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Phytohormonal regulation of S-adenosylmethionine synthetase and S-adenosylmethionine levels in dwarf pea epicotyls

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A significant stimulation (2- to 2.5-fold) of AdoMet synthetase was witnessed in glibberellicd acid (GA_3, 1 μM)-treated epicotyls of the dwarf pea (*Pisum sativum*). This was accompanied by a 2.4-fold increase in the endogenous pool of S-adenosylmethionine. Both abscisic acid (10 μM) and cycloheximide (20 μg/ml) inhibited the GA_3-mediated enhancement of AdoMet synthetase activity. Three isozymes of AdoMet synthetase were detected in GA_3-treated epicotyls, whereas a single activity peak was observed in controls. Thus, GA_3 seems to control the induction of two new isozymes of AdoMet synthetase in the dwarf pea. By contrast, the tall pea exhibited three isozymes of AdoMet synthetase even in the absence of GA_3 treatment. High concentration of L-methionine (2 mM) mimicked the GA_3-elicited induction of two new isozymes of AdoMet synthetase in dwarf pea epicotyls.

AdoMet synthetase; Enzyme regulation; Isozyme; S-Adenosylmethionine; Gibberellic acid (GA_3); L-Methionine

1. INTRODUCTION

The ubiquitous occurrence of AdoMet synthetase has been reported in eukaryotes. The enzyme has been shown to catalyze the synthesis of S-adenosylmethionine from the substrates ATP and L-methionine with the release of pyrophosphate and inorganic orthophosphate [1]. AdoMet synthetase has been purified from *Saccharomyces cerevisiae* [2], *Escherichia coli* [3], several animal tissues [4–7] and wheat embryos [8]. The cDNA clones of AdoMet synthetase have been isolated from rat liver [9] and *Arabidopsis thaliana* [10], whereas genomic clones have been isolated from *Escherichia coli* [11] and *Saccharomyces cerevisiae* [12]. Two genes encoding this enzyme (SAM I and SAM II) have been identified in *S. cerevisae* [13] and *Arabidopsis* [10]. The stimulation of AdoMet synthetase by high concentrations of L-methionine (2 mM) has been reported in *S. cerevisae* [13], rat liver [14] and wheat embryos [8]. Cycloheximide blocked the methionine-induced AdoMet synthetase activity in wheat embryos. In yeast cells, a transcriptional control has been envisaged for the induction of AdoMet synthetase by methionine, as it correlated with the increased levels of AdoMet synthetase mRNA [13]. However, a post-transcriptional regulation of AdoMet synthetase was reported in germinated wheat embryos, where the de novo synthesis of this enzyme occurs from its stored mRNA [8]. So far, no attempt has been made to study the hormonal control of AdoMet synthetase both in animal and plant cells. We now report a phytohormonal (GA_3) regulation of AdoMet synthetase and the modulation of S-adenosylmethionine levels in epicotyls of dwarf pea. The induction of two new isozymes of Adomet synthetase seem to be under the control of GA_3. High concentrations of L-methionine mimicked the regulatory response of GA_3 in the induction of two new isozymes of AdoMet synthetase. We propose that the increase of AdoMet synthetase activity which is correlated to the appearance of two peaks of AdoMet synthetase activity by chromatography on DE-52 seems to be due to regulatory action of GA_3 and controls the levels of the endogenous pool of S-adenosylmethionine.

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Abbreviations: ABA, abscisic acid; AdoMet synthetase, S-adenosylmethionine synthetase; CHI, cycloheximide; C_3, gibberellic acid; 5′MTA, 5′methylthioadenosine; mRNA, messenger RNA; PCA, perchloric acid; PEG, polyethylene glycol; PVP, polyvinyl polypyrrolidone; SAM (or AMe), S-adenosylmethionine

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]methyl methionine (spec. act. 85 Ci/mmol) was purchased from Amersham. L-methionine, SAM, 5′MTA and ATP were products of Sigma. [^{14}C]methylmethionine (spec. act. 37.8 mCi/mmol) was procured from BARC, India.

