Senshu-Pak ODS 4253D column (25 cm × 10 mm i.d.) as reported previously (Nakayama et al. 1989) to give 32 fractions.

Dwarf Rice Assay. Each fraction from the RP-HPLC was assayed by the dwarf rice (*Oryza sativa* L. cv. Tan-ginbozu) microdrop method (Murakami 1968) at 30°C under continuous white light (3.2 Wm^{-2}).

Radioimmunoassay (RIA). An aliquot of each HPLC fraction was methylated with ethereal diazomethane and used for the assay. The assay was done in triplicate at a concentration of 2 g fresh wt equivalent per assay tube using anti-GA_1- and anti-GA_{20}-antisera according to the methods of Yamaguchi et al. (1987, 1990).

Bond-Elut [diethylamino (DEA)] Treatment. Each sample was dissolved in MeOH (1 ml) and loaded onto a Bond Elut (DEA) cartridge (Analytichem International). The cartridge was eluted twice with MeOH (2 ml), and then twice with 0.5% acetic acid (HOAc) in MeOH (2 ml). The combined eluates with 0.5% HOAc in MeOH were evaporated to dryness.

Gas Chromatography–Mass Spectrometry (GC-MS). Each sample for GC-MS was converted into methyl ester trimethylsilyl ether (MeTMSi) and analyzed using a JEOL DX-303 GC-MS system fitted with a fused silica capillary column DB-1 (15 m × 0.258 mm i.d., J&W Scientific Inc., CA, USA). Samples were injected onto the column at 120°C in a splitless mode. Two min after injection, the column temperature was programmed at 16°C/min to 216°C with 5-min isothermal hold at 216°C, and subsequently at 8°C/min to 280°C with 10 min isothermal hold at the end of the program. Head pressure of He-carrier gas was 64 kPa.

Lettuce Seed Germination Assay

The Grand Rapids seeds were punctured with a sharp tungsten needle (Inoue 1990). The 20 punctured seeds were sowed in a polystyrene well (30 mm i.d.) containing 1 ml of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (1mM, pH 6.1) with or without GAs. Incubation was conducted at 25°C in the dark, and the percentage of seed germination was scored 2 days after sowing.

Quantitative Analysis

Internal Standards. Preparations of [1,2,2,3,6-$^2H_5$]GA_1 and [1,2,2,3,6-$^2H_5$]GA_{20} were reported previously (Endo et al. 1989). [17,17-$^2H_2$]GA_{19} was supplied by Prof. L. N. Mander of the Australian National University.

Light Sources and Filters. Red light (5.0 Wm^{-2}) was obtained from 20 W fluorescent tubes (FL20S BRN/18, Toshiba, Ltd., Japan) placed behind a red sheet (Torayglass no. 130, Toray, Ltd., Japan). Far-red (4.6 Wm^{-2}) light was obtained from 20 W fluorescent tubes (FL20S FR74, Toshiba, Ltd., Japan) placed behind a Delglass A900 sheet (Asahikasei, Ltd., Japan). Dim green safe light was obtained from 40 W fluorescent tubes (Toshiba FLR 40 G/A; Toshiba Ltd., Japan) wrapped doubly with viridian and sunny-orange plastic films (cutting sheet 431C and 222C, respectively; Nakagawa Chemical, Japan).

Plant Materials. Mature lettuce seeds were obtained from South Pacific Seeds in Australia in 1992 and stored at 4°C until used. Ten grams each of mature seeds 6 months after harvest were incubated in a plastic box (295 mm × 220 mm, 43 mm in depth) containing 100 ml of MES buffer at 25°C in the dark for 3 h. Light treatments were then carried out as follows (Fig. 1): irradiation with far-red light (10 min; FR seeds); irradiation with far-red light (10 min) and red light (10 min) successively (FR/R seeds); irradiation with far-red light (10 min), red light (10 min) and far-red light (10 min) successively (FR/R/FR seeds). Immediately after the light treatments, 40 ml of MES buffer was removed, and the resultant light-treated seeds were incubated in the dark at 25°C. The seeds were harvested at the chosen times (0, 3, 6, and 8 h after the light treatment for FR seeds; 3, 6, and 8 h for FR/R seeds; and 6 h for FR/R/FR seeds). All the above procedures were carried out under dim green safe light. Forty grams of the seeds were used for each quantitative analysis. The time course of germination of FR and FR/R seeds was determined as follows. Of the seeds incubated under the same conditions as described above, about 100 seeds were taken out at random at the chosen times (17, 21, 25, 29, and 44 h for FR seeds; 5, 9, 13, 17, 21, 25,

