J Plant Growth Regul (1983) 2:165–175

Journal of
Plant
Growth
Regulation

The Biosynthesis of the Gibberellin Precursor *Ent*-Kaurene in Cell-Free Extracts and the Endogenous Gibberellins of Japanese Morning Glory in Relation to Seed Development

Gerard W. M. Barendse,[1] André Dijkstra,[1] and Thomas C. Moore[2]

[1]Department of Botany, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands.
[2]Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331 USA

Received April 13, 1983; accepted July 19, 1983

Abstract. The endogenous levels of gibberellins (GAs) determined by a combined HPLC-bioassay procedure and the formation of *ent*-kaurene, an immediate GA precursor, in cell-free extracts were studied in relation to seed development in *Pharbitis nil* Choisy cv. Violet. Three biologically active GA fractions were obtained, tentatively identified as GA_3, GA_5/GA_{20}, and a GA fraction, possibly GA_{19} and/or GA_{44}, which all increased in activity during early seed development and subsequently declined during maturation of the seeds. The total endogenous GA level reached its maximum at 19 days after anthesis, just before the seeds had attained their maximum fresh weight at about 23 days after anthesis. Similarly, the *ent*-kaurene synthesizing capacity showed a rapid increase during the period of rapid growth of the seeds, followed by a decline during maturation.

A direct relationship between the endogenous GA levels and the *ent*-kaurene synthesizing capacity of a particular tissue was indicated.

Ent-kaurene is a key intermediate in the GA biosynthetic pathway, and its formation can be studied in cell-free extracts from different organs of higher plants. The biosynthesis of *ent*-kaurene from [^{14}C]-mevalonate has been demonstrated in cell-free extracts of endosperm of wild cucumber (*Marah macrocarpus*) (Graebe et al. 1965, Upper and West 1967, Oster and West 1968); *Marah oreganus* (Coolbaugh and Hamilton 1976); *Cucurbita maxima* (Graebe

1969); immature fruits and seeds, shoot tips, and colyledons of peas (*Pisum sativum*) (Anderson and Moore 1967, Graebe 1968, Coolbaugh and Moore 1971a, b, Coolbaugh et al. 1973, Ecklund and Moore 1974, Moore and Ecklund 1974); suspensor of *Phaseolus coccineus* (Ceccarelli et al. 1979); seedlings of castor bean (*Ricinus communis*) (Robinson and West 1967) and sunflower (*Helianthus annuus*) (Shen-Miller and West 1982); seedling shoots of *Robinia pseudoacacia* (Nowak and Brown 1979); germinating seeds of tomato and cell suspension cultures of tobacco (Yafin and Shechter 1975); germinating seeds of *Triticum aestivum* and *Secale cereale* (Schilling et al. 1974); developing seeds, shoot tips, and leaves of Japanese morning glory (*Pharbitis nil*) (Barendse and Moore 1981); and coleoptiles of *Zea mays* (Hedden and Phinney 1979, Mellon and West, 1979).

The available data in the literature indicate a correlation between the capacity for *ent*-kaurene formation *in vitro* and the reported GA levels *in vivo* in developing seeds, e.g. *Marah macrocarpus, Lupinus succulentus, Phaseolus vulgaris* (Corcoran and Phinney 1962), *Pharbitis nil* (Ogawa 1963), and peas (Coolbaugh and Moore 1969). However, a direct comparison of *in vitro ent*-kaurene biosynthesis and extracted endogenous GA has not yet been carried out. Therefore, the aim of this study was to investigate the *ent*-kaurene synthesizing capacity concurrent with the endogenous GA levels in developing seeds of Japanese morning glory (*Pharbitis nil* cv. Violet).

Materials and Methods

Plant Material

In order to obtain developing seeds at several stages, the plants of *Pharbitis nil* Choisy cv. Violet were grown in a growth chamber at 25°C under 8 h short days to induce flowering.

Light was supplied with Sylvania VHO fluorescent tubes maintaining approximately 10,000 lux at plant level. The flower buds were tagged at anthesis and the immature seeds harvested at 3 to 4 day intervals starting 9 days after anthesis. The number of seeds per 5 g was determined. The seeds were frozen at −20°C for future analysis.

