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Effect of methyl ester of jasmonic acid and benzylaminopurine on growth and protein profile of excised cotyledons of *Cucurbita pepo* (zucchini)

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Abstract

Treatment of excised marrow (*Cucurbita pepo* L., zucchini) cotyledons with methyl ester of jasmonic acid (MeJA) had no effect on their growth in darkness. On the other hand, MeJA induced the synthesis of three polypeptides (69, 60 and 43 kDa) and stimulated the accumulation of other polypeptides (97.4 and 53 kDa). These changes in the polypeptide profile were accompanied by a suppression of total protein and RNA synthesis as well as the activity of nuclear RNA polymerases. In contrast to MeJA, N^6-benzylaminopurine (BAP) significantly enhanced cotyledon growth and stimulated protein and RNA synthesis. Furthermore, BAP, when applied together with MeJA, was able to counteract some effects of MeJA including the appearance of specific MeJA-induced polypeptide bands.

Additional key words: endogenous nuclear RNA polymerases, polyacrylamide gel electrophoresis, protein and RNA synthesis.

Introduction

Jasmonic acid (JA) and its methyl ester (MeJA) are widespread in the plant kingdom and may play a regulatory role in different development-related processes. When applied exogenously they can affect germination, production of viable pollen, fruit ripening, tuber formation and senescence (Koda 1992, Sembdner and Parthier 1993, Creelman and Mullet 1997). JA and MeJA are regarded as signalling substances, responsible for the activation of signal transduction pathways in response to different kinds of biotic and abiotic stresses (for a review see Wasternack and Parthier 1997).

It has been shown that jasmonates and cytokinins can counteract during leaf

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Abbreviations: AAM - amino acid mixture; BAP - benzylaminopurine; MeJA - methyl ester of jasmonic acid; PAGE - polyacrylamide gel electrophoresis; PMSF - phenylmethylsulphonylfluoride, SDS - sodiumdodecylsulphate, TCA - trichloroacetic acid.

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senescence (Weidhase *et al.* 1987a) or in the growth responses of different plants (Ueda *et al.* 1981, Ueda and Kato 1982). Besides, exogenous JA was able to alter the endogenous cytokinins in potato stem node cultures in favour of metabolically active cytokinin ribosides (Dermastia *et al.* 1994). On the other hand, cytokinins were found to be indispensable for the control of endogenous content of JA and salicylic acid (SA) in relation to their signalling role as inducers of pathogenesis-related (PR) proteins (Sano *et al.* 1996).

Excised *Cucurbitaceae* cotyledons have been characterized as an useful tool for studying the effect of exogenous cytokinins (Longo *et al.* 1979, Kulaeva 1981, Letham and Palni 1983). In the present work we used this model system in order to study the specific effects of exogenously applied MeJA and its interaction with benzylaminopurine (BAP).

Materials and methods

Plant material and cotyledon treatment: Seeds of *Cucurbita pepo* L. (zucchini) cv. Cocozelle, were soaked for 4 h in tap water and germinated on a moistened filter paper for 96 h in darkness at 28 °C. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water and kept in darkness for further 24 h in order to decrease endogenous cytokinin and abscisic acid (ABA) contents. Then the cotyledons were incubated on distilled water, or aqueous solutions of MeJA (1, 10 or 100 μM), BAP (45 μM) or their mixture (45 μM MeJA + 45 μM BAP) in darkness for 1 - 4 d. All experiments were carried out in darkness in order to eliminate the stimulatory effect of light on the growth and development of the excised cotyledons which can interfere with the cytokinin action.

Protein extraction and SDS-PAGE: Frozen cotyledons were ground with mortar and pestle in extraction buffer containing 50 mM HEPES-NaOH, pH 8.0, 330 mM sorbitol, 2 mM KNO_3, 2 mM EDTA, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, 20 mM NaCl, 2 mM phenylmethylsulphonylfluoride (PMSF). All steps of protein extraction procedure were carried out at 4 °C. The homogenate was then centrifuged at 10 000 *g* for 30 min. Aliquots of the supernatant were precipitated with ice-cold acetone and SDS-PAGE was performed according to Laemmli (1970). Polypeptides were stained with Coomassie Brilliant Blue *R250*; 40 μg proteins were charged per slot. Total protein content was determined according to Lowry *et al.* (1951).

