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E. Szabo · A. Thelen · M. Petersen

Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*

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Abstract Suspension cultures of *Coleus blumei* (Lamiaceae) treated with either an elicitor preparation from the culture medium of the phytopathogenic oomycete *Pythium aphanidermatum* or with methyl jasmonate enhanced accumulation of rosmarinic acid approximately threefold. The specific activities of phenylalanine ammonia lyase and rosmarinic acid synthase were also enhanced after addition of the fungal elicitor. The addition of methyl jasmonate transiently increased activities of phenylalanine ammonia lyase and hydroxyphenylpyruvate reductase, whereas the activity of rosmarinic acid synthase was not stimulated and the activity of tyrosine aminotransferase was slightly and constantly enhanced. Methyl jasmonate stimulated rosmarinic acid accumulation not only when added directly to the culture medium, but also when it could reach the cells only via the gas phase.

Key words *Coleus blumei* (Lamiaceae) · Fungal elicitor · Methyl jasmonate · Constitutively accumulated defense compound · Rosmarinic acid

Abbreviations *HPPR* Hydroxyphenylpyruvate reductase · *MJ* Methyl jasmonate · *PAL* Phenylalanine ammonia lyase · *RA* Rosmarinic acid · *RAS* Rosmarinic acid synthase · *TAT* Tyrosine aminotransferase

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E. Szabo · A. Thelen · M. Petersen (✉)[1]
Institut für Entwicklungs- und Molekularbiologie der Pflanzen,
Heinrich-Heine-Universität Düsseldorf,
Universitätsstr. 1, D-40225 Düsseldorf, Germany

Present address:
[1] Institut für Pharmazeutische Biologie,
Philipps-Universität Marburg,
Deutschhausstr. 17A, D-35037 Marburg, Germany
Fax: +49-6421-285828
e-mail: petersen@mailer.uni-marburg.de

Introduction

Rosmarinic acid (RA) is one of the most abundant caffeic acid esters, occurring mainly in species of the Lamiaceae and Boraginaceae. This ester of caffeic acid and 3,4-dihydroxyphenyllactic acid is commonly found in all plant organs and is accumulated constitutively. The biological activity of RA is described as antibacterial, antiviral, and antioxidative (Parnham and Kesselring 1985).

The accumulation of RA has been intensively investigated in suspension cultures of *Anchusa officinalis* (Razzaque and Ellis 1977; De-Eknamkul and Ellis 1984, 1985a, b) and *Coleus blumei*. In suspension cultures of *C. blumei*, RA accumulates up to a content of 20% of the cell dry weight when the cells are cultured in medium with a high (4%) sucrose content (Petersen and Alfermann 1988; Gertlowski and Petersen 1993). In the medium used for maintenance of the cell cultures with only 2% sucrose, RA content remains at a basic level of about 2% of cell dry weight.

All eight enzymes involved in RA biosynthesis have been identified and characterized in cell cultures of *C. blumei* (Petersen et al. 1993). The activities of these enzymes are coordinately regulated parallel to the accumulation of RA in the cultures. The enzymes involved in the formation of RA precursors from phenylalanine and tyrosine exhibit maximal activities around day 6 of the culture period in medium with 4% sucrose, whereas the ester-forming enzyme, RA synthase (RAS), has its highest activity at day 8, followed by the maximal accumulation of RA around day 10 of the cultivation period.

Fungal elicitors, mostly derived from the cell walls of either the plant cell or the pathogen, are known to induce the de novo synthesis of antimicrobial compounds, the phytoalexins, which are involved in the defense against phytopathogenic microorganisms. Another group of secondary compounds involved in defense against pathogens accumulate constitutively. Among these, a number of derivatives of the phenylpropanoid pathway are found in plant cells. Because of its antimicrobial activities, RA could serve as such a constitutively accumulated defense com-

pound against pathogens, but also against herbivores because of its tannin-like properties.

Fungal elicitors, the accumulation of phytoalexins, and the accumulation of jasmonic acid or its derivatives have been shown to be interconnected. In cell cultures of different plant species, the transient accumulation of *cis*-jasmonic acid could be demonstrated after addition of fungal elicitors, followed by accumulation of phytoalexins (Müller et al. 1993). Exogenous addition of jasmonates to plant cell cultures was followed by the accumulation of phytoalexins (Gundlach et al. 1992). For these reasons, jasmonates are supposed to play a role in the elicitation process and/or the signal transduction leading to gene activation and finally to defense responses.