Enzyme Preparations

Five-g portions of plant material were homogenized in 0.1M phosphate buffer, pH 7.1, containing dithiothreitol (2mM) and wet PVP (1 g per g material), in a Sorvall omnimixer, usually 2 × 1 min at maximum speed. In general, 4 ml of phosphate buffer was used per g seeds. The brei was filtered through four layers of cheesecloth, the filtrate centrifuged at 12,000 *g* (10,000 rpm in Sorvall SS-34) for 10 min, and the supernatant centrifuged at 100,000 *g* (40,000 rpm in Beckman 50Ti) for 2 h. This supernatant was used as the enzyme preparation.

Enzyme Incubations

A typical enzyme incubation mixture consisted of 1 ml enzyme preparation, 200 µl ATP (2 µmole), 200 µl Mn^{++}/Mg^{++} (8 µmole), 100 µl glycerol, and 25 µl DL-2-[^{14}C]-mevalonic acid DBED salt (0.02 µmole:1 µCi). The incubations were carried out in triplicate for 2 h at 30°C. (At this time the enzyme reaction is still linear.) The reactions were stopped by adding 2 ml acetone and extracted three times with 3 ml of benzene-acetone (2:1, v/v) mixture. The combined extracts were evaporated to dryness under vacuum, subsequently dissolved in 100 µl acetone, applied on thin-layer Silica gel 60 (Merck), and developed in n-hexane. Authentic *ent*-kaurene was used as reference, and the corresponding radioactive spots were usually visualized by exposure in a radiochromatogram camera (LKB 2105). The authenticity of the spots has been further characterized by development of the thin layers in several solvent systems as described previously (Coolbaugh and Moore 1971b) and by high performance liquid chromatography using authentic as well as [^{14}C]-*ent*-kaurene, produced with the *Cucurbita* endosperm system (Graebe 1969) as references.

The spots containing radioactive *ent*-kaurene were scraped into 6-ml counting vials, to which 0.5 ml Lumasolve and 5 ml Lipoluma (Baker) was added. The vials were counted in a Philips liquid-scintillation analyser (PW 4540).

Protein Determination

Protein determinations were made on portions of each enzyme preparation by the Bio-Rad assay after Bradford, using bovine serum albumin as a standard.

Extraction and Purification of Endogenous GAs

The seeds, in 5-g portions, were homogenized in methanol in a Sorvall omnimixer. The residue after filtration was twice reextracted with methanol. The combined methanol extract was evaporated to a small volume of approximately 15 ml under vacuum and subsequently forced through a Sep-pak C cartridge. The filtrate was then evaporated to dryness and kept at −20°C until further analysis by reversed-phase, high-performance liquid chromatography (HPLC).

HPLC Analysis of GAs

The separation of GAs took place on a HPLC system from Water Associates consisting of two pumps (model 600-A), a solvent programmer (model 660), a universal injector (UK6), variable wavelength detector (model 450), radial compression system (RCM-100) containing a Radial-pak A cartridge, guard column, Omniscribe recorder (Houston Instruments), and a fraction collector (Pharmacia, Frac 3000). The analysis was carried out essentially according to previously described procedures (Barendse et al. 1980).

The extract residue was taken up with 30% methanol in 0.01 M H_3PO_4 and subsequently injected into the HPLC system. The GAs were eluted and separated by a gradient run in 20 min from 30% MeOH, 0.01 M H_3PO_4, pH 2.5, to 35% MeOH, 0.01 M H_3PO_4, pH 7.5. The flow was 2.5 ml/min and detection of the references (GA_3 and GA_{20}) took place at 206 nm. One-min fractions were collected, neutralized, evaporated to dryness, and stored at −20°C until the bioassay.

Dwarf Rice Bioassay

Seeds of the dwarf rice cv. Tan-ginbozu were obtained from Prof. Y. Murakami, Japan. The fractions were tested essentially according to the "microdrop" method of Murakami (1968). The seeds were germinated in Petri dishes with tap water (refreshed after 24 h) at 30°C under fluorescent illumination at 2,000 lux.

The germinated seeds were selected for homogeneity, planted on 0.75% agar-agar in boxes, and grown for another 48 h. The extract residues were taken up in 1 ml ethanol/water (1:1, v/v) and 1 µl solution was applied to five plants per treatment. After 5 days the length of the second leaf sheath was measured and the GA activity related to standards of GA_3 and expressed as GA_3 equivalents.

Results

Endogenous GA Determination

Immature seeds are known to contain high levels of endogenous GAs, and immature seeds of *Pharbitis nil* have been shown to attain their maximum level of extractable GAs just before reaching maximum fresh weight at about 20 days after anthesis (Murakami 1961). Therefore, immature seeds at this stage were used for the initial experiments to analyze their endogenous GAs as well as their *ent*-kaurene synthesizing capacity in order to optimize conditions.