2.2. *Source of enzyme*

Pea seeds (*Pisum sativum*, dwarf var. HF P-4 and tall garden pea) were surface-sterilized with $HgCl_2$ solution (0.02%, 10 min) and washed extensively with sterile distilled water. The seeds were then imbibed in sterile distilled water for 10 h at 25°C. The soaked seeds were germinated in the dark for two days and then transferred to the light

(25°C). The four-day-old seedlings were sprayed with the following phytohormones: GA_3 (1 μM), GA_3 (1 μM) + ABA (10 μM) and etherel (2 μM–2 mM). Cycloheximide (20 μg/ml) was also sprayed along with GA_3 (1 μM). Pea epicotyls (1 cm segment from apex) were excised after 48 h of treatment. Pea epicotyls, excised from four-day-old seedlings, were also incubated (24 h at 25°C) in sterile solution of L-methionine (2 mM), prepard in phosphate buffer (10 mM, pH 7.0) and sucrose (2%). Chloramphenicol (50 μg/ml) was added to the incubation medium as a bactericidal agent. The effect of CHI (20 μg/ml) was tested in L-methionine-treated epicotyls.

2.3. *Preparation of enzyme fraction*

Excised pea epicotyls were homogenized in Tris-HCl buffer (50 mM, pH 7.6) containing $MgCl_2$ (10 mM), Na-EDTA (0.1 mM) and β-mercaptoethanol (5 mM). Sand and PVP (2%) were added during homogenization of the tissue. The homogenate was centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was subjected to ammonium sulphate fractionation (30–60% satn.) and dialysed overnight. The desalted ammonium sulphate fraction was used for the assay of AdoMet synthetase. Protein was estimated according to the procedure of Bradford [15].

2.4. *Assay of AdoMet synthetase*

The assay mixture contained Tris-HCl buffer (100 mM, pH 7.6), $MgCl_2$ (20 mM), KCl (70 mM), freshly reduced glutathione (8 mM), unlabelled L-methionine (1 mM), [^{3}H]methyl methionine (0.5 μCi) and ammonium sulphate fraction precipitate (200–800 μg protein) in a final volume of 125 μl. The reaction mixture was incubated for 10 min at 30°C. An aliquot (40 μl) of the assay mixture was plated on P-81 paper discs (H^+ form) and dried under infra-red light [16]. The unreacted [^{3}H]methionine was removed by washing the filter discs with several aliquots of phosphate buffer (5 mM, pH 7.0). The filter discs were transferred to scintillation vials and soaked in ammonium hydroxide solution (1 ml, 1.5 M). Radioactivity was measured in Triton X-100 toluene scintillation fluid.

2.5. *DE-52 ion-exchange column chromatography*

The desalted ammonium sulphate fraction (30–60% satn., 250 mg protein at a concentration of 5 mg/ml) was allowed to bind to the DE-52 matrix (50 ml), equilibrated with Tris-HCl buffer (20 mM, pH 7.6), $MgCl_2$ (10 mM), Na-EDTA (0.1 mM), β-mercaptoethanol (5 mM) and glycerol (10%). The column was washed with two bed volumes of equilibrating buffer to remove unbound proteins. The bound proteins were eluted with a salt gradient (0–0.4 M ammonium sulphate, 4 bed volumes). Fractions of 10 ml each were collected and concentrated against PEG (M.Wt. 20 000). The desalted enzyme fraction (250 μg protein each) was employed for the assay of AdoMet synthetase activity.

2.6. *Quantification of endogenous pool of S-adenosylmethionine*

Pea epicotyls (10 g) were clipped from control and GA_3 (1 μM)-treated seedlings and incubated (24 h, 25°C) in phosphate buffer (30 ml, 10 mM, pH 7.0) containing sucrose (2%), β-mercaptoethanol (5 mM), [^{14}C]methyl methionine (60 μCi/set) with and without GA_3 (1 μM). The tissue (10 g) was homogenized in chilled PCA (5%). The homogenate was centrifuged at 10 000 × *g* for 15 min. The pellet fraction of control and GA_3-treated tissue was dried in an oven (24 h) for determining the dry weight of the tissue. The supernatant fraction was neutralized with $KHCO_3$ (2 M solution). The neutralized supernatant fraction (200 μl) from control and GA_3-treated samples was plated on P-81 discs, dried and washed extensively with phosphate buffer (5 mM, pH 7.0). Heat-treated supernatant fraction (100°C for 10 min, pH 7.0) served as a blank. The levels of ^{14}C-labelled S-adenosylmethionine (DPM) were expressed per gram dry weight of tissue.

