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Promotion by Gibberellic Acid of Polyamine Biosynthesis in Internodes of Light-Grown Dwarf Peas[1]

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ABSTRACT

When gibberellic acid (GA_3; 5-35 micrograms per milliliter) is sprayed on 9-day-old light-grown dwarf Progress pea (*Pisum sativum*) seedlings, it causes a marked increase in the activity of arginine decarboxylase (ADC; EC 4.1.1.9) in the fourth internodes. The titer of putrescine and spermidine, polyamines produced indirectly as a result of ADC action, also rises markedly, paralleling the effect of GA_3 on internode growth. Ammonium (5-hydroxycarvacryl) trimethyl chloride piperidine carboxylate (AMO-1618; 100-200 micrograms per milliliter) causes changes in the reverse direction for enzyme activity, polyamine content, and growth. GA_3 also reverses the red-light-induced inhibition of ADC activity in etiolated Alaska pea epicotyls; this is additional evidence for gibberellin-light interaction in the control of polyamine biosynthesis. The enzyme ornithine decarboxylase (ODC; EC 4.1.1.17), an alternate source of putrescine arising from arginine, is not increased by GA_3 or by AMO-1618.

The results support the hypothesis that ADC and polyamine content are important regulators of plant growth.

The naturally occurring diamines, triamines, and tetraamines, collectively referred to as PAs[3], have been implicated for more than a decade in processes controlling cellular growth in microbial and animal systems (3, 9). More recently, evidence has begun to accumulate that these substances may also play a similar role in plants (1, 5, 16, 26). PAs arise in animal and microbial systems mainly through the decarboxylation of ornithine, and the activity of the enzyme ODC is known to rise rapidly and sharply following the application of physical or chemical agents that stimulate growth (4, 8). Inasmuch as blockage of the rise in ODC activity by appropriate inhibitors prevents the expected induced growth stimulation, it would appear that PA synthesis is causally linked to the induced growth promotion. In plants, where both ADC and ODC may be operative, some similar evidence has been adduced (12, 21), although the absence of appropriate competitive inhibitors for ADC that do not simultaneously block protein synthesis makes the proof of causal linkage more difficult to establish. In some tissues, the activity of SAMDC, which provides aminopropyl groups for the conversion of the diamine Put to the triamine Spd and the tetraamine Spm, seems also to be linked to growth rate, as evidenced by the consequences of its inhibition by MGBG (28).

Recently we (10) reported that ADC activity in buds and epicotyls of etiolated Alaska pea seedlings is altered in opposite directions following conversion of phytochrome from Pr to Pfr. This behavior parallels the opposite regulatory effects of this pigment on the growth of these different organs (13, 25). Since GA_3 is known to reverse the effects of red light in etiolated peas (17) and to stimulate stem growth, especially in dwarf cultivars (7, 23), we decided to investigate the influence of GA on the control of polyamine biosynthesis. This report deals with the effect of GA on stem growth as related to ADC and ODC activities and endogenous polyamine levels in light-grown dwarf Progress pea seedlings.

MATERIALS AND METHODS

Plant Materials. Seeds of a dwarf variety of Progress pea (*Pisum sativum*; Asgrow, Inc.) were imbibed overnight in tap water and sown in bottom-perforated plastic containers in coarse vermiculite. They were subirrigated twice daily with a solution of 1.2 g/l Hyponex (Hydroponics Chemicals Co., Copley, OH) in a temperature-controlled room at 24°C, with a 16-h photoperiod of about 2,000 ft-c of mixed daylight fluorescent-incandescent light (about 9:1 energy ratio). Nine days after sowing, experimental plants were selected for uniformity and received spray treatment.

Application of GA_3 and AMO-1618. Nine-day-old plants were sprayed with GA_3 (Sigma; 5, 20, or 34.6 μg/ml) or AMO-1618 (Calbiochem; 100 or 200 μg/ml). All solutions were prepared in 0.03 M phosphate buffer (pH 6.4) containing 0.05% pluronic L101 (Wyandotte Chemical Corp.) to aid penetration. Buffer solution with pluronic L101 was used as a control. Two days after spraying, the plants were harvested for enzyme assays and determination of polyamine levels.