Reversed-phase HPLC has proved to be very effective in separating GAs in plant extracts without derivatization (Barendse et al. 1980, Jones et al. 1980, Lin and Heftmann 1981, Koshioka et al. 1983). HPLC alone is an insufficiently rigorous detection method and, therefore, it has to be combined with a definite qualitative assay such as a specific bioassay or specific physical methods. We have preferred the use of the dwarf rice bioassay, which is specific as well as very sensitive to GAs.

Fig. 1 represents the results of such a combined HPLC-bioassay analysis of seeds harvested 19 days after anthesis. Three biologically active regions, i.e. I, II, and III, were obtained in the elution histogram of which I co-elutes with GA_3 and II with GA_5 and GA_{20}. The endogenous GAs of immature seeds of *Pharbitis nil* have been reinvestigated by Jones et al. (1980), and they have confirmed the presence of GA_3, GA_5, GA_{17}, GA_{20}, and GA_{29} by gas-liquid chromatography-mass spectrometry. In addition, they identified two new GAs, GA_{19} and GA_{44}, in extracts of immature seeds. Using reversed-phase HPLC,

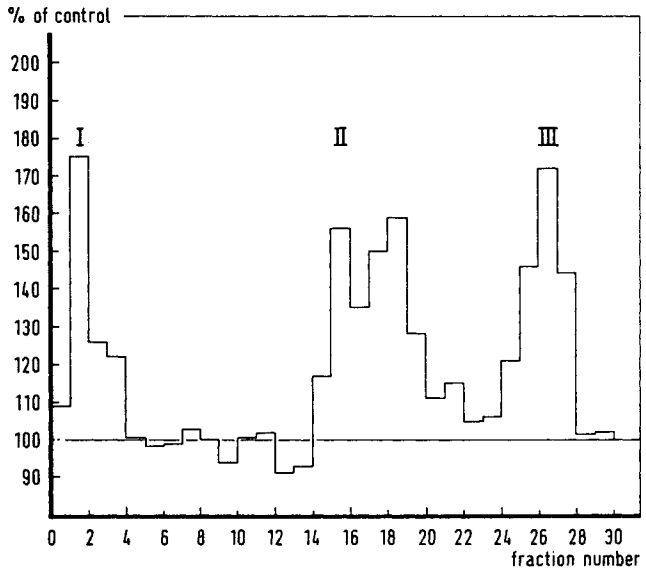

Fig. 1. Combined HPLC-bioassay analysis of GAs in an extract of immature *Pharbitis nil* seeds at 19 days after anthesis. Biological activity of the collected fractions obtained with the dwarf rice bioassay. I = GA_3-like, II = GA_5/GA_{20}-like, III = unidentified GA.

they obtained two major and one minor biologically active fractions. One of the major biologically active fractions was identified as GA_3; the second contained GA_5 and GA_{20}, while the minor fraction consisted of GA_{44} and GA_{19}. The sequence of elution that was obtained by reversed-phase HPLC for the endogenous GAs of *Pharbitis nil* was, respectively, GA_{29}, GA_3, GA_5/GA_{20}, GA_{44}/GA_{19}, and GA_{17}.

With our reversed-phase HPLC procedure (Barendse et al. 1980) the same sequence of elution was obtained, e.g. GA_{29}, GA_3, GA_5, and GA_{20}. Authentic samples of GA_{17}, GA_{19}, and GA_{44} were not available. GA_{29} has very little biological activity in the dwarf rice bioassay (Reeve and Crozier 1975) and has the closest retention to GA_3 in HPLC with regard to the known GAs in *Pharbitis*.

The biologically active fraction I was further analysed by HPLC under isocratic solvent delivery, i.e. 30% methanol, 0.01 M H_3PO_4, pH 3.5, which resolved the retention times of GA_{29} and GA_3 even better. Under these conditions, when only a few related GAs are eluted, definitely excluding the other known GAs of *Pharbitis*, fraction I is clearly associated with GA_3 and/or GA_1. Since GA_1 has not been identified in *Pharbitis*, fraction I must be identical to GA_3.

In a similar way, it was established that fraction II contained both GA_5 and GA_{20}, since under isocratic HPLC, i.e. 35% methanol 0.01 M H_3PO_4, pH5.5, which resolved GA_5 and GA_{20} completely, two peaks of biological activity were also obtained, as is indicated in Fig. 1.