***In vivo* labelling of proteins and RNA:** The incorporation of [^{14}C]-amino acid mixture (AAM) (*Amersham*, Buckinghamshire, England) in newly synthesised proteins was determined using the filter disc method according to Mans and Novelli (1961) with modifications. Cotyledons from different variants with equal fresh mass were incubated with 185 kBq cm^{-3} [^{14}C]-AAM for 4 h. Cotyledons were homogenized in the extraction buffer and the homogenate was centrifuged for 30 min at 10 000 *g*. Aliquots of the supernatants (0.1 cm^3) were pipetted onto *Whatman No. 1* filter paper discs and precipitated with 5 and 10 % TCA. Hydrolysis of aminoacyl-tRNAs was

performed in 5 % TCA for 20 min at 90 °C and the discs were subsequently washed with cold ethanol, ethanol:ether (3:1) and ether. Radioactivity was counted in a liquid scintillation counter (*Beckman LS 1801*, Irvine, USA).

In vivo RNA synthesis was determined using the procedure for estimation of RNA content according to Klyachko *et al.* (1979) with modifications. Cotyledons from different variants were labelled with 370 kBq cm^{-3} [^{3}H]-uridine (ÚVVVR, Prague, Czech Republic) for 4 h and homogenized in the extraction buffer. The homogenate was centrifuged for 30 min at 10 000 *g* and aliquots of the supernatants containing total cell RNA were pipetted onto *Whatman No. 1* filter paper discs and precipitated with 10 % and 5 % TCA. The discs were subsequently washed with cold ethanol, ethanol:ether (3:1) and ether. Radioactivity was counted in the same liquid scintillation counter.

Isolation of nuclei: Nuclei were isolated according to the procedure described by Ananiev and Karagyozov (1984). Cotyledons were grounded in a mortar with precooled buffer A containing 20 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 20 mM KCl, 0.6 M sucrose, 30 % glycerol, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. After centrifugation at 600 *g* for 10 min at 4 °C, the nuclear pellet was suspended in buffer A containing 0.2 % Triton X-100. The nuclei were pelleted through a cushion of buffer A with 1.37 M sucrose (4 500 *g*, 15 min). The final nuclear pellet was suspended in buffer B containing 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 60 % glycerol, and 20 mM 2-mercaptoethanol. DNA contents of nuclear suspensions were determined according to the method of Burton (1956).

Endogenous nuclear RNA-polymerase activity: was determined as described by Ananiev *et al.* (1987). Nuclei were incubated in a reaction mixture (0.075 cm^3) containing: 40 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 50 mM $(NH_4)_2SO_4$; 10 mM 2-mercaptoethanol; 0.4 mM each of ATP, GTP, CTP; 7 μM [^{3}H]UTP (185 GBq $mmol^{-1}$), nuclei (10 μg of nuclear DNA) and 20 % glycerol. After an incubation period of 10 min at 25 °C, the whole reaction mixture was pipetted on Whatman No. 1 filter paper discs presoaked in 0.2 M EDTA, precipitated with 10 % TCA and processed as described by Ananiev and Karagyozov (1984).

Results and discussion

Treatment of excised marrow (*C. pepo*) cotyledons with MeJA in darkness had no significant effect on their fresh mass accumulation during 72 h in the concentration range tested. In contrast to MeJA, BAP or BAP + MeJA strongly stimulated the growth of isolated cotyledons (Fig. 1*A*). Our results confirm the well-known effect of exogenous cytokinins on the fresh mass accumulation in isolated cotyledons (Klyachko *et al.* 1979, Longo *et al.* 1979, Letham and Palni 1983). The cotyledon dry mass gradually decreased after a lag-period of 24 h (Fig. 1*B*). Similar results were reported for other dicots (Bewley and Black 1978). MeJA did not cause any significant changes in the dry mass at the three concentrations used and BAP

markedly decreased dry mass of cotyledons after 72-h treatment. Contradictory data exist in literature indicating that jasmonates can either inhibit the growth of different plant tissues (Ueda and Kato 1982, Ueda *et al.* 1994), or they can promote growth (Montague 1997). As we did not find any inhibitory effect of MeJA on the growth of excised cotyledons in darkness, it can be concluded that MeJA has no significant growth regulating activity in this biological system in the absence of light.