Enzyme Extraction. Seven complete excised fourth internodes from light-grown Progress pea seedlings were homogenized in prechilled mortars, with 1 ml 100 mM phosphate buffer (pH 7.0) for ADC, or 100 mM Tris-HCl buffer (pH 7.1) for ODC activity. The homogenates were centrifuged at 27,000*g* for 15 min at 4°C, and the supernatant fractions were used for enzyme assays and protein estimations.

Enzyme Assays. ADC activity was determined by measuring the release of $^{14}CO_2$ from [U-^{14}C]-L-arginine (New England Nuclear; 300 mCi/mmol) as described earlier (10). For ODC assay, a similar method was used with DL-[1-^{14}C]ornithine (New England Nuclear; 54.9 mCi/mmol) as substrate. For the pH optima and cofactor requirement experiments, the components of the reaction mixture were as described in the text. Protein in the crude enzyme was determined according to Lowry *et al.* (20), using BSA as a standard.

Extraction and Measurement of Polyamines. Seven complete excised fourth or fifth internodes were extracted with 1 ml 5% perchloric acid. The homogenates were centrifuged at 27,000*g* for

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[3] Abbreviations: PA, polyamine; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SAMDC, *S*-adenosyl-methionine decarboxylase; Put, putrescine; Spd, spermidine; Spm, spermine; MGBG, methylglyoxal-*bis*-guanylhydrazone; AMO-1618, ammonium (5-hydroxycarvacryl) trimethyl chloride piperidine carboxylate.

FIG. 1. Effect of GA and AMO-1618 on the internode elongation in light-grown Progress pea seedlings. Left, Control; center, GA (5 μg/ml) treatment; right, AMO-6618 (100 μg/ml) treatment. Application of GA and AMO-1618 was by spray, given to 9-day-old seedlings. The picture was taken 10 days after application.

15 min at 4°C. Aliquots of the supernatant fraction (0.2 ml) were added to a mixture of 0.4 ml of dansyl-Cl (Sigma; 5 mg/ml acetone) and 0.2 ml of a saturated solution of Na_2CO_3. After incubation overnight, the dansylated products were extracted with 0.5 ml benzene. Fifty μl benzene extract were then spotted on a silica gel thin layer plate (Whatman; LK6D), and thin layer chromatograms were developed with chloroform:triethylamine (25:2, v/v). The fluorescent spots, located in a dark chamber with ultraviolet light, were scraped off and eluted with ethyl-acetate (Baker), and the dansylated polyamines were measured in an Aminco-Bowman spectrophotofluorimeter with an activating wavelength of 350 nm and a fluorescent wavelength of 495 nm. Standard curves permitted calculation of concentration equivalents.

Replication of Experiments. The data presented are from single experiments which are representative of 2 to 4 experiments or their means, each performed in duplicate or triplicate. For experiments using 7-day-old dark-grown Alaska pea seedlings, procedures of plant-enzyme assay, red-light treatment, and application of GA are the same as those described earlier (10); in this case, however, the spraying of GA was usually performed 30 min before red irradiation.

RESULTS

pH-Optimum and Cofactor Requirement. Optimum activity for ADC in crude extracts from etiolated pea epicotyls was obtained at pH 7.0 when a range of pH 5.8 to 8.0 phosphate buffer was used. The activity was higher in phosphate than in Tris buffer and, in both cases, decreased dramatically with increase in pH. This is in contrast to the enzyme from *Escherichia coli* (6), which showed optimum activity at pH 5.25 and only slight activity at pH 6.0, and from *Lathyrus sativus* seedlings, which had an optimum at pH 8.5. Like ADC, the ODC activity in the crude extracts decreased sharply with increase in pH ranging from 6.5 to 8.0 phosphate buffer and 7.1 to 8.5 Tris-HCl buffer. ODC activity at pH 7.1 with Tris-HCl buffer was higher than it was with phosphate buffer, and, hence, this buffer and pH were used for measuring the comparative activities.

