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Gibberellins in Embryo-Suspensor of *Phaseolus coccineus* Seeds at the Heart Stage of Embryo Development[1]

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ABSTRACT

Gibberellins (GAs) in suspensors and embryos of *Phaseolus coccineus* seeds at the heart stage of embryo development were analyzed by combined gas chromatography-mass spectrometry (GC-MS). From the suspensor four C_{19}-GAs, GA_1, GA_4, GA_5, GA_6, and one C_{20} GA, GA_{44}, were identified. From the embryo, five C_{19}-GAs GA_1, GA_4, GA_5, GA_6, GA_{60} and two C_{20} GAs, GA_{19} and GA_{44} were identified. The data, in relation to previous results, suggest a dependence of the embryo on the suspensor during early stages of development.

Several contributions (1, 16, 22) concerning the endogenous GAs[2] in various plant species have highlighted the fact that the GA status of a plant is rather complicated. Therefore there is the need for a more complete picture of the GA concentration in the specific plant tissue studied, if the physiology of this hormonal group is to become more clear.

The developing seed of *Phaseolus coccineus* is a good demonstration of how complex the GA status can be: at least 12 GAs have been identified in the various seed components. Our work with this species has recently been dedicated to the identification, quantification, and biosynthesis of GAs in specific parts of the seed in an attempt to elucidate their role in the embryo-suspensor system during seed development.

With few exceptions, the first division of the zygote in both monocots and dicots originates two cells. The basal cell (micropylar) generates the suspensor, while the other cell develops into the embryo proper. The suspensor is almost ubiquitous in angiosperms. Its structure and morphology vary from rudimentary (few small and/or giant cells; sometimes only one) to massive (many giant cells; in *P. coccineus* about 200 cells). According to an old interpretation, the suspensor has the function of pushing the embryo into the endosperm to facilitate its nutrition. On the other hand, a massive suspensor develops in some plants which are characterized by minimal endosperm development (*Sherardia, Tropaeolum, Medicago, Phaseolus*). These massive suspensors show conspicuous cell growth through chromosome endoreduplication or, much less frequently, multinucleation (10).

Earlier, a hypothesis (21) was put forward that the suspensor might take over the function of the endosperm. To support this idea, Corsi (8) showed that the *Eruca sativa* embryo, excised at the early stage, could grow *in vitro* if attached to the suspensor. Meanwhile, at the late heart stage, the embryo could be easily grown *in vitro* even though deprived of the suspensor. One postulated function attributed to the suspensor is the synthesis of hormones and their supply to the growing embryo (15). To test this hypothesis, analysis of GA-like activities in embryo and suspensors were conducted at two different stages of embryogenesis: heart shaped (stage A) and cotyleonary (stage B) embryo. High levels of GA-like substances were found in both tissues (2), but even by extracting 2000 suspensors only GA_1 was identified (3). The hypothesis that the suspensor could be a site of GA synthesis was confirmed by work done with a cell-free system derived from it. This cell-free system demonstrated the incorporation of MVA into kaurene and of kaurene into 7 β-hydroxykaurenoic acid, and finally 7 β-hydroxykaurenoic into GA_1, GA_5, and GA_8 (5, 6).

The question was then posed as to whether suspensor and embryo proper would contain other native GAs, besides GA_1, at these two stages of embryogenesis. Initially we analyzed suspensors and embryos at stage B of embryogenesis. These results showed the presence of six GAs in the suspensor and five GAs in the embryo (18, 19).

In this paper we report the amount and the type of GAs present in suspensor and embryo at stage A (heart-shaped embryo) of *P. coccineus*, together with a general discussion about GAs in this system.

MATERIALS AND METHODS

Plant Material

Plants of *Phaseolus coccineus* of the same white-seeded variety used in our previous studies on GAs (18, 19) were grown in the field. Embryos and suspensors were removed under a stereoscopic microscope from seeds 4 to 6 mm long (heart-shaped embryo). Collection was carried out during two consecutive seasons (1985–1986). All material was kept in an ice bath during the isolation of embryos and suspensors from the seeds, which were stored at −20°C until required.

