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Phenylmethylsulphonyl fluoride inhibits the formation of jasmonate-induced proteins in cotyledons of *Cucurbita pepo* (zucchini)

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Abstract

Phenylmethylsulphonyl fluoride (PMSF), a well known inhibitor of both thiol- and serine-type proteases, in aqueous solutions either alone or with the plant growth regulators, methyl ester of jasmonic acid (MeJA) and N^6-benzyl-aminopurine (BAP), significantly inhibited the growth of excised *Cucurbita pepo* L. (zucchini) cotyledons. SDS-PAGE analysis of the protein profiles showed that PMSF suppressed the gradual decline of the main 20 - 25 kDa polypeptide group and the low molecular mass polypeptides (below 15 kDa) while leupeptine was not able to affect the electrophoretic pattern of cotyledon proteins. On the other hand, in the presence of PMSF, the content of the polypeptides with higher molecular mass including the 97.4 kDa polypeptide and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (55 kDa) decreased. Besides, when applied together with MeJA, PMSF prevented the appearance of the jasmonate-induced polypeptides (JIPs; 69, 60 and 43 kDa) thus suggesting that JIPs are synthesized from aminoacids released during the breakdown of cotyledon storage proteins.

Additional key words: benzylaminopurine, leupeptine, methyl jasmonate, polypeptide profile, storage protein metabolism.

Introduction

Jasmonic acid (JA), its methyl ester (MeJA) and related compounds, called jasmonates are ubiquitously occurring plant growth regulators (Meyer *et al.* 1984). Upon exogenous application, they exert diverse physiological effects such as inhibition of germination and growth, promotion of leaf senescence, induction of stomata closure, acceleration of fruit ripening and induction of potato tuber formation (for reviews see Koda 1992, Sembdner and Parthier 1993). Jasmonates exert most of their effects through an up-regulation and a down-regulation of the expression of a variety of plant genes, whether applied externally or upon a rise in their endogenous contents in response to abiotic or biotic stresses (Wasternack and Parthier 1997). Proteins encoded by specific jasmonate-induced genes, so-called jasmonate-induced polypeptides (JIPs), include predominantly stress protectants involved in plant defensive reactions (for reviews see Reinbothe *et al.* 1994, Wasternack and Parthier 1997). Due to their high sensitivity to exogenous cytokinins, excised cotyledons from plants of *Cucurbitaceae* family have been explored for studying the regulatory action of these phytohormones (Kulaeva 1982, Letham and Palni 1983, Bewley and Black 1985, Stoynova-Bakalova *et al.* 2001). Some of the jasmonate effects in this specific assay system have been described in our previous works (Ananieva and Ananiev 1998, 1999, 2000).

Mobilization of stored reserves in cotyledons of dicots is essential as the degradative products are utilized as both the substrate and the energy source for the growing seedling. In the seeds of *Cucurbita* sp., the insoluble 11S-type globulin comprising more than 90 % of total protein content, is hydrolyzed to a limited extent in the first 4 d of germination by globulin-specific proteinases to produce acidic (33 kDa) and basic (22 kDa) polypeptides (Hara *et al.* 1976, Hara and Matsubara 1980). These

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Abbreviations: BAP - N^6-benzylaminopurine; LSU - large subunit of Rubisco; MeJA - methyl ester of jasmonic acid; PAGE - polyacrylamide gel electrophoresis; PMSF - phenylmethylsulphonyl fluoride; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS - sodiumdodecylsulphate; SSU - the small subunit of Rubisco.

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authors have also shown that during the later stages of germination these polypeptides are further degraded by means of proteases synthesised *de novo*, to a number of small polypeptides with lower molecular mass.

PMSF and leupeptine are known as inhibitors of both thiol- and serine-type proteases in plants (Mikola *et al.* 1986). It has been proved that PMSF can inhibit the germination of lettuce seeds as well as of seeds of some other plant species (*e.g. Cucurbita moschata, Phaseolus vulgaris*). In addition, PMSF was the only inhibitor from several protease inhibitors tested including leupeptine which had an inhibitory effect on the breakdown of the storage peptides (32 - 39 kDa) (Takeba 1990).

In the present study, we investigated the effects of PMSF and leupeptine on the growth of excised *C. pepo* cotyledons and the changes in the polypeptide spectrum of their total soluble proteins. Special interest deserved the question whether MeJA-induced polypeptides (JIPs) could be accumulated upon MeJA treatment in the presence of the protease inhibitors tested.