The effect of yeast extract as a fungal elicitor or of methyl jasmonate (MJ) on the accumulation of RA has been described for cell cultures of two species of the Lamiaceae and Boraginaceae, namely *Orthosiphon aristatus* (Sumaryono et al. 1991; Sumaryono and Proksch 1993) and *Lithospermum erythrorhizon* (Mizukami et al. 1992, 1993). Yeast extract enhanced RA accumulation up to ten-fold in cell cultures of *O. aristatus* independent of the growth stage. Highest RA levels were reached 72–96 h after addition of yeast extract to the cell cultures. In cell cultures of *L. erythrorhizon*, RA accumulation was stimulated two to three fold by yeast extract, with maximum levels 24 h after addition. The addition of 100 μM methyl jasmonate led to a ten-fold stimulation of RA accumulation in *Lithospermum* cells after 48–72 h.

In this paper, we report on the influence of an elicitor preparation from the culture medium of *Pythium aphanidermatum*, a ubiquitous phytopathogenic oomycete, and of MJ on the accumulation of RA in suspension cultures of *C. blumei* and the activities of selected enzymes involved in RA biosynthesis. MJ was not only directly added to the culture medium, but also via the gas phase as vapor.

Materials and methods

Plant material

Suspension cultures of *C. blumei* were initiated and subcultured in CB2 medium as described by Petersen and Alfermann (1988). For elicitation experiments, 10 ml of 7-day-old suspensions was added to 25 ml of CB2 medium in 100-ml Erlenmeyer flasks. Fungal elicitor preparations or MJ were added aseptically (see below) after a precultivation period of, usually, 3 days.

Elicitor preparation

The oomycete *P. aphanidermatum* (kindly given to us by Prof. Dr. H. U. Seitz, University of Tübingen) was maintained on a medium according to Gamborg et al. (1968) with 3% sucrose and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and solidified by 1% agar-agar. For the preparation of the fungal elicitor, a piece of the mycelium (approx. 1 cm^2) was added to 50 ml liquid medium (as above but without agar-agar) and cultivated on a gyratory shaker (120 rpm) in the dark. The cell-free medium was harvested after a cultivation period of 7 days, concentrated to 20% of the original volume under vacuum, adjusted to pH 6.0 with HCl and autoclaved at 117 °C for 20 min prior to addition to the plant cell suspensions. The sugar content of the elicitor preparations was determined by the phenol sulfuric acid method according to Dubois et al. (1956) and was expressed as glucose equivalents.

Addition of elicitor or MJ

Appropriate volumes of the elicitor preparations from *P. aphanidermatum* were aseptically added to suspension cultures of *C. blumei* routinely at day 3 of the culture period. Control cultures received the same volume of CB2 medium adjusted to pH 6.0. MJ (Serva, Heidelberg, Germany) was prepared as stock solution in ethanol, filter-sterilized and added in suitable amounts to suspension cultures of *C. blumei* routinely at day 3 of the culture period. The final concentration of ethanol in MJ-treated and control cultures was 0.28%.

Application of MJ via the gas phase

A 35-ml volume of cell suspension of *C. blumei* was cultured in the upper compartment of a two-tier flask (total volume 500 ml; modified after Stuart and Street 1971). The lower compartment contained 35 ml of 0.1 M potassium phosphate buffer pH 5.6. The gas phases of both compartments were connected by a "chimney" and had a total volume of 430 ml. The whole system was sealed in a gas-tight manner by aluminum foil and Parafilm. MJ was added to the buffer in the lower compartment at day 3 of the culture period in concentrations of 0–2 mM. In this system, MJ could reach the *C. blumei* cells only via the gas phase.

Determination of culture parameters

Suspension cells were harvested at specific times after addition of the fungal elicitor preparation or MJ by suction filtration. The fresh weight of the cells was recorded and aliquots of 0.5 g were freeze-dried for determination of dry weight and RA content. The medium was used to determine the pH and conductivity by appropriate electrodes, and the refractive index as a measure of sugar content.

Protein extraction

Freshly filtered cells were homogenized three times for 30 s by an Ultraturrax homogenizer (Janke & Kunkel, Freiburg, Germany) together with 20% of the fresh weight Polyclar AT (Serva, Heidelberg, Germany) and 1 ml 0.1 M potassium phosphate buffer pH 7.0 or 7.5 with 1 mM dithiothreitol. The homogenate was centrifuged for 20 min at 48 000 *g* at 4 °C. The supernatant was used to determine enzyme activities. Protein concentrations in the enzyme preparations were determined according to Bradford (1976) with bovine serum albumin (1 mg/ml) as a standard.