Fraction III elutes immediately after fraction II. Jones et al. (1980) showed that GA_{44}/GA_{19} eluted immediately after GA_5/GA_{20}.

GA_{19} is biologically active in the dwarf rice bioassay; whether GA_{44} is active in this assay is not known. GA_{17}, the remaining endogenous GA of *Pharbitis*,

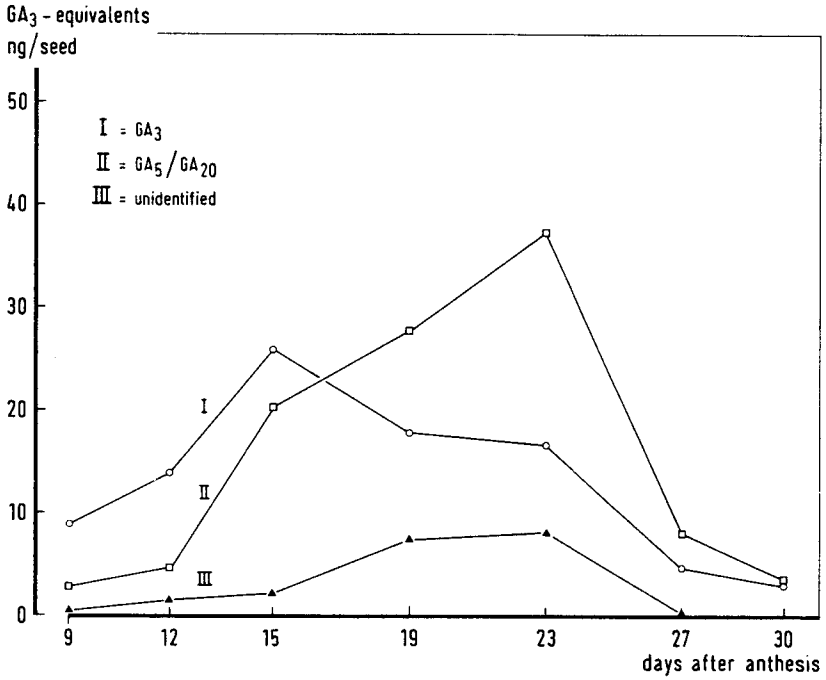

Fig. 2. Changes in the activities of the three fractions I, II, and III (See Fig. 1) during seed development of *Pharbitis nil* determined by the combined HPLC-bioassay procedure.

elutes after GA_{44}/GA_{19} and possesses little or no biological activity. Thus it is highly likely that fraction III represents GA_{44} and/or GA_{19}.

As will be shown, fractions I and II represent the major biologically active fractions during seed development, while the activity of fraction III is comparatively much lower. This also concurs completely with the results of Jones et al. (1980).

In the above-described manner, developing seeds were analysed at different time intervals after anthesis for the respective biological activities of I, II, and III. The results in Fig. 2 represent the average of two independent experiments. It shows that all three fractions consecutively increase during seed growth, reach a maximum, and decrease during the development of the seeds. However, the GA_3 fraction I peaks at 15 days, the GA_5/GA_{20} fraction II peaks at 23 days, and III somewhere between 19 and 23 days after anthesis.

In Fig. 3 the total GA-like activity has been calculated from the results in Fig. 2 and compared with the increase in fresh weight during seed development.

A comparison of the total GA_3 equivalents, expressed in ng/seed, with the fresh weight shows that the total level of endogenous GA_3 increases during the period of increase in fresh weight and reaches its maximum before the maximum in fresh weight at 23 days after anthesis, followed by a subsequent decrease during seed maturation. The total of GA_3 equivalents, expressed as μg/g seed, demonstrates that the concentration of GA_3 in the seed tissue is