Materials and methods

Plants and growth conditions: Seeds of *Cucurbita pepo* L. (zucchini), cv. Cocozelle, were germinated for 96 h in darkness at 28 °C. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water for another 24 h in order to reduce the endogenous cytokinins content. Then the cotyledons were incubated on distilled water (control) or aqueous solutions of 45 μM MeJA and 45 μM BAP in the presence or absence of PMSF (1 mM). In some experiments another protease inhibitor, leupeptine (1 mM), was also applied instead of PMSF. The solutions containing the inhibitors were renewed every 12 h. All experiments were carried out in darkness at 27 °C. Measurements were done at 24, 48 and 72 h. Fresh mass accumulation was expressed as an increase in fresh mass per cotyledon measured at 24, 48 and 72 h, referred to the initial value at time zero (prior to treatment).

Protein extraction and SDS-PAGE: Frozen cotyledons were ground with a mortar and a 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 and 2 mM PMSF. The homogenate was then centrifuged at 10 000 *g* for 30 min. An aliquot of the supernatant was mixed with an equal volume of sample buffer containing 80 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 2 % β-mercapto-ethanol and 5 mM EDTA. The samples were heated for 5 min in boiling water. Proteins were separated by SDS-PAGE on 12 % gels according to Laemmli (1970). Each lane was loaded with 40 μg of protein and the gels were run at 150 V. Polypeptides were stained with Coomassie Brilliant Blue R-250. Protein content was determined according to Lowry *et al.* (1951).

Protein labelling and autoradiography: Cotyledon proteins were labelled *in vivo* with 280 kBq cm^{-3} [^{14}C]-amino acid mixture (*Amersham*, Buckinghamshire, England) in the presence or absence of the tested compounds for the last 4 h of the respective incubation period. Total soluble proteins were extracted, separated by SDS-PAGE and transferred electrophoretically from the polyacrylamide gel onto a nitrocellulose membrane. The transfer buffer contained 25 mM Tris-HCl (pH 8.3), 190 mM glycine and 20 % methanol (v/v). The electrotransfer was performed in ice-cold bath at 90 V for 120 min or at 28 V overnight. The autoradiograms were developed after 4 - 6 d of exposure at room temperature.

Results and discussion

Treatment of excised marrow cotyledons grown for 72 h in darkness with 45 μM MeJA had no significant effect on their fresh mass accumulation over the whole period of treatment (Fig. 1) which confirmed the lack of effect of MeJA on the growth of cotyledons in darkness shown previously (Ananieva and Ananiev 1999). On the other hand, the application of BAP stimulated strongly the growth of cotyledons. This result is in accordance with the well known effect of exogenous cytokinins to increase the fresh mass and the size of excised cotyledons of different species (Letham 1971, Bewley and Black 1985). PMSF either alone or with both plant growth regulators, decreased the growth of cotyledons by approximately 40 - 45 % as compared to controls during 72 h treatment. On the other hand, PMSF maintained the relative dry mass of excised cotyledons at a higher level in all treatments as compared to the respective controls (data not shown).

The polypeptide profile upon MeJA treatment was characterized by the accumulation of several abundant polypeptides with relative molecular masses of 97.4, 69, 60, 53 and 43 kDa (Fig. 2*A*) as previously shown (Ananieva and Ananiev 1999). By means of inhibitory analysis with cycloxehimide it was earlier proved that the 69, 60 and 43 kDa polypeptides were MeJA-inducible (jasmonate-induced polypeptides, JIPs), whereas the