ADC activity was enhanced about 50% by 0.4 mM pyridoxal phosphate added either during homogenization or to the assay mixture. Thus, pyridoxal phosphate appears to be a cofactor for ADC of pea, as it is in other plants (27, 29) and in *E. coli* (6). The enzyme activity is not enhanced either by the reducing agent DTT or by EDTA, suggesting that neither sulfhydryl nor metal groups are required for its activity. ODC showed a similar cofactor requirement and properties, but the optimal concentration of pyridoxal phosphate in reaction mixture was 0.8 mM.

Effect of GA_3 and AMO-1618 on ADC and ODC Activities. It is well known that GA_3 elicits dramatic stem elongation when applied to light-grown dwarf peas (Fig. 1) (7). In general, AMO-1618 works in the opposite direction, but its effects cannot be interpreted in terms of GA alone, inasmuch as it also inhibits the formation of steroids. It is, thus, gratifying that similar opposite effects of GA_3 and AMO-1618 on ADC activity were found in fourth internodes of light-grown Progress pea seedlings. Figure 2 shows that a single spray treatment with 5 μg/ml GA_3 enhanced ADC activity more than 3-fold as compared to the control, while 34.6 μg/ml GA_3, presumably slightly supraoptimal, caused a 250% increment in ADC activity. Conversely, 100 μg/ml AMO-1618 produced a slight inhibition of ADC activity.

It is both surprising and significant that GA_3 treatment significantly decreased ODC activity in fourth internodes (Fig. 3). AMO-1618 also inhibited ODC activity even more strongly than it inhibited ADC activity. Thus, ADC, but not ODC, responds to GA_3 treatment in a direction that makes sense for polyamine levels and growth effects (see below).

Effects of GA_3 and AMO-1618 on Polyamine Levels in Internodes and Their Correlation with Growth. Table I shows that GA induces about 1.5 and 3.0 times as much elongation in fourth and

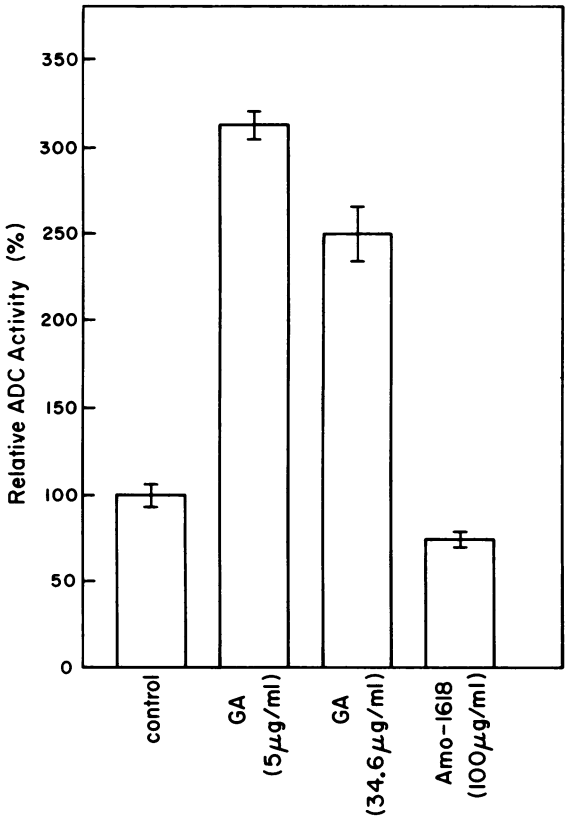

FIG. 2. Effect of GA and AMO-1618 on ADC activity in fourth internodes of light-grown Progress pea seedlings. Measurements from a single representative experiment were made 2 days after GA and AMO-1618 treatments. The SE are in the range of 4.46 to 16.20.