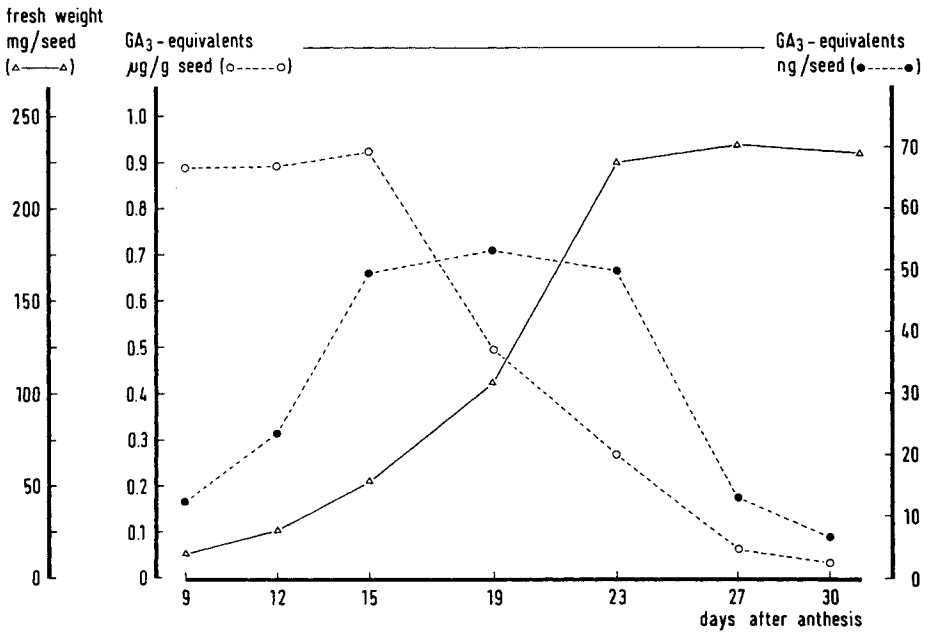

Fig. 3. The total endogenous GA content of *Pharbitis nil* seeds, either expressed as µg/g seed or as ng/seed, in comparison with the increase in fresh weight of seeds during their development.

high during early seed development and decreases in the later stages of seed development.

Ent-*Kaurene Synthesis in Cell-Free Extracts*

Enzymes were prepared from developing seeds at different time intervals after anthesis. Each enzyme extraction was made as equal as possible by homogenizing 5 g of material in 20 ml 0.1 M phosphate buffer, pH 7.1, containing dithiothreitol and PVP, except for mature seeds, which needed somewhat more buffer for extraction. Fig. 4 shows the change in the *ent*-kaurene synthesizing capacity during seed development. The capacity to synthesize *ent*-kaurene increases slowly during early seed development between 9 and 17 days after anthesis and is followed by a rapid increase concurrent with the rapid increase in fresh weight of the seeds between 17 and 23 days after anthesis. During maturation of the seeds, when there is no further increase in fresh weight, i.e. between 23 and 30 days after anthesis, the *ent*-kaurene synthesizing capacity gradually drops concurrent with the decrease in gibberellin activity shown in Fig. 3. In another experiment, the *ent*-kaurene synthesizing capacity of developing seeds at different stages was compared with that of shoot tips and young and mature leaves. The results shown in Table I confirm the very high *ent*-kaurene synthesizing capacity of immature seeds at 20 days after anthesis, particularly when expressed as dpm/g fresh weight.

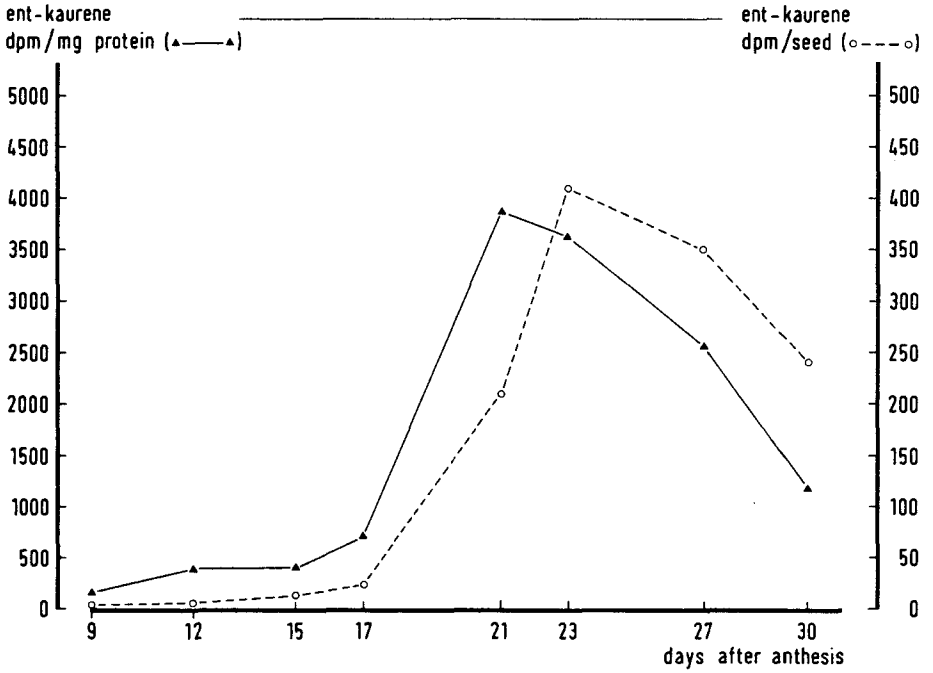

Fig. 4. The *ent*-kaurene synthesizing capacity in cell-free extracts from seeds of *Pharbitis nil* during their development.