Extraction and Purification

Frozen embryos (5,100, 9.3 g f.w.) and suspensors (10,000 2.1 g f.w.) were homogenized in cold 80% (v/v) aqueous methanol and stirred for 12 h at 4°C before centrifugation at 2,500*g* for 10 min. The pellets were separated from the

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[2] Abbreviation: GA(s), gibberellin(s); f.w., fresh weight; MVA, mevalonic acid; EtOAc, ethyl acetate; SiO_2, silica-gel; TMSi, trimethylsilyl; TMSi-TMSi, trimethylsilyl ester ether; Me-TMSi, methyl ester trimethylsilyl ether.

supernatant and reextracted twice. The combined supernatants were reduced to the aqueous phase and the pH adjusted to 2.8. The aqueous phase from each extract was then partitioned four times against EtOAc. The EtOAc extracts, reduced to a small volume, were adsorbed onto a small quantity of SiO_2 for SiO_2 partition chromatography, which was carried out as previously described (3). After the fractions were collected the columns were washed with methanol; the methanol fractions are referred to as MeOH wash in the figures. The fractions showing GA-like activity from the suspensor extracts were dried under vacuum and derivatized for GC and GC-MS without any further purification. However, the active fractions from the embryo extract were further purified on C_{18} reverse phase HPLC prior to derivatization as described by (13).

Bioassay

Dwarf-rice (*Oryza sativa* L., cv Tan-ginbozu) and barley endosperm α-amylase (*Hordeum vulgare* L., cv Himalaya) were used for detecting GA-like activity (11, 14).

Derivatization, GC, and GC-MS

SiO_2 and HPLC fractions containing GA-like activity were dried and derivatized by adding a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane (5:1:1). Some samples were methylated with a saturated solution of diazomethane in acetone and silylated using the mixture reported above. The derivatized samples were injected onto a 3% OV-1 GC packed column (19). GC-MS was performed on a Hewlett-Packard 5992B equipped with GC, jet separator and operating at 70 eV. GC conditions were as follows: glass column (90 × 0.2 cm i.d.) 2% OV-1; carrier gas He at 25 mL min^{-1}; oven temperature program of 200 to 300°C at 5°C min^{-1}. The compounds were identified as the TMSi ester TMSi ether and/or Me ester TMSi ether in the derivatized samples by comparison of their mass spectra with reference spectra obtained under the same conditions, and/or with published mass spectral data.

RESULTS AND DISCUSSION

An aliquot (1/80) of the fractions collected from SiO_2 partition chromatography were bioassayed. Figure 1, a and b, shows the localization of the GA-like activity in the suspensor extract using the barley endosperm α-amylase and dwarf rice microdrop bioassay, respectively. A wide zone of the chromatogram showed GA-like activity. The bioactive fractions were collected, pooled, and named as shown in Figure 1a; fractions 16–20: S_1, 21–25: S_2, 26–30: S_3, 31–35: S_4, 36–41: S_5. After derivatization the samples were analyzed by analytical and preparative GC. GA-like activities were located from the preparative GC trace by α-amylase bioassay of individual peaks after hydrolysis of the TMSi-TMSi. The samples were then analyzed by GC-MS and repetitive scans were taken in those peaks that had shown GA-like activity in the preparative GC eluate. The following GAs were identified as TMSi-TMSi: GA_4, GA_5 (trace) in sample S_1; GA_1, GA_5, GA_6, GA_{44} in sample S_2; GA_1 in sample S_3 and S_4; GA_1 (trace) in sample S_5