accumulation of the 97.4 kDa and 53 kDa polypeptides was strongly stimulated by MeJA (Ananieva and Ananiev 1998). BAP caused a gradual decrease in total soluble protein content and especially in the middle (20 - 25 kDa) and low molecular mass (below 15 kDa) polypeptides (Fig. 2*A*). MeJA also caused a gradual decrease in the content of the 20 - 25 kDa polypeptides but to a lesser extent (Fig. 2*A*). The radioactivity in the cotyledons treated for 24 h with MeJA was incorporated mainly into the 43 kDa polypeptide while after 48 h the incorporation of the label was observed extremely in the jasmonate-affected polypeptides (97.4, 69, 60, 53 and 43 kDa), thus pointing to the *de novo* synthesis of this set of polypeptides (Fig. 3*A*,*B*). Besides, no radioactivity was observed in the LSU of Rubisco both in the control and MeJA-treated cotyledons (Fig. 3*B*) although it still remained one of the dominating polypeptides on the SDS gels (Fig. 2*A*). By contrast, cotyledons treated with BAP for 24 h incorporated radioactive aminoacids almost exclusively into the LSU of Rubisco (Fig. 3*A*) whereas after 48 h the incorporation was rather reduced (Fig. 3*B*). No radioactivity was observed in the 20 - 25 kDa and the low molecular mass polypeptides indicating that these polypeptides are not newly synthesized.

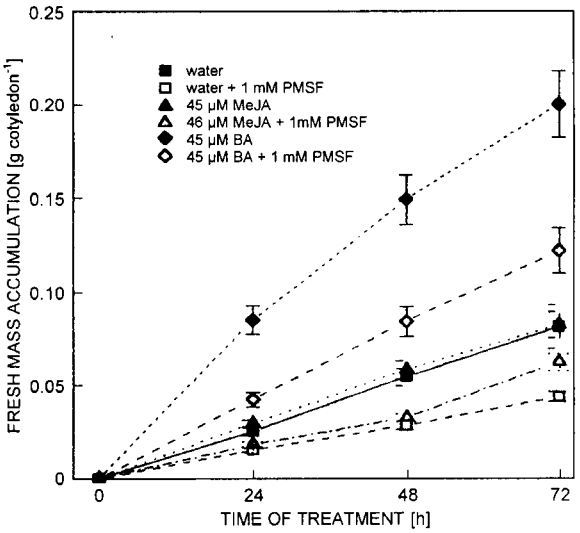

Fig. 1. Effects of MeJA and BAP applied at a concentration of 45 μM with or without PMSF (1 mM) on the growth of excised *C. pepo* cotyledons in darkness. Growth is expressed as the accumulation of fresh mass per cotyledon for different periods of time, referred to the initial value at time zero (fresh mass per cotyledon prior to treatment). Values represent the mean of three different experiments ± SE, each performed with 10 cotyledons.

Therefore, our results showed that in the excised *C. pepo* cotyledons, exogenous MeJA triggered specific quantitative and qualitative changes in the spectrum of total soluble proteins. It is well known that JIPs accumulate in a variety of species (Sembdner and Parthier 1993) and can be involved in defence functions of plant cells against different biotic and abiotic stresses (Reinbothe *et al.* 1994, Wasternack and Parthier 1997).

Our results showed also lack of radioactivity incorporation in the LSU of Rubisco in the MeJA-treated cotyledons, indicating that the synthesis of this nuclear-