2.7. *Characterization of the reaction product*

The ^{3}H-labelled reaction product of the enzyme assay was purified on Dowex (Na^+) by the procedure given in [17]. The purified product (putative SAM) was chemically characterized by its conversion into 5′MTA by the procedure given in [18]. The in vivo labelled [^{14}C]S-adenosylmethionine was also chemically characterized by its conversion into 5′MTA [18].

3. RESULTS

3.1. *Regulation of AdoMet synthetase by GA_3*

Spray application of GA_3 (1 μM) to dwarf pea seedlings (four-day-old) brought about a 2- to 2.5-fold stimulation of AdoMet synthetase activity over that of the control epicotyls (Fig. 1). The GA_3-induced AdoMet synthetase activity was completely nullified by the simultaneous presence of ABA (10 μM) (Table I). However, spray application of ABA (10 μM) alone had no inhibitory effect on AdoMet synthetase activity. De novo protein synthesis seemed necessary for GA_3 stimulation of the enzyme activity, since CHI (20 μg/ml) completely inhibited the hormone-mediated response (Table I). A characteristic difference in the isozymic pattern of AdoMet synthetase was witnessed in control and GA_3-treated dwarf pea epicotyls. A single activity peak of AdoMet synthetase was observed in control epicotyls. In contrast, the hormone-treated tissue revealed three distinct isozymes, one of which coincided with the activity peak of the control enzyme (Fig. 2). Thus the appearance of two new isozymes seems to be under the direct control of GA_3. With a view to determine the physiological significance of GA_3-induced isozymes in dwarf pea epicotyls, we examined the isozymic pattern of AdoMet synthetase in the tall pea, which has inherently high levels of endogenous GA_3. Interestingly, the epicotyls of the tall

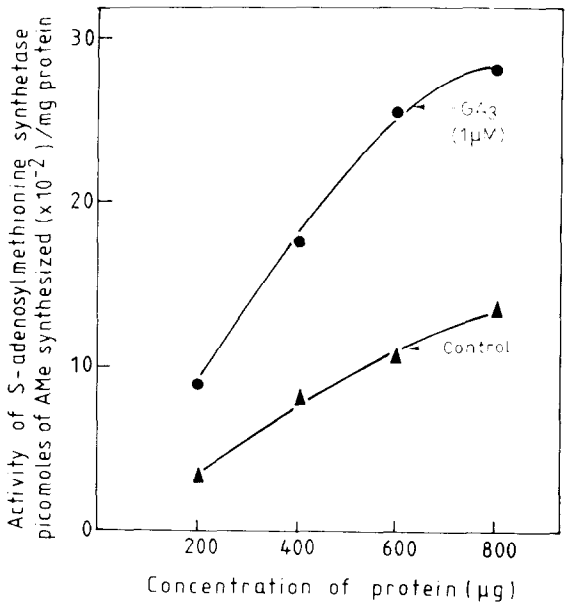

Fig. 1. Stimulation of AdoMet synthetase activity by gibberellic acid (GA_3) in dwarf pea epicotyls. The enzyme activity in control and GA_3-treated epicotyls is expressed as a function of protein concentration in ammonium sulphate fraction precipitate (30–60% satn.).

Table I

Regulation of AdoMet synthetase by gibberellic acid (GA_3) and its counteraction by abscisic acid and cycloheximide in dwarf pea epicotyls

Treatment	AdoMet synthetase activity (pmol of SAM synthesized/mg protein)	Relative activity
Control	1900	1.0
GA_3 (1 μM)	4644	2.44
GA_3 (1 μM) + ABA (10 μM)	1778	0.94
GA_3 (1 μM) + CHI (20 μg/ml)	1485	0.78

Four-day-old pea seedlings were sprayed with GA_3 (1 μM), GA_3 (1 μM) + ABA (10 μM) and GA_3 (1 μM) + CHI (20 μg/ml). The enzyme activity was assayed in dialysed ammonium sulphate fraction precipitate (30–60% satn.), prepared from control and treated epicotyls clipped after 48 h of treatment.

variety exhibited three isozymes of AdoMet synthetase by DE-52 ion-exchange chromatography that are comparable to the isozymes of GA_3-treated dwarf pea epicotyls (data not presented). Thus the induction of two additional isozymes of AdoMet synthetase by GA_3 in dwarf peas could by considered a typical biochemical response observable in nature. Unlike the dwarf pea, there was no stimulation of AdoMet synthetase activity by GA_3 in epicotyls of the tall pea (data not presented).