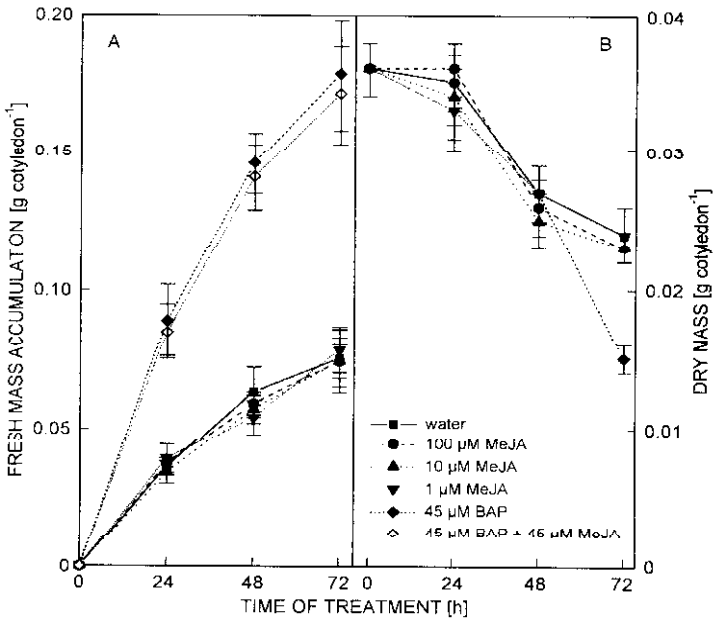

Fig. 1. Effects of MeJA (1, 10 and 100 μM), BAP (45 μM) and BAP + MeJA (45 μM + 45 μM) on the growth (*A*) and the decrease in dry mass (*B*) of isolated cotyledons grown in the dark. Growth is expressed as the accumulation of fresh mass per cotyledon for different periods of time, referred to the initial value at time zero (prior to treatment). *Bars* indicate SE of the means, obtained from four different experiments.

As shown by SDS-PAGE, five abundant polypeptides (97.4, 69, 60, 53 and 43 kDa) were accumulated upon MeJA-treatment (Fig. 2). With exception of the 97.4 kDa polypeptide, the other were absent in controls. The corresponding bands were visible already 24 h after MeJA addition and accumulation of polypeptides increased with duration of treatment (Fig. 2). Furthermore, MeJA caused a gradual decrease of several bands in the region of 20 - 25 kDa (representing cotyledons reserve proteins - globulins) together with a group of lower molecular mass polypeptides (below 20 kDa), especially after 96 h treatment (Fig. 3), when the latter polypeptides almost disappeared. By means of inhibitory analysis with cycloheximide (inhibitor of protein synthesis) and cordycepin (inhibitor of mRNA synthesis), we have recently shown that three of the polypeptides (69, 60 and 43 kDa) are specifically induced upon MeJA-treatment and their synthesis is regulated at the level of transcription (Ananieva and Ananiev 1998), whereas the accumulation of the

97.4 and 53 kDa polypeptides is only strongly stimulated by the presence of MeJA. The molecular mass of 97.4 kDa polypeptide was determined by separation in 8 % SDS-PAGE using *BioRad* high molecular markers (data not shown). Our data on the polypeptide profile of soluble proteins are consistent with the results of other authors showing that MeJA-treatment can trigger a rapid shift in the spectrum of proteins resulting in *de novo* synthesis of new polypeptides in different plant tissues (Weidhase *et al.* 1987b, Sembdner and Parthier 1993). The set of these abundant polypeptides is specific for our experimental system as it differs from the polypeptides reported for other plant tissues (Weidhase *et al.* 1987b, Mueller-Uri *et al.* 1988, Reinbothe *et al.* 1992).

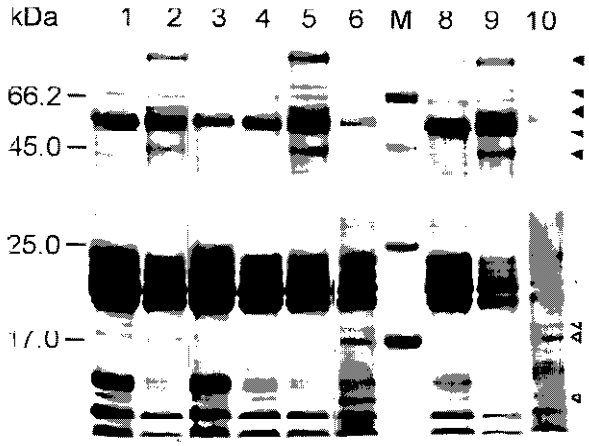

Fig. 2. Polypeptide profiles of total soluble proteins extracted from isolated marrow cotyledons floated on distilled water, 45 μM MeJA and 45 μM BAP in the dark. Polypeptides were separated by 12 % SDS-PAGE. Key to *lane numbers*: 24 h incubation on: water (1), MeJA (2), BAP (3); 48 h incubation on: water (4), MeJA (5), BAP (6); 72 h incubation on: water (8), MeJA (9), BAP (10). *Solid arrowheads* indicate the positions of MeJA-affected 97.4, 69, 60, 53 and 43 kDa polypeptides. *Open arrowheads* indicate BAP-affected polypeptides. M - molecular mass marker proteins.