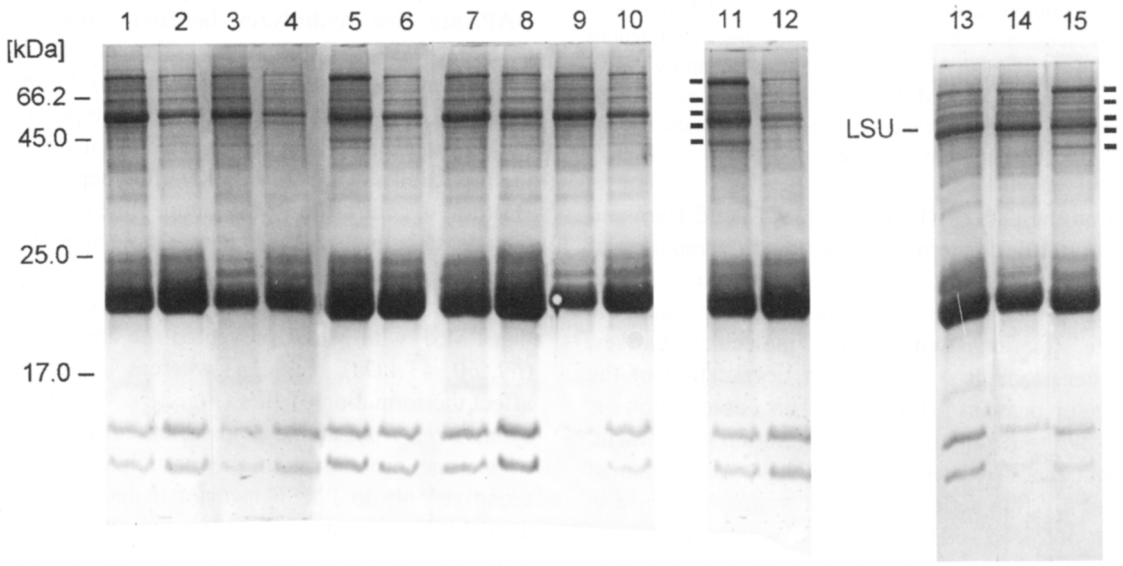

Fig. 2. Polypeptide profiles of total soluble proteins extracted from excised *C. pepo* cotyledons floated on water, MeJA (45 μM) or BAP (45 μM) with or without PMSF (1 mM) or leupeptine (1 mM) instead of PMSF in darkness. Polypeptides were electrophoretically separated by 12 % SDS-PAGE. *A* - 48 h incubation on: water (*lane 1*), water and 1 mM PMSF (*2*), BAP (*3*), BAP and 1 mM PMSF (*4*), MeJA (*5*) and MeJA and 1 mM PMSF (*6*), 72 h incubation on: water (*7*), water and 1 mM PMSF (*8*), BAP (*9*), BAP and 1 mM PMSF (*10*), MeJA (*11*) and MeJA and 1 mM PMSF (*12*). *B* - 48 h incubation on: water and 1 mM leupeptine (*13*), BAP and 1 mM leupeptine (*14*), MeJA and 1 mM leupeptine (*15*). The positions of the MeJA-affected 97.4, 69, 60 53 and 43 kDa polypeptides are indicated by lines. LSU - the large subunit of Rubisco (55 kDa). Molecular mass markers are shown on the left.

encoded protein has already ceased at the 24 h of treatment (Fig. 3). The jasmonate effect to turn off genes involved in photosynthesis (*e.g.*, LSU and SSU of Rubisco) thus leading to characteristic senescence symptoms such as loss of chlorophyll and degradation of Rubisco has been well documented (Reinbothe *et al.* 1993, 1997). On the other hand, it has been proved that cytokinins stimulate the biosynthesis of a number of cytoplasmic and chloroplast enzymes including Rubisco (Letham and Palni 1983, Lerbs *et al.* 1984). Our result indicating incorporation of radioactivity in the LSU of Rubisco (Fig. 3) is in good agreement with these earlier data.

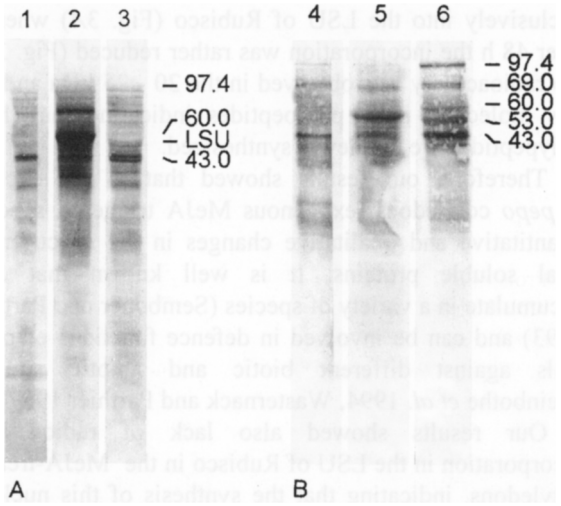

Fig. 3. Autoradiograms of polypeptides *in vivo* labelled with [^{14}C]-amino acid mixture from excised *C. pepo* cotyledons floated for 24 h (*A*) or 48 h (*B*) on water (*lanes 1* and *4*), BAP (45 μM) (*lanes 2* and *5*) or MeJA (45 μM) (*lanes 3* and *6*). The positions of the MeJA-affected polypeptides are indicated by lines. LSU - the large subunit of Rubisco (55 kDa).