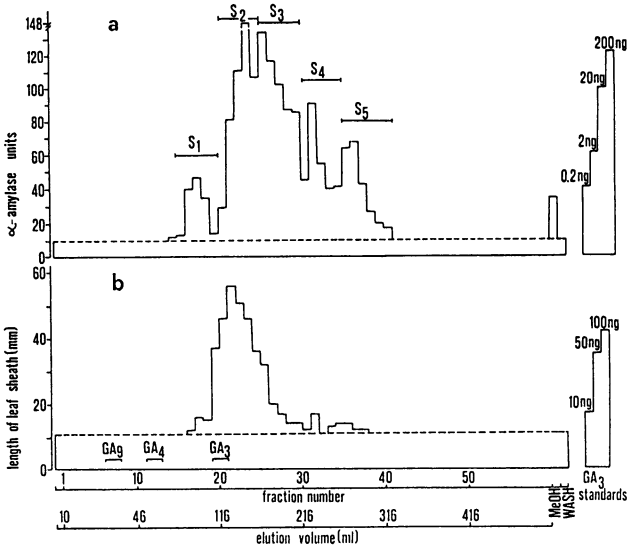

Figure 1. Histograms of GA-like activity from SiO_2 partition chromatography of the acidic EtOAc fraction from the suspensor of *P. coccineus*. Histograms were obtained with aliquot of 1/80 of each fraction tested on the barley endosperm α-amylase bioassay (a), and on dwarf rice bioassay (b). Fractions 16–20: S_1, 21–25: S_2, 26–30: S_3, 31–35: S_4, 36–41: S_5. Elution of authentic standards of GA_9, GA_4, and GA_3 are shown by (——).

(see Table I for relative intensities of the characteristic ions and the retention times of TMSi-TMSi GAs).

Figure 2, a and b, shows the localization of the GA bioactivity in the embryo extract after SiO_2 partition chromatography using barley endosperm α-amylase and dwarf rice microdrop bioassays, respectively. The active fractions were collected, pooled, and named as shown in Figure 2a: fractions 9–20: E_1, 21–26: E_2, 27–32: E_3, 33–38: E_4, 39–45: E_5. These five samples were further purified by reversed phase C_{18} HPLC. The biologically active fractions from HPLC analysis were located by α-amylase and dwarf rice bioassays and analyzed by GC and GC-MS. The following GAs were identified as TMSi-TMSi: GA_4, GA_5, GA_6, GA_{44} in sample E_1; GA_1 in sample E_4 and E_5 (see Table I for relative intensities of the characteristic ions and the retention times of TMSi-TMSi GAs). GC-MS analysis of the biologically active peak of sample E_2 gave a mass spectrum as TMSi-TMSi with the following major ions and relative intensities (retention time 6.0 min): 564 (M^+, 54), 549 (19), 447 (86), 433 (29). This peak was trapped by preparative GC followed by hydrolysis of the TMSi, methylated and silylated. GC-MS analysis of this peak as Me-TMSi gave a spectrum with the following major ions and relative intensities (retention time 5.1 min): 506 (M^+, 61), 491 (9), 447 (10), 375 (42), 321 (6) nearly identical to that of GA_{60} Me-TMSi (J MacMillan, personal communication; and ref. 12).

GC-MS analysis of the active peak of sample E_3 gave a mass spectrum as TMSi-TMSi with the following major ions and relative intensities (retention time 6.7 min): 578 (M^+, 13), 563 (23), 550 (22), 488 (12), 460 (40), 432 (49). After trapping, the peak was hydrolyzed, methylated, and silylated. GC-MS analysis of this peak as Me-TMSi gave a spectrum with the

Table I. *GC-MS Relative Intensities of the Characteristic Ions and the Retention Times of TMSi-TMSi GAs Identified in Suspensors and Embryos of P. coccineus of Stage A and for Some Authentic GAs*

Where no authentic GAs were available, published data for mass spectral analyses are presented.