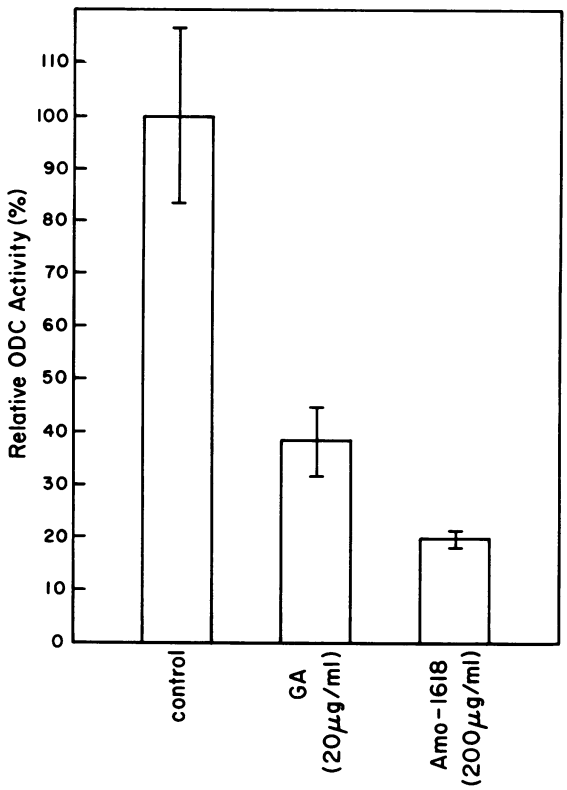

FIG. 3. Effect of GA and AMO-1618 on ODC activity in fourth internodes of light-grown Progress pea seedlings. Measurements from a single representative experiment were made 2 days after GA treatment. The SE are in the range of 1.83 to 16.94.

fifth internodes, respectively, as it does in controls. By contrast, AMO-1618 inhibited elongation by about 10%.

Levels of Put, Spd, and Spm, determined by TLC in fourth and fifth internodes after length measurements had been made, revealed a close correspondence between internode growth and polyamine content. As shown in Table II, Put and Spd increase about 1.5-fold after GA treatment and decline about 15% in fourth internodes after AMO-1618 application. In fifth internodes, GA treatment caused much stronger increase of Put and Spd levels that paralleled the increased internode elongation (Tables I and II). Similarly, AMO-1618 produced parallel decreasing effects on Put and Spd levels and on elongation. Spm seemed not to correlate well with growth; for this polyamine, the differences induced by GA_3 and AMO-1618 are slight, both in fourth and fifth internodes (Table II).

GA Effect on the Red-Light-Induced Inhibition of ADC Activity in Etiolated Alaska Pea Epicotyls. We have shown previously (10) that transformation of phytochrome from Pr to Pfr decreases the growth and ADC activity in epicotyls of etiolated Alaska pea seedlings in a parallel fashion. Table III shows that the inhibition of ADC activity by red light can be completely reversed by GA_3 and that GA_3 has no effect on ADC activity in dark-grown plants. Thus, in both green and etiolated pea seedlings, gibberellin acts opposite to Pfr phytochrome.

DISCUSSION

A clear and marked stem elongation in response to applied GA_3 has made the intact light-grown dwarf pea plant a convenient system for the investigation of the possible relation between GA action and polyamine metabolism. To date, there is scant information on possible correlations between GA and polyamines,

Table I. *Effect of GA and AMO-1618 on the Growth in Length of Internodes of Light-Grown Progress Pea Seedlings*

Measurements were made 2 days after GA and AMO-1618 treatments.

Treatment	Growth in Length			
	Fourth internodes		Fifth internodes	
	mm	%	*mm*	%
Control	9.78 ± 0.24	100	6.68 ± 0.51	100
GA (20 μg/ml)	16.03 ± 0.59	165	21.50 ± 0.73	322
AMO-1618 (200 μg/ml)	9.18 ± 0.42	94	5.62 ± 0.50	84

despite the fact that the similar effects of both classes of compounds are well established (5). Our results show that, of all the PAs, the level of Put is most markedly raised by GA treatment. Inasmuch as this diamine arises from arginine via either ADC or ODC, the activities of these enzymes were studied in reference to the effects of applied GA and AMO-1618. The promotion of ADC activity by GA in intact Progress pea internodes resembles that in excised cucumber cotyledons (29). Unlike in many animal systems (2, 3, 30) and some plant systems (16), ODC activity is not related to growth rate in our pea system. Furthermore, the inhibition of ODC activity by both GA and AMO-1618 indicates the existence of a complex regulatory mechanism for PA biosynthesis in this plant.