Incubation of cotyledons with BAP (Fig. 2) decreased the content of the 20 - 25 kDa polypeptides. Similar results were obtained with cultured melon cotyledons where exogenous cytokinins enhanced the mobilization of reserve proteins with the same molecular masses (Leshem *et al.* 1994). In addition, BAP caused a selective repression of 97.4 kDa polypeptide. On the other hand, BAP stimulated the accumulation of two polypeptides - 17 and 18 kDa, as well as a polypeptide below 12 kDa (Fig. 2). Treatment of cotyledons with the mixture of MeJA and BAP prevented the appearance of 60 kDa polypeptide and inhibited strongly the accumulation of 97.4, 53 and 43 kDa polypeptides (Fig. 3). It is obvious that BAP can neutralize almost completely the effect of MeJA on the formation of specific MeJA - induced polypeptides. In contrast to senescing barley leaves where BAP was unable to prevent or restore the formation of specific polypeptides (Weidhase *et al.* 1987b), in the case of excised marrow cotyledons BAP completely eliminated the MeJA-induced polypeptides. These differences could be explained by

the specificity of the excised cotyledons which are reserve organs and not yet developed into primary cotyledonary leaves or they are probably due to the different endogenous levels of cytokinins.

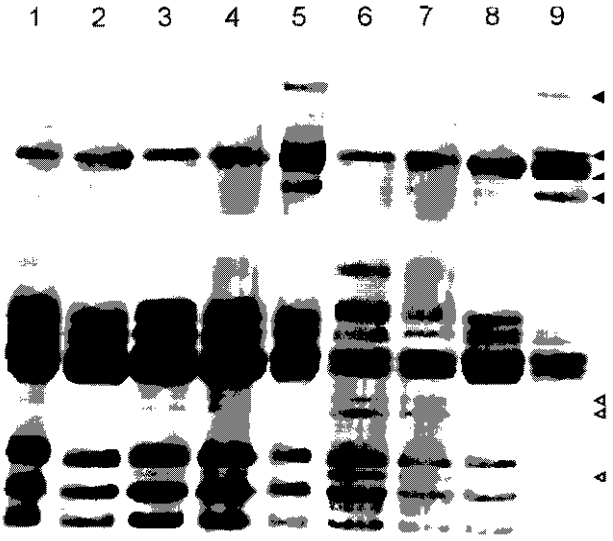

Fig. 3. Polypeptide profiles of total soluble proteins extracted from isolated cotyledons floated on distilled water, MeJA (45 μM), BAP (45 μM) or their combination. Key to *lane numbers*: 24 h incubation on: water (1), MeJA (2), BAP (3), MeJA + BAP (4); 48 h incubation on: MeJA (5), BAP (6), MeJA + BAP (7); 96 incubation on: water (8), MeJA (9). *Solid arrowheads* indicate the positions of MeJA-affected 97.4, 60, 53 and 43 kDa polypeptides. *Open arrowheads* indicate BAP-affected polypeptides.

Treatment of cotyledons with MeJA inhibited the rate of incorporation of [^{14}C]-AAM by 22 % after 24 h. On the contrary, BAP strongly stimulated protein synthesis with a maximum of stimulation measured at the 12-h treatment (60 %), followed by a marked decline (Fig. 4*A*). The combination of MeJA and BAP had no effect on [^{14}C]-incorporation rate. Therefore, BAP was able to counteract the inhibitory effect of MeJA on protein synthesis. On the other hand, no differences between MeJA, BAP and their mixture were observed when the incorporation was expressed as percentage of total uptake (Table 1). These results indicated that the rates of incorporation upon MeJA and BAP-treatments decreased proportionally to the rate of uptake of labelled amino acids by the cotyledons (data not shown).