Gibberellin or Sample	Retention Time	Mass Spectra	Identity
	min	*m/z values*	
TMSi-TMSi GA_5	5.2	474 (M^+, 100), 459 (31), 357 (85), 299 (28), 207 (20)	
Samples S_1, S_2, E_1 (see Figs. 1 and 2)	5.2	474 (M^+, 100), 459 (30), 357 (71), 299 (23), 207 (15)	GA_5
TMSi-TMSi GA_4	5.4	476 (M^+, 17), 461 (32), 386 (28), 347 (12), 342 (22), 224 (100)	
Samples S_1, E_1 (see Figs. 1 and 2)	5.4	476 (M^+, 21), 461 (45), 386 (44), 347 (12), 342 (26), 224 (100)	GA_4
TMSi-TMSi GA_6 (see ref. 19)	6.0	490 (M^+, 91), 475 (30), 405 (18), 373 (100), 303 (32), 235 (16), 207 (21)	
Samples S_2, E_1 (see Figs. 1 and 2)	6.0	490 (M^+, 84), 475 (25), 405 (19), 373 (100), 303 (35), 235 (15), 207 (14)	GA_6
TMSi-TMSi GA_1	7.1	564 (M^+, 100), 549 (26), 447 (63), 207 (27)	
Samples S_2-S_5, E_4, E_5 (see Figs. 1 and 2)	7.1	564 (M^+, 100), 549 (26), 447 (73), 207 (15)	GA_1
TMSi-TMSi GA_{44} (see ref. 19)	8.3	490 (M^+, 44), 475 (15), 373 (100), 296 (18), 207 (62), 208 (26)	
Samples S_2, E_1 (see Figs. 1 and 2)	8.3	490 (M^+, 51), 475 (16), 373 (100, 296 (13), 207 (33), 208 (14)	GA_{44}

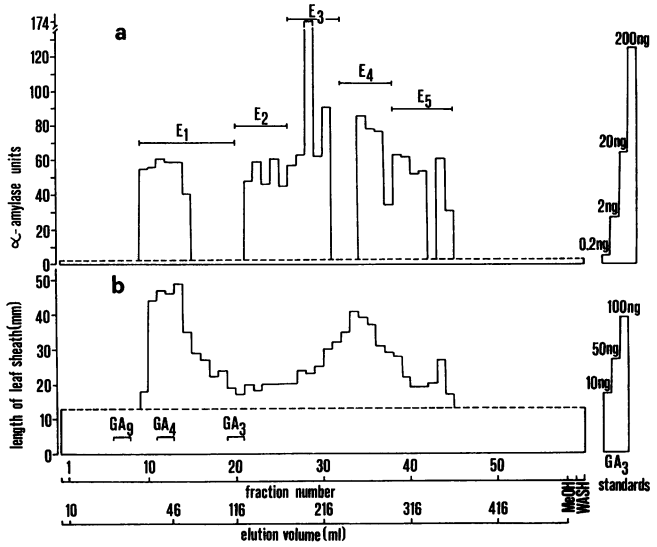

Figure 2. Histograms of GA-like activity from SiO_2 partition chromatography of the acidic EtOAc fraction from embryos of *P. coccineus*. Histograms were obtained with aliquot of 1/80 of each fraction tested on the barley endosperm α-amylase bioassay (a) and dwarf rice bioassay (b). Fractions 9–20: E_1, 21–26: E_2, 27–32: E_3, 33–38: E_4, 39–42: E_5. Elution of authentic standards of GA_9, GA_4, and GA_3 are shown by (——).

following major ions and relative intensities (retention time 6.2 min): 462 (M^+, 11), 447 (4), 434 (100), 431 (9), 402 (29), 375 (40), 374 (50) nearly identical to that of GA_{19} (4).

Rough estimates of the amounts of GAs identified in the suspensor and embryo at the heart stage (Table II) were made

Table II. *Gibberellins and Their Approximative Concentrations in Suspensor and Embryo of P. Coccineus at the Heart Stage (stage A) of Embryo Development*

Gibberellins	Gibberellin Concentration	
	Suspensor	Embryo
	μg/g f.w.	
C_{19}-GAs		
GA_1	51	4.1
GA_4	12.8	1.6
GA_5	29.8	3.8
GA_6	+[a]	+
GA_{60}		+
C_{20}-GAs		
GA_{19}		+
GA_{44}	13.2	0.6

[a] Identified but not quantified.

by comparing the GC peak areas with those obtained from GC trace of several standard (GA_1, GA_4, GA_5) run under identical condition.