Table 1. GAs identified by GC-MS analysis of their MeTMSi derivatives in the mature seeds of *Lactuca sativa* L. cv. Grand Rapids.

Rt on HPLC (min)	Identified GA	KRI	Principal ions and relative abundance (% base peak)
12–17	GA_1	2663	506 (M^+, 100), 491 (10), 448 (26), 376 (39), 313 (6)
	3-*epi*-GA_1	2775	506 (M^+, 100), 491 (8), 448 (26), 459 (10), 376 (20), 313 (5)
	GA_{77}[a]	2631	506 (M^+, 53), 491 (9), 416 (41), 403 (100), 390 (18), 357 (17), 347 (24)
20–22	GA_{20}	2482	418 (M^+, 100), 403 (15), 390 (7), 375 (52), 301 (15)
22–24	GA_{17}	2572	492 (M^+, 100), 460 (48), 432 (48), 401 (26), 373 (45)
	GA_{19}	2590	462 (M^+, 7) 434 (100), 402 (22), 375 (44), 374 (49), 345 (22)

KRI, Kovats retention index.
[a] Since GA_{77}-MeTMSi and 12-*epi*-GA_{77}-MeTMSi showed the very close KRI values and essentially identical mass spectra (KRI of 12-*epi*-GA_{77}-MeTMSi, 2624), KRI value of MeTMSi derivative of the native compound was carefully compared with those of GA_{77}-MeTMSi and 12-*epi*-GA_{77}-MeTMSi.

29, and 44 h for FR/R seeds) after the light treatments and the percent of germination was determined.

Extraction and Purification. Each sample of plant material was extracted three times with methanol (400 ml), and the internal standards ([$^2H_5$]GA_1, 50 ng; [$^2H_2$]GA_{19}, 100 ng; [$^2H_5$]GA_{20}, 1 μg) were added to each methanol extract. The AE fraction from each extract was purified by a polyvinylpolypyrrolidone (PVPP; 3 g) column as described in Toyomasu et al. (1992). The purified sample was dissolved in 1.5 ml of MeOH-H_2O (2:1, vol/vol) and loaded on a Sep-Pak (ODS) cartridge (Waters, Inc.). The cartridge was eluted three times with 2 ml of MeOH-H_2O (4:1, vol/vol). The Sep-Pak eluate was dissolved in MeOH (0.6 ml) and loaded on a column of Sepralyte (DEA) (Analytichem International, 0.6 g). The column was eluted with 6 ml of MeOH, and then with 12 ml of 0.5% HOAc in MeOH. The eluate was combined and subjected to ODS-HPLC as described above. The fractions of retention time (*Rt*) 10–16 min (for GA_1) and *Rt* 17–26 min for (GA_{19} and GA_{20}) were separately purified on a Senshu-Pak $N(CH_3)_2$-3151N column (15 cm × 8 mm i.d.), eluted with 0.05% HOAc in MeOH at a flow rate of 3 ml/min at 50°C (*Rt* 9–15 min for GA_1; *Rt* 13–23 min for GA_{19} and GA_{20}).

Gas Chromatography-Selected Ion Monitoring (GC-SIM). The operating conditions were the same as those of GC-MS described in Qualitative Analysis. Ions monitored were as follows: for GA_1/[$^2H_5$]GA_1, m/z 511, 506, 496, 491, 449, and 448; for GA_{20}/[$^2H_5$]GA_{20}, m/z 423, 418, 408, 403, 376, and 375; and for GA_{19}/[$^2H_2$]GA_{19}, m/z 464, 462, 436, 434, 404, and 402. The contents of GA_1, GA_{19}, and GA_{20} in the respective original extract were determined from the peak area ratios at m/z 506/511 (GA_1), 434/436 (GA_{19}), and 418/423 (GA_{20}) by reference to the calibration curves. The other ions were monitored to confirm the identification of the compounds analyzed.