As total RNA in the plant cell consists mainly of ribosomal RNA, the results on the total RNA synthesis (Fig. 4*B*) reflect the effects of the two growth regulators tested predominantly on the synthesis of rRNA. Treatment of cotyledons with MeJA inhibited the rate of incorporation of [^{3}H]-uridine in a similar manner as the [^{14}C]-AAM incorporation rate. BAP enhanced the rate of RNA synthesis by 50 % already 6 h from the onset of treatment. Similarly to protein synthesis, the maximum of the cytokinin stimulatory effect was registered after 12-h treatment, however, the enhancement was much more pronounced (1.3-fold as compared to the control).

Table 1. Effect of MeJA (45 μM), BAP (45 μM) and MeJA + BAP on the incorporation rates of [^{14}C]-labelled amino acids into total protein and [^{3}H]-uridine in the total RNA of excised marrow cotyledons

Cotyledon treatment	Incorporation [% of uptake]			
	[^{14}C]-AAM		[^{3}H]-uridine	
	12 h	24 h	12 h	24 h
water	81	79	85	82
MeJA	83	76	82	79
BAP	84	80	80	79
MeJA + BAP	83	84	89	85

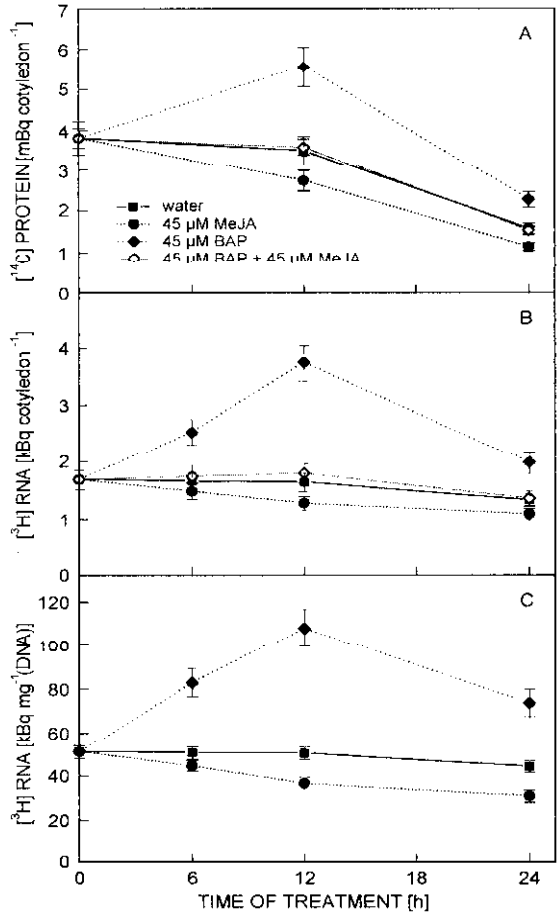

Fig. 4. Effects of MeJA (45 μM), BAP (45 μM) or their combination on: incorporation of [^{14}C]-labelled amino acids in total soluble protein (*A*); [^{3}H]-uridine incorporation into total cell RNA (*B*); endogenous RNA-polymerase activity in nuclei isolated from excised marrow cotyledons (*C*). *Bars* indicate SE of the means obtained from three different experiments.

These results showed that BAP was more effective in stimulation of RNA synthesis than protein synthesis in the isolated cotyledons. Besides, BAP could again counteract the MeJA effect. Again, no differences between MeJA and BAP were observed when the incorporation values were expressed as percentage of total uptake (Table 1). Therefore, it could be suggested that the uptake capacity of the cotyledons as well as the redistribution of radioactive precursors for RNA synthesis between intracellular pools were affected by both plant growth substances in a similar manner as the incorporation rate. In order to eliminate the interference effect of endogenous intracellular pools of precursors of RNA synthesis we investigated further the activity of total endogenous RNA polymerases in nuclei isolated from excised cotyledons after incubation in tested solutions (Fig. 4*C*). The results showed a close similarity of the effects of MeJA and BAP on nuclear RNA polymerase activity with their action on the synthesis of RNA (Fig. 4*B*). Therefore, the inhibition of RNA synthesis by MeJA could be considered as a consequence of decreased activity of the endogenous nuclear RNA polymerases rather than a lowered rate of uptake of the radioactive precursors.

Summing up, our results indicate that MeJA is able to cause both qualitative and quantitative changes in the protein and RNA metabolism in the excised cotyledons of *C. pepo* (zucchini). The counteraction of cytokinins to some of the effects induced by MeJA indicates that they can play a role of antagonists to MeJA in the regulation of cotyledon development.

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