In Table II we report the distribution and the approximate amounts of GAs identified in suspensor and embryo at the heart stage of embryo development. The total amounts (about 100 $\mu g \cdot g^{-1}$ f.w.) of bioactive GAs (GA_1, GA_4, GA_5, GA_{44}) (Table II) identified in suspensor of stage A are not significant different from those of suspensor of stage B (19). The main difference between suspensor of stage A and B is due a great accumulation of the GA_8 in stage B (19). The suspensor at stage A is metabolically very active and all the GAs are in their bioactive form; only when the suspensor gets older (stage B) are some of the bio-active GAs (GA_1 and/or GA_5) con-

verted to GA_8 which is 2β-hydroxylated and relatively inactive.

Although total GA content between embryos of stage A and B, is very similar, qualitative differences have been observed. These may, however, be due to different purification procedures. Thus, we now find two new GAs in the young embryo: the C_{20} GA_{19} and the C_{19} GA_{60}. GA_{60} is 1,13 hydroxylated, and thus for the first time we note the natural occurrence of a 1-β hydroxy GA in *P. coccineus* seeds. However 1 β-hydroxy GAs have been identified in developing grain of *Triticum aestivum* (wheat) in seeds of *Pyrus malus* (apple) (12) and in immature seeds of *Calonyction aculeatum* (9).

As far as dependence on the suspensor is concerned we do not yet know if these embryo GAs are provided by the suspensor. It is clear that developing cotyledons excised from 16 mm long seeds have acquired the ability to biosynthesize their own GAs (23); whether this ability exists in the embryo from its initial stage of growth (first division of the zygote), or is acquired later, still needs to be clarified as stated by other authors (16). The amount of biologically active GAs per individual structure in the *P. coccineus* embryo-suspensor system change with the stage of development (Table III); from stage A to stage B the embryo f.w. increases of about 30-fold while its total GA concentration shows an 80-fold increase; this result could indicate that the ability to produce GAs progressively increases with embryo growth, and a dependence on the suspensor for large amounts of GA could be excluded. On the other hand, at stage A the embryo is already a structure whose f.w. is 10-fold higher than that of the suspensor, but their GA contents are comparable and therefore an embryo dependence on the suspensor could still be hypothesized. *In vitro* culture study of the embryo suspensor system has shown, that the embryo proper became independent rather early, *e.g.* after it reached a length of 2 mm (seeds of 6 mm or more) (7); our data on endogenous GAs refer to heart-stage embryos longer than 2 mm. Recent results on the effect of the suspensor and GA_3 on *Phaseolus vulgaris* embryo protein synthesis (24), have shown that the suspensor strongly stimulates protein synthesis in the embryo at the early heart stage, and this effect can be mimicked by GA_3.

Considering the above it seems clear that to determine whether GA controls seed growth, much attention has to be paid to the GA content of the suspensor cell, this for those species with a massive suspensor rich in GAs, *e.g. Tropaeolum majus* and in *Cytisus laburnum* (17, 20). However, as recently stated (16), the small size of the suspensor, and of the embryo at the first development stages renders the study of the role of the suspensor rather complex.

Table III. *Fresh Weight and Amounts of Biologically Active Gibberellins (GA_8 has not been considered) per Individual Structure in P. coccineus Embryo Suspensor System at Two Stages—Heart (A) and Cotyledonary (B)— of Development*

	f.w.	GA
		ng
Stage A		
Suspensor	0.21	22
Embryo	1.81	18
Stage B		
Suspensor	0.19	27
Embryo	55.56	1472

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