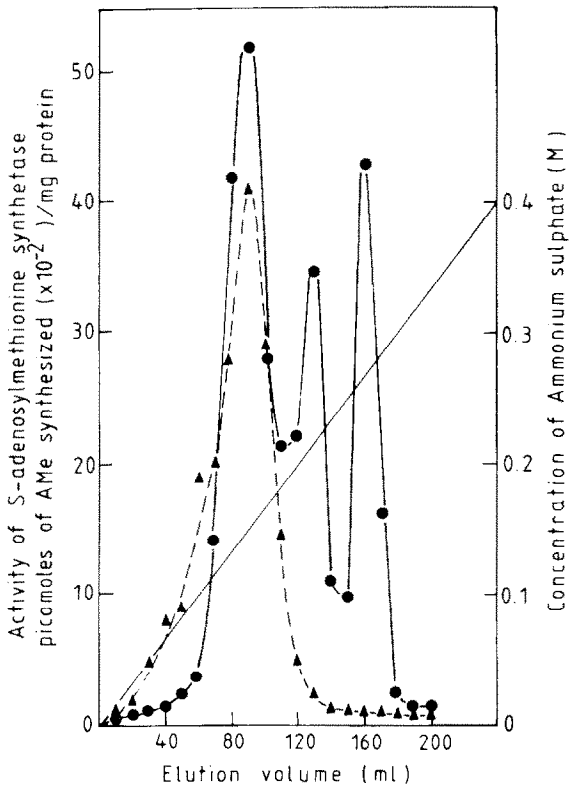

Fig. 2. Induction of isozymes of AdoMet synthetase by gibberellic acid (GA_3) in dwarf pea epicotyls. The DE-52 enzyme fraction showed a single activity peak in controls, while three isozymes were observed in GA_3-treated epicotyls. Control (▲---▲), GA_3 (●—●).

Table II

Stimulatory effect of GA_3 on the endogenous pool of S-adenosylmethionine in dwarf pea epicotyls

Treatment	[^{14}C]S-adenosylmethionine (DPM/g dry wt. of tissue)	Relative activity
Control	3.6×10^5	1.0
GA_3 (1 μM)	8.7×10^5	2.42

Four-day-old pea seedlings were sprayed with GA_3 (1 μM). The control and GA_3-treated excised pea epicotyls (10 g) were incubated in a solution of [^{14}C]methionine (60 μCi) for labelling the endogenous pool of S-adenosylmethionine. The levels of [^{14}C]S-adenosylmethionine were quantified in control and GA_3-treated epicotyls (See text).

Table III

Stimulation of AdoMet synthetase activity by L-methionine and its counteraction by cycloheximide in dwarf pea epicotyls

Treatment	AdoMet synthetase activity (pmol of SAM synthesized/mg protein)	Relative activity
Control	1535	1.0
L-methionine (2 mM)	3141	2.05
L-methionine (2 mM) + CHI (20 μg/ml)	1094	0.7

Pea epicotyls were excised from four-day-old seedlings and were incubated in the presence of L-methionine (2 mM) and L-methionine (2 mM) + CHI (20 μg/ml) for 24 h at 25°C. AdoMet synthetase activity was assayed in dialysed ammonium sulphate fraction precipitate (30–60% satn.), prepared from control and treated epicotyls.

We then designed experiments to ascertain whether the hormonal regulation of AdoMet synthetase affects the endogenous levels of S-adenosylmethionine. This was achieved by measuring the incorporation of [^{14}C]methyl methionine into the labelled [^{14}C]SAM in vivo, both in control and GA_3-treated dwarf pea epicotyls. We did observe a relative abundance of [^{14}C]SAM in hormone-treated epicotyls (2.4-fold) as compared to the controls (Table II). It appears that the hormonal control of isozymes of AdoMet synthetase does influence the levels of SAM. Thus a positive correlation was observed between the rise in the activity of AdoMet synthetase and the high pool of SAM in hormone-treated dwarf pea epicotyls.