Results and Discussion

Qualitative Analysis

A methanol extract from mature Grand Rapids seeds was fractionated to give an AE fraction. The AE fraction was subjected to RP-HPLC after Sep-Pak (ODS) treatment. Gibberellin activity of eluates from the HPLC was estimated by dwarf-rice assay and RIA. Based on the GA-like activity, three pooled fractions of *Rt* 12–17 min, 20–22 min, and 22–24 min were obtained. These three fractions were separately purified by Bond-Elut (DEA) treatment, and were derivatized and analyzed by full-scan capillary GC-MS. Gibberellin A_1, 3-*epi*-GA_1, GA_{17}, GA_{19}, GA_{20}, and GA_{77} were identified by comparison of the Kovats retention indices and mass spectra of their MeTMSi derivatives with those of authentic samples (Table 1). The major GAs were 3-*epi*-GA_1, GA_{17}, GA_{19}, and GA_{20}, and minor ones, GA_1 and GA_{77}. Non-13-hydroxylated GAs such as GA_4 and GA_9 were not detected even by highly sensitive RIA using anti-GA_1- and anti-GA_{20}-antisera, which showed high cross-reactivity with GA_4 and GA_9, respectively. Of the identified GAs, 3-*epi*-GA_1 could be an artefact from GA_1, because GA_1 epimerizes under alkaline conditions to give a mixture of GA_1 and 3-*epi*-GA_1 in the ratio of 1:3 (Cross et al. 1961). However, in the extract of the seeds the ratio of GA_1 to 3-*epi*-GA_1 was less than 1:5. Furthermore, in Quantitative Analysis, [$^2H_5$]GA_1 added as an internal standard did not epimerize to [$^2H_5$]3-*epi*-GA_1 during the purification process. Thus, it is concluded that 3-*epi*-GA_1 is naturally occurring in the lettuce seeds. Natural occurrence of 3-*epi*-GA_1 has been reported by several workers (Jones and Zeevaart 1980; Pearce et al. 1987; Nakayama et al. 1990). On the basis of the current knowledge of GA biosynthesis, it is suggested that the major GA biosynthetic pathway in the lettuce seeds is the early-13-hydroxylation pathway leading to GA_1 (Fig. 2). In lettuce seedlings, the same biosynthetic pathway also functions (Waycott et al. 1991; Toyomasu et al. 1992). The immediate precursor of 3-*epi*-GA_1 is probably GA_{20}, because in lettuce seedlings [^3H]GA_{20} was converted into