Determination of enzyme activities

The activities of enzymes involved in the biosynthesis of RA [phenylalanine ammonia lyase (PAL), tyrosine aminotransferase (TAT), hydroxyphenylpyruvate reductase (HPPR), RAS] were determined as described by Häusler et al. (1991), Petersen (1991), Petersen et al. (1993) and Kempin (1994). Values are expressed as specific activities (μkat/kg protein).

Determination of RA concentration

Freeze-dried cells were weighed for determination of the cell dry weight and then extracted with 2 ml 70% ethanol in an ultrasonic bath at 70 °C for 20 min. Cell residues were sedimented at 3000 *g* for 10 min. The supernatant was diluted (1 : 10) with 50% methanol/50% water (vol/vol) acidified with 0.01% H_3PO_4. RA concentrations were determined by HPLC: Hypersil ODS column (290×46 mm; particle size 5 μm), eluent 50% methanol/50% water acidified with 0.01% H_3PO_4, spectrophotometric detection at 333 nm. Authentic RA was used as a reference substance at a concentration of 25 μM.

Results and discussion

Variation in elicitor and MJ concentrations

Different concentrations of MJ and *Pythium* elicitor were added to suspension cultures of *C. blumei* at day 3 of the cultivation period, which was determined to be the optimal time for elicitation of RA accumulation. MJ concentrations were varied between 0 and 500 μM, the concentrations of the fungal elicitor between 0 and 0.7 mg glucose equivalents per milliliter of suspension. The highest stimulating effects on PAL activity 24 h after addition of MJ and fungal elicitor preparations or RA accumulation 48 h after elicitation were observed at a concentration of 50 μM MJ and 280 μg glucose equivalents of the *Pythium* elicitor per milliliter of culture. Growth measured as fresh weight and dry weight accumulation were slightly reduced by the addition of MJ and the fungal elicitor preparation. This was also reflected by a slower decrease of conductivity and sugar content (measured by refractive index) in the medium of treated cultures.

After elicitation with MJ or *Pythium* elicitor, the color of the medium in which the *C. blumei* cells were cultured changed to blue-gray. The nature of the compound causing this color change could not be identified. However, a bluish color of the culture medium was reported for cell cultures of lavender (Banthorpe et al. 1989). The compound responsible for this color was identified as a decarboxylated RA. This compound was also found in cell cultures of *O. aristatus* after elicitation with yeast extract (Sumaryono et al. 1991; Sumaryono and Proksch 1993).

Possible excretion of RA from the cells into the culture medium, which is often described for compounds formed after elicitation (Eilert et al. 1985; Kurz et al. 1988), could not be measured, because very active peroxidases are present in the culture medium of *C. blumei* suspension cultures which rapidly destroy RA (unpublished results).

Time course of RA accumulation and activities of enzymes involved in its biosynthesis after addition of *Pythium* elicitor or MJ

After addition of 100 μM MJ or *Pythium* elicitor (81 μg glucose equivalents per milliliter culture) to cell cultures of *C. blumei* at day 3 of the culture period, the RA content of the cells as well as the activities of enzymes involved in RA biosynthesis were monitored for 2 days.

Addition of Pythium elicitor preparation

After addition of *Pythium* elicitor, the specific activity of PAL was enhanced from 6 until 44 h after elicitation in comparison to control cultures (Fig. 1). A similar pattern was observed for the activity of RAS (Fig. 1). Highest stimulation of both enzyme activities was found around 20 h after elicitation; PAL activity was enhanced about fourfold

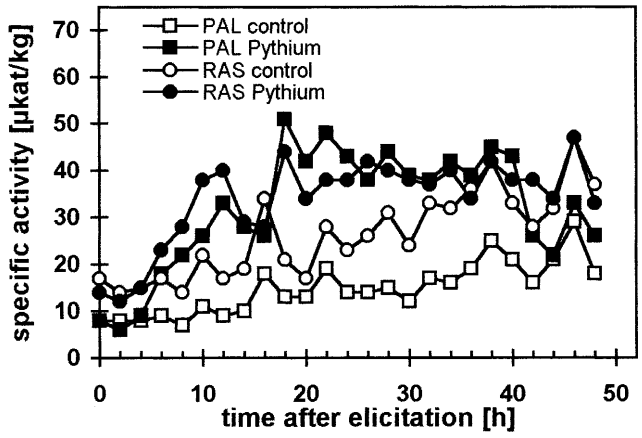

Fig. 1 Time course of specific activities of PAL and RAS in protein extracts from suspension cultures of *Coleus blumei* after addition of an elicitor preparation of *Pythium aphanidermatum*, in comparison to control cultures