Shoot tips also possess a relative high capacity for *ent*-kaurene synthesis. The capacity of young leaves is comparable to that of very young seeds.

On a fresh weight basis, mature leaves have a low *ent*-kaurene synthesizing capacity due to their lower protein content.

Discussion

Coolbaugh and Moore (1969) reported apparent changes in rate of *ent*-kaurene synthesis in cell-free extracts from pea seeds during seed development and that those changes could also be related to previously reported endogenous GAs. Similarly, Moore and Ecklund (1974) showed that the ontogenetic changes in growth rate of pea seedlings could be related to the *ent*-kaurene synthesizing capacity of cell-free extracts from those seedlings.

The results described in this paper on the endogenous GAs and the *ent*-kaurene synthesizing capacity in developing seeds of *Pharbitis nil* elaborate the above-mentioned reports on peas. These results provide clear evidence that both the *ent*-kaurene biosynthetic capacity in cell-free extracts and the endogenous GAs *in vivo* are related to seed development, i.e. they increase rapidly during the stage of rapid increase in fresh weight and decrease during maturation in *Pharbitis nil*.The endogenous GA levels described in this paper concern the determination of the so-called free GAs. It is known, however,

Table 1. Formation of *ent*-kaurene from [^{14}C]-mevalonic acid in cell-free enzyme preparations from different organs of *Pharbitis nil* Choisy cv. Violet.

Plant organ	Number of organs/10g	*Ent*-kaurene	
		dpm/mg protein	dpm/g fresh weight
Immature seeds at:			
10 days after anthesis	360	532	2999
20 days after anthesis	175	4866	19464
30 days after anthesis	97	4790	6706
Mature seeds	200	223	4683
Shoot tips	150	1508	6936
Young leaves	85	551	2755
Mature leaves	8	600	450

that in immature seeds of *Pharbitis nil* a part of the GAs occurs as conjugates (Yokota et al. 1969a, 1969b, 1970). Barendse (1971) has shown that at the early stages of seed development (15 and 18 days after anthesis) the conjugated GA content is low, and much lower than the free GA content; at 25 days after anthesis, both the conjugated and free GA content were found to be high and the conjugated GA content nearly as high as the free GA content, whereas in mature seeds the conjugated GA content, though lower than in immature seeds at 25 days after anthesis, has surpassed the level of free GAs. These results indicated a progressive conjugation of the synthesized free GAs during seed development. Among the conjugated GAs present in immature *Pharbitis* seeds, e.g. GA_3-, GA_8-, GA_{26}-, GA_{27}-, and GA_{29}-glucopyranoside (see Bearder 1980), GA_3-glucopyranoside is the only conjugate yielding a biologically active GA upon hydrolysis. Thus the major part of the high GA conjugate content at 25 days after anthesis determined previously has to be attributed to GA_3-glucopyranoside.

Taking this fact into account, the maximum at 15 days after anthesis of the GA_3 fraction I does not reflect the actual maximum in GA_3 biosynthesis. The latter concurs more likely with the maximum content of GA conjugates at 25 days after anthesis. This would also shift the maximum of total endogenous GA toward about 25 days after anthesis and not 19 days as shown in Fig. 3, which represents only the free GAs. This is more in accordance with the maximum of the *ent*-kaurene synthesizing capacity at 23 days after anthesis, which one would expect to peak before the endogenous GA level.

Furthermore, the described total endogenous GA is composed of three GA activities that do not peak at the same stage during seed development, i.e. GA_3 activity, which peaks at 15 days after anthesis; GA_5/GA_{20} activity, which peaks at 23 days after anthesis; as well as the GA activity peaking between 19 and 23 days after anthesis. Thus the rate at which different GAs are being synthesized varies consecutively during seed development. This seems to be a common phenomenon in developing seeds as it has also been shown for developing pea seeds (Frydman et al. 1974, Sponsel 1980) in which the biosynthesis of GA_9 reached its maximum level just before that of GA_{17} and GA_{20}; they, in turn, reach their maximum before that of GA_{29}. In addition to syn-

thesis, metabolism of the GAs may occur, e.g. GA_{20} may be metabolized in plants to GA_{29} (Frydman and MacMillan 1975).