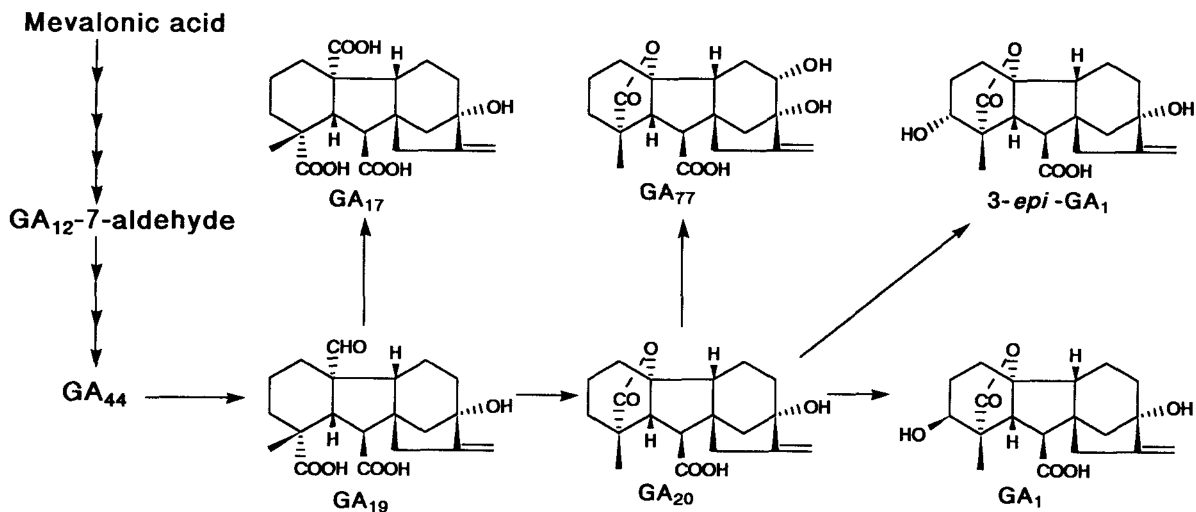

Fig. 2. Hypothetical biosynthetic pathway of the GAs identified from mature seeds of *Lactuca sativa* L. cv. Grand Rapids.

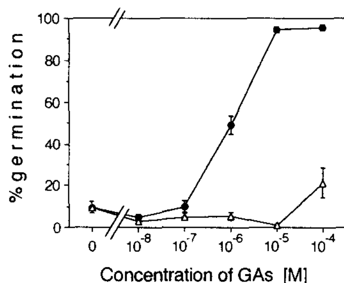

Fig. 3. Effects of GA_1 (●) and GA_{20} (△) on germination of punctured lettuce (cv. Grand Rapids) seed in the dark. Each GA was given from the start of imbibition. Germination was determined 2 days after sowing. Each point represents the average percent of germination ± SE of 10 replicates.

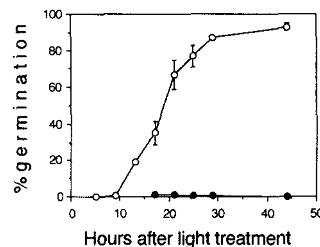

Fig. 4. Time course of germination of lettuce seeds under different light conditions. Each point represents the average percent of germination ± SE of three replicates. -●-, FR seeds; -○-, FR/R seeds.

$[^3H]GA_1$- and $[^3H]3$-*epi*-GA_1-like substances, but conversion of $[^3H]GA_1$ into $[^3H]3$-*epi*-GA_1 was not observed (Toyomasu et al. unpublished results).

Effects of GA_1 and GA_{20} on the Lettuce Seed Germination

Since it is well established in other plant species that GA_1 controls shoot elongation (Phinney and Spray 1982; Suzuki et al. 1981; Ingram et al. 1984; Toyomasu et al. 1992), GA_1 is probably physiologically active in germination of lettuce seeds. To test this hypothesis, the effects of GA_1 and its possible precursor, GA_{20}, were investigated on the germination of punctured lettuce seeds (Inoue 1990) in the dark. As shown in Fig. 3, GA_{20} was active at a concentration of 10^{-4} M, while GA_1 was active at concentrations of 10^{-7} M and higher. These results suggest that GA_1 is responsible for induction of lettuce seed germination. The low activity of exogenously applied GA_{20} may be due to its low conversion rate into GA_1 in the dark.

Quantitative Analysis

We investigated the changes of levels of endogenous GAs in the lettuce seeds incubated under different light conditions. In our preliminary experiment, the lettuce seeds irradiated with red light at any time within 3 h after sowing exhibited the same time course of percentage of germination as those irradiated with red light 3 h after sowing, suggesting that at least 3-h incubation was necessary for the lettuce seeds to respond fully to red light. Red light irradiation for 10 min was long enough to induce seed germination. Far-red light irradiation for 10 min was long enough to inhibit germination in the dark completely and to cancel the effect of red light.