In addition, our kinetic study (Y.-R. Dai and A. W. Galston, in preparation) shows that, in the fourth internodes of light-grown Progress pea seedlings, the stimulation of ADC activity by GA_3 appears 6 h after its application, and two peaks of the stimulation have been observed at 9 h and at about 27 h. On the other hand, the promotion of the length growth of 4-h internodes by GA_3 does not occur until 12 h. A distinct promotion appears at 24 h and

Table II. *Effect of GA and AMO-1618 on Polyamine Content of Internodes of Light-Grown Progress Pea Seedlings*

Measurements were made 2 days after GA and AMO-1618 treatments (in triplicate).

Treatment	Putrescine				Spermidine				Spermine			
	Internode 4		Internode 5		Internode 4		Internode 5		Internode 4		Internode 5	
	nmol/internode	%	*nmol/internode*	%	*nmol/internode*	%	*nmol/internode*	%	*nmol/internode*	%	*nmol/internode*	%
Control	13.60 ± 0.50	100	5.39 ± 0.18	100	54.89 ± 3.74	100	21.74 ± 0.32	100	18.94 ± 1.07	100	13.68 ± 1.06	100
GA (20 μg/ml)	21.66 ± 1.50	159	15.33 ± 2.87	284	92.54 ± 2.39	169	39.37 ± 5.19	183	23.15 ± 0.81	122	17.99 ± 1.69	132
AMO-1618 (200 μg/ml)	12.08 ± 0.00	89	4.75 ± 1.31	88	46.46 ± 2.65	85	19.14 ± 0.71	88	17.30 ± 0.61	91	12.97 ± 2.12	95

Table III. *Effect of GA and Red Light on ADC Activity in Etiolated Alaska Pea Seedlings*

Measurements were from a single representative experiment made 6 hrs after red irradiation.

Treatment	ADC Activity	Relative Activity
	cpm × 10^{-3}/*mg protein*	%
Dark	55.49 ± 6.62	100
Red	36.66 ± 5.14	66
Dark + GA (5 μg/ml)	53.73 ± 1.50	97
Red + GA (5 μg/ml)	57.32 ± 1.80	103

continues to increase with time up to 36 h. The results suggest that an increment of ADC activity preexists before the stimulation of length growth, possibly implicating a cause-and-effect relationship between GA-induced promotion in polyamine biosynthesis and growth.

The correspondence of GA effects on ADC activity and stem elongation in the light-grown Progress pea system is comparable to the phytochrome effect on ADC activity and epicotyl elongation in the etiolated Alaska pea system (10). Actually, the dwarfism of light-grown Progress peas can be considered a result of photoinhibition of stem elongation, inasmuch as, in the dark, Progress pea behaves like the normal Alaska pea. Thus, we have to confront an old and unsolved question, *i.e.* the meaning of the reversal by GA of photoinhibition of stem elongation. Lockhart (17), noting the reversal of red-light inhibition of Alaska pea stem growth by applied GA, postulated that light acts through inhibition of gibberellin synthesis (18). However, the data of other authors could not confirm this hypothesis (14, 15, 22). Our results (Fig. 2 and Table III) make it possible that both the reversal of photoinhibition of elongation by GA in the etiolated Alaska pea system and the promotion of elongation by GA in light growth Progress pea system depend on the same mechanism, *i.e.* one mediated via polyamines and their biosynthesis.

It is noteworthy that PA level per unit fresh weight or dry weight is fairly constant, but PA per organ or starting tissue weight changes dramatically. This suggests a regulatory role for PA levels in growing tissue. A similar analysis was made by Suresh *et al.* (29), using paired cotyledons as a basis for comparison. The control by GA of polyamine biosynthesis and the correlation between PA increase and level of induced growth compels further examination of the possible role of polyamines in controlling the growth of plant cells.

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