3.2. *Regulation of AdoMet synthetase by L-methionine*

About 2-fold stimulation of AdoMet synthetase activity was observed in dwarf pea epicotyls in response to L-methionine (2 mM). The simultaneous presence of CHI (20 μg/ml) nullified the regulatory role of methionine, thereby indicating the requirement of de novo protein synthesis for enzyme induction (Table III). However, ABA (10 μM) failed to inhibit methionine-stimulated activity of AdoMet synthetase (data not presented). It is interesting to observe that the concentration of L-methionine (2 mM) for the optimum stimulation of AdoMet synthetase is roughly 2000-fold more than the concentration of GA_3 (1 μM). The

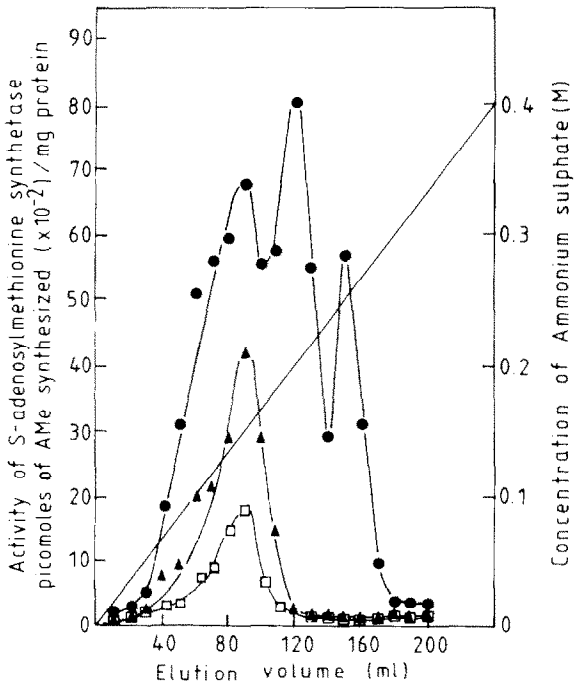

Fig. 3. Induction of isozymes of AdoMet synthetase by L-methionine in dwarf pea epicotyls. The figure depicts the isozymic pattern of AdoMet synthetase in DE-52 fraction prepared from control, L-methionine (2 mM)- and L-methionine (2 mM) + CHI (20 μg/ml)-treated pea epicotyls. Two additional isozymes were witnessed in methionine-treated pea epicotyls that were completely extinguished by CHI treatment. Control (▲—▲), L-methionine (●—●), L-methionine + CHI (□—□).

methionine-treated epicotyls also showed three distinct isozymes of AdoMet synthetase (Fig. 3). Thus the isozymic pattern of AdoMet synthetase in response to methionine is strikingly similar to that observed in GA_3-treated dwarf pea epicotyls. Further, it was observed that cycloheximide treatment strongly repressed the methionine-induced isozymes of AdoMet synthetase (Fig. 3).

Methionine is known to be a precursor of ethylene, a gaseous phytohormone [19]. Thus it could be argued that the administration of relatively high concentration of L-methionine to excised pea epicotyls could result in the build-up of a high pool of ethylene in the epicotyls that could regulate AdoMet synthetase activity. We tested this possibility by treating pea epicotyls with different concentrations of Etherel (2 μM–2 mM). Etherel (2-chloro ethyl phosphonic acid) is a synthetic growth regulator which undergoes spontaneous hydrolysis in plant tissues and in aqueous solution to yield ethylene and phosphoric acid. Etherel, however, failed to stimulate AdoMet synthetase activity, thereby ruling out the regulatory role of endogenous levels of ethylene in methionine-treated pea epicotyls. Nevertheless, Etherel-treated dwarf pea epicotyls showed significant stimulation of peroxidase activity [20].

Both methionine and GA_3-regulated AdoMet synthetase activity was a tissue specific response and was confined only to the apical zone of the pea epicotyls. The basal part of epicotyls and root tissue showed no stimulation of AdoMet synthetase in response to GA_3 and L-methionine.