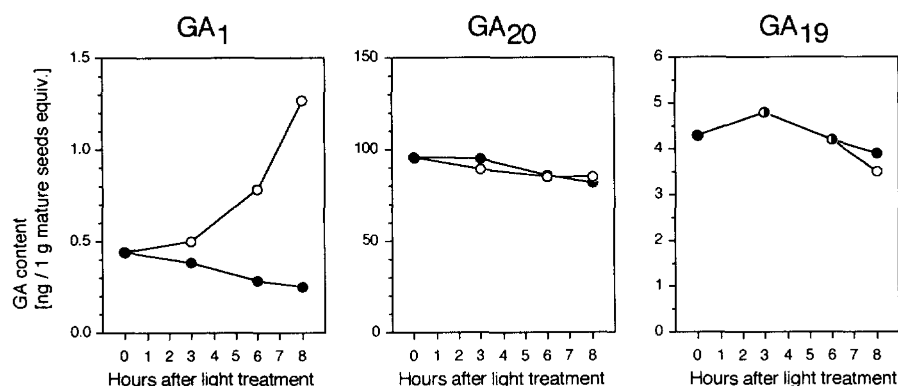

Fig. 5. Changes of endogenous levels of GAs in the seeds of lettuce during incubation under different light conditions. The levels of GAs were measured by GC-SIM using [^{2}H]GAs as internal standards. -●-, FR seeds; -○-, FR/R seeds.

Table 2. Effects of R light irradiation and FR light irradiation on endogenous levels of GA_{19}, GA_{20}, and GA_1 in the seeds of Grand Rapids 6 h after the light treatment.

Sample	Endogenous level of GA (ng/1 g mature seeds equivalent)		
	GA_{19}	GA_{20}	GA_1
FR seeds	4.2	86	0.28
FR/R seeds	4.2	85	0.78
FR/R/FR seeds	4.2	85	0.25

With respect to FR and FR/R seeds, the data shows in detail the levels of GAs from 6 h time-point on Fig. 5. The levels of GAs in FR/R/FR seeds were determined by GC-SIM using [^{2}H]GAs as internal standards.

Based on the above information, the plant materials for quantitative analysis of GAs were prepared as shown in Fig. 1, and GA_1, GA_{19}, and GA_{20} in extracts of the seeds were quantified by GC-SIM using the corresponding deuterated GAs as internal standards. FR seeds were used as the control, because far-red light irradiation suppressed the small percent of germination observed in the complete dark. The time course of germination of the lettuce seeds under different light conditions are shown in Fig. 4. The FR/R seeds began to germinate 9–13 h after the light treatment and the FR seeds did not germinate until 44 h. The FR/R/FR seeds also did not germinate until 44 h after the light treatment (data not shown). In this study, the levels of GAs in the lettuce seeds were determined from 0–8 h after the light treatments, because we focused on the changes of levels of GAs in the seeds before germination.

The AE fraction from each sample of plant material was prepurified by PVPP, Sep-Pak (ODS), and Sepralyte (DEA) treatments in that order, and then fractionated by RP-HPLC. Portions of the fractions of *Rt* 10–16 min and *Rt* 17–26 min were further purified by $N(CH_3)_2$-HPLC for quantification of GA_1 and $GA_{19/20}$, respectively. The purified extracts were derivatized and then subjected to GC-SIM. The level of GA_1 in the FR seeds decreased slightly during incubation, but the level of GA_1 in the FR/R seeds increased during incubation and reached levels of about 3 and 5 times that of the FR seeds (dark control) 6 h and 8 h after a brief red light irradiation, respectively (Fig. 5). Table 2 shows the GA levels in FR, FR/R, and FR/R/FR seeds 6 h after the light treatments. The results indicates that a brief far-red light irradiation given after the red light irradiation completely canceled the effect of red light. The endogenous levels of GA_{19} and GA_{20} were not affected by the light treatments until 8 h after the light treatments (Fig. 5 and Table 2). The above results show that a brief irradiation of red light induced the increment of a physiologically active GA (GA_1) in the lettuce seeds. We cannot conclude whether or not the increase of GA_1 is sufficient to cause the germination. However, since red light irradiation caused an increase of GA_1 and the seed germination, it is considered that the increased GA_1 plays an important role on canceling the suppression of germination by the fruit wall.

Acknowledgment. We thank Professor L. N. Mander of the Australian National University for the generous gift of [$^2H_2$]GA_{19}.