In our study, neither GA_{29} nor GA_{27} was detected, since they have very little activity in the dwarf rice bioassay.

Shoot tips are also known as active sites of GA synthesis; their relatively high *ent*-kaurene synthesizing capacity confirms this fact.

When expressed on a protein basis, the *ent*-kaurene synthesizing capacity of young and mature leaves do not differ significantly; however, on the basis of fresh weight the young leaves are more active, since they contain more protein/g fresh weight. Similarly, the apparent low *ent*-kaurene synthesizing capacity on a protein basis of very young immature seeds or that of mature seeds nevertheless appears to represent a considerable GA-synthesizing capacity on a fresh weight basis. This is also confirmed by the relatively high level of endogenous GAs expressed on a fresh weight (μg/g) basis during early seed development (Fig. 3). This is, therefore, a clear illustration of the fact that the level of endogenous GAs in a particular plant organ is determined not only by the relative capacity to synthesize *ent*-kaurene per unit of protein, but also by the protein content per unit of fresh weight.

References

Anderson JD, Moore TC (1967) Biosynthesis of (−)-kaurene in cell-free extracts of immature pea seeds. Plant Physiol 42:1527–1534

Barendse GWM (1971) Formation of bound gibberellins in *Pharbitis nil*. Planta 99:290–301

Barendse GWM, Moore TC, (1981) Biosynthesis of the gibberellin precursor *ent*-kaurene in cell-free extracts of Japanese morning glory in relation to seed and seedling development. In: Cooke AR and Abdel-Rahman M (eds) Proceedings, 8th Annual Meeting of the Plant Growth Regulator Society of America, pp 124–131

Barendse GWM, Van de Werken PH, Takahashi N (1980) High-performance liquid chromatography of gibberellins. J Chrom 198:449–455

Bearder JR (1980) Plant hormones and other growth substances: Their background, structures and occurrence. In: MacMillan J (ed) Hormonal regulation of development. I. Encyclopedia of plant physiology, new series, vol. 9. Springer Verlag, pp 9–112

Ceccarelli N, Lorenzi R, Alpi A (1979) Kaurene and kaurenol biosynthesis in cell-free system of *Phaseolus coccineus*. Phytochem 18:1657–1658

Coolbaugh RC, Hamilton R (1976) Inhibition of *ent*-kaurene oxidation and growth by α-cyclopropyl-α- (p-methoxyphenyl)-5-pyrimidine methyl alcohol. Plant Physiol 57:245–248

Coolbaugh RC, Moore TC (1969) Apparent changes in rate of kaurene biosynthesis during development of pea seeds. Plant Physiol 44:1364–1367

Coolbaugh RC, Moore TC (1971a) Localization of enzymes catalysing kaurene biosynthesis in immature pea seeds. Phytochem 10:2395–2400

Coolbaugh RC, Moore TC (1971b) Metabolism of kaurene in cell-free extracts of immature pea seeds. Phytochem 10:2401–2412

Coolbaugh RC, Moore TC, Barlow SA, Ecklund PR (1973) Biosynthesis of *ent*-kaurene in cell-free extracts of *Pisum sativum* shoot tips. Phytochem 12:1613–1618

Corcoran MR, Phinney BO (1962) Changes of gibberellin-like substances in developing seed of *Echinocystis, Lupinus,* and *Phaseolus*. Physiol Plantarum 15:252–262

Ecklund PR, Moore TC (1974) Correlations of growth rate and de-etiolation with rate of *ent*-kaurene biosynthesis in pea (*Pisum sativum* L.). Plant Physiol 53:5–10

Frydman VM, MacMillan J (1975) The metabolism of gibberellins A_9, A_{20}, A_{29} in immature seeds of *Pisum sativum* cv. Progress No. 9. Planta 125:181–195

Frydman VM, Gaskin P, MacMillan J (1974) Qualitative and quantitative analysis of gibberellins throughout seed maturation in *Pisum sativum* cv. Progress No. 9. Planta 99:290–301

Graebe JE (1968) Biosynthesis of kaurene, squalene and phytoene from mevalonate-2-^{14}C in a cell-free system from pea fruits. Phytochem 7:2003–2020