Based on the data of Hara *et al.* (1976) and Hara and Matsubara (1980), concerning the gradual degradation of globulins in the seeds of *Cucurbita* sp., it can be assumed that the 20 - 25 kDa polypeptide group and the polypeptide fractions with lower molecular masses, represent the products of proteolytic degradation of the seed storage proteins (Fig. 2). This assumption is supported by the results of the incorporation experiment. It is evident that BAP causes the highest stimulation of protein mobilization reflected by the gradual decrease in the content of 20 - 25 kDa polypeptides with duration of treatment. This result is in accordance with the well known effect of endogenous cytokinins to promote storage proteins mobilization in cotyledons of different dicots through stimulation of the proteolytic activity (Gepstein and Ilan 1979, Muñoz *et al.* 1990) as well as after exogenous cytokinin treatment in monocots (Angelova *et al.* 2002). As was previously shown (Ananieva and Ananiev 1999), MeJA stimulated storage protein degradation as assessed by the gradual decrease in the quantity of the 20 - 25 kDa polypeptides and the polypeptides with lower molecular mass, thus suggesting an effect similar to cytokinins. It has been reported that JA stimulates the activity of basic and acidic lipases as well as of proteases hydrolyzing the proteins in embryos, isolated from apple seeds (Ranjan and Lewak 1992).

PMSF hindered the gradual decline in the quantity of the 20 - 25 kDa and two low molecular mass polypeptides (below 15 kDa) in the control cotyledons as well as after treatment with MeJA and BAP (Fig. 2*A*) which is obviously due to its inhibitory action on peptidases participating in the breakdown of storage proteins. Similar data showing that the incorporation of PMSF in the nutrient media with either IAA or BAP inhibited the disappearance of a polypeptide group in the region 20 - 25 kDa as evidenced by SDS-PAGE and Western blot analyses of total soluble proteins from excised melon cotyledons cultured *in vitro* were obtained by Leshem *et al.* (1994). They also showed that 10^{-5} M BAP in the medium enhanced the disappearance of the 20 - 25 kDa polypeptide group. Our results showed also that the content of the 97.4-kDa polypeptide and the LSU of Rubisco (55 kDa) decreased in the presence of PMSF upon all treatments (Fig. 2), thus suggesting that PMSF can inhibit some types of proteases in the cells, while others remain active. Similar data reported by Takeba (1990) showed that PMSF inhibited the activities of two types of peptidases during imbibition of lettuce seeds: a carboxypeptidase that hydrolyzes haemoglobin and BAPAase that hydrolyzes benzoyl-L-arginine *p*-nitro-anilide (BAPA), but had no effect on the activity of another endopeptidase (an acid protease). Leupeptine can inhibit likewise PMSF thiol- and serine-type of proteases, but in the excised zucchini cotyledons it did not inhibit the breakdown of the 20 - 25 kDa polypeptides (Fig. 2*B*). Besides, in contrast to PMSF, leupeptine did not stimulate the degradation of the higher molecular mass polypeptides.

Of particular interest in the present work is the finding that PMSF with MeJA prevented the appearance of JIPs (69, 60, 43 kDa), (Fig. 2*A*) whereas leupeptine did not affect the formation of JIPs (Fig. 2*B*). Therefore, JIPs are synthesized from amino acids released during hydrolysis of storage proteins with the participation of proteases sensitive only to PMSF and not to leupeptine. Data are available in literature showing that the MeJA-induced senescence of barley leaf segments was accompanied by proteolytic degradation of chloroplast proteins (Weidhase *et al.* 1987, Reinbothe *et al.* 1993). The released amino acids might be utilized for the biosynthesis of cytosolic JIPs (Reinbothe *et al.* 1993). Therefore, the exchange of chloroplast proteins can be considered as a necessary prerequisite for rapid JIP formation. On the other hand,

Roloff *et al.* (1994) demonstrated that the amino acids derived from jasmonate-induced Rubisco degradation were not directly used in the synthesis of JIP-23, the most abundant JIP of barley leaves. In the present work, using the specific assay system of excised cotyledons, we extend and complement these results by presenting evidence for the relationship between the synthesis of JIPs and the breakdown of storage proteins.

In conclusion, our results indicate that MeJA can affect protein metabolism in excised marrow cotyledons in two directions: on the one hand, it is involved in the degradation of storage proteins in a way similar to cytokinins, and on the other hand, it induces the synthesis of specific polypeptides. PMSF, a well known inhibitor of both thiol- and serine-type proteases, causes an inhibition of fresh mass accumulation and prevents the appearance of JIPs, thus suggesting a close relationship between the synthesis of JIPs and the breakdown of storage proteins in the excised cotyledons.

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