3.3. *Chemical characterization of the reaction product*

The in vivo labelled [^{14}C]S-adenosylmethionine was chemically characterized. Heat treatment of putative [^{14}C]SAM yielded 97% of [^{14}C]-5′methylthioadenosine. This proved the authenticity of ^{14}C-labelled SAM which is synthesized in vivo by the incorporation of [^{14}C]methionine (Table IV). The in vitro synthesized putative ^{3}H-labelled SAM catalyzed by AdoMet synthetase was also chemically characterized by its conversion into 5′MTA by heat treatment. About 93% of labelled reaction product was converted into 5′MTA (Table IV).

4. DISCUSSION

The present investigation has revealed that AdoMet synthetase activity is regulated by GA_3 (1 μM) in dwarf pea epicotyls. This stimulatory response of GA_3 was nullified by abscisic acid (10 μM). Cycloheximide (20 μg/ml) strongly inhibited the GA_3-stimulated AdoMet synthetase activity, thereby suggesting the requiremen

Table IV

Chemical characterization of putative S-adenosylmethionine synthesized in vivo and in vitro assay of AdoMet synthetase in dwarf pea epicotyls

Treatment	In vivo synthesis of ^{14}C-labelled SAM		In vitro synthesis of ^{3}H-labelled SAM	
	Radioactivity in the zone of		Radioactivity in the zone of	
	SAM (DPM)	5′MTA (DPM)	SAM (DPM)	5′MTA (DPM)
Control reaction product	7235	538	5432	239
Heat-treated reaction product	612	7012	591	5053

The in vivo and in vitro synthesized putative labelled S-adenosylmethionine was purified on Dowex (Na$^+$). An aliquot of the neutralized fraction (pH 7.0) was heated at 100°C for 10 min for its chemical conversion into 5′MTA. The control and heat-treated samples were chromatographed on Whatman 3MM for the separation of labelled SAM and labelled 5′MTA.

of de novo protein synthesis. Whereas a single activity peak of AdoMet synthetase was observed in control epicotyls, the GA_3-treated tissue revealed three isozymes. Thus, the quantitative regulation of AdoMet synthetase by GA_3 (2- to 2.5-fold stimulation) was also associated with the induction of two distinct isozymes at the qualitative level. The true significance of this observation became apparent when we observed the occurrence of three isozymes of AdoMet synthetase in epicotyls of the tall pea, without GA_3-treatment. Conceivably, the induction of AdoMet synthetase isozymes by GA_3 in light-grown dwarf peas could be considered a true biochemical response, in view of the prevalence of a similar pattern of isozymes in light-grown tall peas in nature.

In *E. coli*, mutants of the structural gene for AdoMet synthetase have been constructed by in vitro mutagenesis of a plasmid-borne met_K gene. These conditionally defective mutants, when grown in minimal medium, had a 200-fold less intracellular level of S-adenosylmethionine at non-permissive temperatures. However, the mutants grew normally on a yeast extract-based rich medium even at non-permissive temperatures. The S-adenosylmethionine pool and AdoMet synthetase activity in extracts of mutant and wild-type strains were similar at 30°C and 40°C of growth temperature. These observations revealed that an alternate form of AdoMet synthetase was expressed in nutritionally rich medium. This gene, designated as met_X, encodes another isozyme of AdoMet synthetase in mutant strains grown in rich medium [21]. At present, the precise nature of chemical stimulus that expresses the met_X gene in mutant strains remains elusive.

It is interesting to note that high levels of substrate L-methionine (2 mM) mimicked the stimulatory response of GA_3 (1 μM) in the modulation of AdoMet synthetase. Methionine-treated epicotyls also showed the induction of two additional isozymes of AdoMet synthetase, a situation comparable to that observed in GA_3-treated epicotyls. Earlier, the regulation of AdoMet synthetase by L-methionine (2 mM) has also been reported in yeast [13], rat liver [14] and wheat embryos [8]. In yeast, the methionine-elicited stimulation of AdoMet synthetase activity is ascribed to the increased levels of its mRNA [13]. However, the hormonal regulation of AdoMet synthetase has so far not been reported in animal cells or even in other plant systems.

The phytohormonal regulation of AdoMet synthetase isozymes in dwarf pea epicotyls could have a physiological significance, since it is accompanied by a parallel rise (2.4-fold) in the levels of the endogenous pool of S-adenosylmethionine in vivo. Further studies will be necessary to show the ubiquitous role of GA_3 in the regulation of AdoMet synthetase and the rise in the levels of SAM in other GA_3-responsive tissues.

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