Graebe JE (1969) The enzymic preparation of |^{14}C|-kaurene. Planta 85:171–174

Graebe JE, Dennis DT, Upper CD, West CA (1965) Biosynthesis of gibberellins. I. The biosynthesis of (−)-kaurene, (−)-kaurenol, and trans-geranylgeraniol in endosperm nucellus of *Echinocystis macrocarpa* Greene. J Biol Chem 240:1847–1854

Hedden P, Phinney BO (1979) Comparison of *ent*-kaurene and *ent*-isokaurene synthesis in cell-free systems from isolated shoots of normal and dwarf-5 maize seedlings. Phytochem 18:1475–1479

Jones MG, Metzger JD, Zeevaart JAD (1980) Fractionation of gibberellins in plant extracts by reverse phase high performance liquid chromatography. Plant Physiol 65:218–221

Koshioka M, Harada J, Takeno K, Norna M, Sassa T, Ogiyama K, Taylor JS, Rood SB, Legge RL, Pharis RP (1983) Reversed phase C_{18} high-performance liquid chromatography of acidic and conjugated gibberellins. J Chrom 256:101–115

Lin J-T Heftmann E (1981) Adsorption and reversed-phase partition high-performance liquid chromatography of gibberellins. J Chrom 213:507–510

Mellon JE, West CA (1979) Diterpene biosynthesis in maize seedlings in response to fungal infection. Plant Physiol 64:406–410

Moore TC, Ecklund PR (1974) Biosynthesis of *ent*-kaurene in cell-free extracts of pea shoots. In: plant growth substances 1973. Hirokawa Publishing Co., Tokyo, pp 252–259

Murakami Y (1961) Paper-chromatographic studies on change in gibberellins during seed development and germination in *Pharbitis nil*. Bot Mag (Tokyo) 74:241–247

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 (Tokyo), 81:33–43

Nowak J, Brown GN (1979) Free and bound gibberellin activities and *ent*-kaurene synthesis during induction of cold hardiness in black locust seedlings. Physiol Plant 45:11–16

Ogawa Y (1963) Gibberellin-like substances occurring in the seed of *Pharbitis nil* Choisy and their change in contents during seed development. Plant Cell Physiol 4:217–225

Oster MO, West CA (1968) Biosynthesis of trans-geranylgeranyl pyrophosphate in endosperm of *Echinocystis macrocarpa* Greene. Arch Biochem Biophys 127:112–123

Reeve DR, Crozier A (1975) Gibberellin bioassays. In: Krisnamoorthy HN (ed) Gibberellins and plant growth. John Wiley & Sons, London, pp 35–64

Robinson DR, West CA (1967) Biosynthesis of (−)-kaurene and other diterpenes in germinating castor bean seeds. Fed Proc 26:454

Schilling G, Podlesak W, Eckert H (1974) The actions of some plant growth inhibitors in wheat studied by cell-free systems. In: Schreiber K, Schütte H-R, Sembdner G (eds) Biochemistry and chemistry of plant growth regulators. Inst Pl Biochem Acad Sci GDR, Halle, pp 17–32

Shen-Miller J, West CA (1982) *Ent*-kaurene biosynthesis in extracts of *Helianthus annuus* L. seedlings. Plant Physiol 69:637–641

Sponsel VM (1980) Metabolism of gibberellins in immature seeds of *Pisum sativum*. In: Skoog F (ed) Plant growth substances 1979. Springer-Verlag, Berlin Heidelberg New York, pp 170–179

Upper CD, West CA (1967) Biosynthesis of gibberellins. II. Enzymic cyclization of geranylgeranyl pyrophosphate to kaurene. J Biol Chem 242:3285–3292

Yafin Y, Shechter I (1975) Comparison between biosynthesis of *ent*-kaurene in germinating tomato seeds and cell suspension cultures of tomato and tobacco. Plant Physiol 56:671–675

Yokota T, Murofushi N, Takahashi N (1969a) Structure of new gibberellin glycosides in immature seeds of *Pharbitis nil*. Tetrahedron Letters 2081–2084

Yokota T, Murofushi N, Takahashi N (1970) Structures of a new gibberellin glucoside in immature seeds of *Pharbitis nil* (3-0-β-glucosyl-gibberellin A_{29}; acid hydrolysis: enzymatic hydrolysis). Tetrahedron Letters 1489–1492

Yokota T, Takahashi N, Murofushi N, Tamura S (1969b) Isolation of gibberellins A_{26} and A_{27} and their glycosides from immature seeds of *Pharbitis nil*. Planta 87:180–184