References

Bewley JD, Black M (1982) Physiology and biochemistry of seeds in relation to germination, vol. 2. Springer-Verlag, Berlin

Borthwick HA, Hendricks SB, Parker MW, Toole EH, Toole VK (1952) A reversible photoreaction controlling seed germination. Proc Natl Acad Sci USA 38:662–666

Cross BE, Grove JF, Morrison A (1961) Gibberrellic acid. XVIII. Some rearrangements of ring A. J Chem Soc X: 2498–2515

De Greef JA, Fredericq H (1983) Photomorphogenesis and hormones. In: Shropshire W Jr, Mohr H (eds) Encyclopedia

of plant physiology, new series, vol. 16A. Springer-Verlag, Berlin, pp 401–427

Durley RC, Bewley JD, Railton ID, Pharis RP (1976) Effects of light, abscisic acid, and ^{6}N-benzyladenine on the metabolism of [^{3}H]gibberellin A_4 in the seeds and seedlings of lettuce, cv. Grand Rapids. Plant Physiol 57:699–703

Endo K, Yamane H, Nakayama M, Yamaguchi I, Murofushi N, Takahashi N, Katsumi M (1989) Endogenous gibberellins in the vegetative shoots of tall and dwarf cultivars of *Phaseolus vulgaris* L. Plant Cell Physiol 30:137–142

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL. MacMillan J (1984) Internode length in *Pisum sativum,* The *Le* gene controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. Planta 160:455–463

Inoue Y (1990) Role of gibberellins in phytochrome-mediated lettuce seed germination. In: Takahashi N, Phinney BO, MacMillan J (eds) Gibberellins. Springer-Verlag, New York, pp 289–295

Jones MG, Zeevaart JAD (1980) The effect of photoperiod on the levels of seven endogenous gibberellins in the long-day plant *Agrostemma githago* L. Planta 149:269–273

Murakami Y (1968) A new rice seedling test for gibberellins, microdrop method, and its use for testing extracts of rice and morning glory. Bot Mag 81:334–343

Nakayama M, Yamane H, Yamaguchi I, Murofushi N, Takahashi N, Katsumi M (1989) Endogenous gibberellins in the shoots of normal- and bush-type *Cucumis sativus* L. J Plant Growth Regul 8:237–247

Nakayama M, Yamane H, Yokota T, Yamaguchi I, Murofushi N, Takahashi N, Nishijima T, Katsura N, Nonaka M, Gaskin P, MacMillan J, Mander LN, Chu A (1990) Endogenous gibberellins in mature seeds of *Raphanus sativus* L. cv. Taibyo-sobutori. Agric Biol Chem 54:837–840

Pearce D, Miller AL, Roberts LW, Pharis RP (1987) Gibberellin-mediated synergism of xylogenesis in lettuce pith cultures. Plant Physiol 84:1121–1125

Phinney BO, Spray C (1982) Chemical genetics and the gibberellin pathway in *Zea mays* L. In: Wareing PF (ed) Plant growth substances. Academic Press, London, pp 101–110

Suzuki Y, Kurogochi S, Murofushi N, Ota Y, Takahashi N (1981) Seasonal changes of GA_1, GA_{19} and abscisic acid in three rice cultivars. Plant Cell Physiol 22:1085–1093

Toyomasu T, Yamane H, Yamaguchi I, Murofushi N, Takahashi N, Inoue Y (1992) Control by light of hypocotyl elongation and levels of endogenous gibberellins in seedlings of *Lactuca sativa* L. Plant Cell Physiol 33:695–701

Waycott W, Smith VA, Gaskin P, MacMillan J, Taiz L (1991) The endogenous gibberellins of dwarf mutants of lettuce. Plant Physiol 95:1169–1173

Yamaguchi I, Nakagawa R, Kurogochi S, Murofushi N, Takahashi N, Weiler EW (1987) Radioimmunoassay of gibberellins A_5 and A_{20}. Plant Cell Physiol 28:815–824

Yamaguchi I, Nakazawa H, Nakagawa R, Suzuki Y, Kurogochi S, Murofushi N, Takahashi N, Weiler EW (1990) Identification and semi-quantification of gibberellins from the pollen and anthers of *Zea mays* by immunoassay and GC/MS. Plant Cell Physiol 31:1063–1069