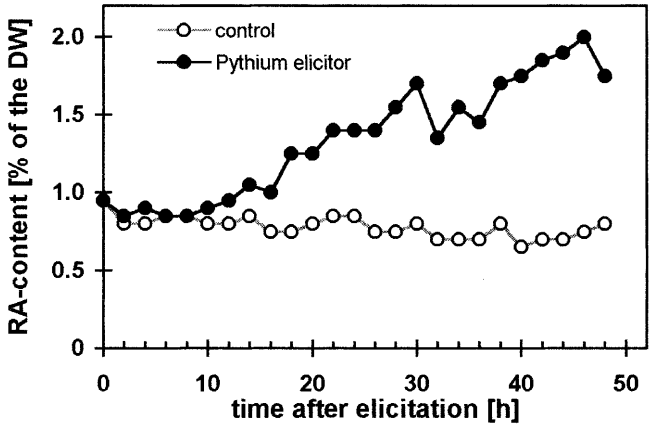

Fig. 2 Time course of RA content in suspension cells of *C. blumei* after addition of an elicitor preparation of *P. aphanidermatum* in comparison to control cultures (*DW* dry weight)

by addition of the *Pythium* preparation, wherease RAS activity was doubled. This increase in PAL activity compared to control levels was much lower than reported for cell cultures of *O. aristatus* after addition of yeast extract (Sumaryono et al. 1991), but comparable to the effects of yeast extract on PAL activity in cell suspensions of *L. erythrorhizon* (Mizukami et al. 1992).

Eight hours after elicitation with the fungal elicitor, the RA content of the *Coleus* cells started to rise, in comparison to the control cultures (Fig. 2). After 46 h the RA content was enhanced from 0.7% of the cell dry weight in control cultures to 2.1% in cells treated with *Pythium* elicitor. Enhanced RA levels could be detected until day 5 after elicitation. Later, the cells entered the death phase and growth as well as RA accumulation ceased. As with the specific activity of PAL, the stimulating effect of the fungal elicitor on RA accumulation in *C. blumei* cells was lower than the effect of yeast extract on cells of *O. aristatus* (Sumaryono et al. 1991), but comparable to the effect of the same elicitor on *L. erythrorhizon* cells (Mizukami et al. 1992).

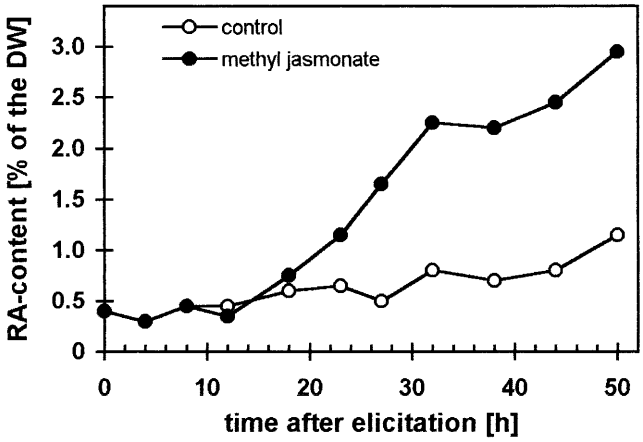

Fig. 3 Time course of RA content in suspension cells of *C. blumei* after addition of 100 µM MJ at day 3 of the culture period, in comparison to control cultures (*DW* dry weight)

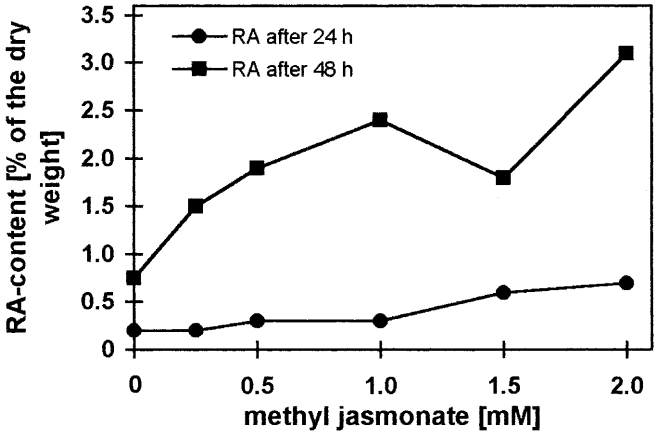

Fig. 5 Enhancement of the RA content in suspension-cultured cells of *C. blumei* after application of MJ via the gas phase (by addition to the lower compartment of two-tier flasks)

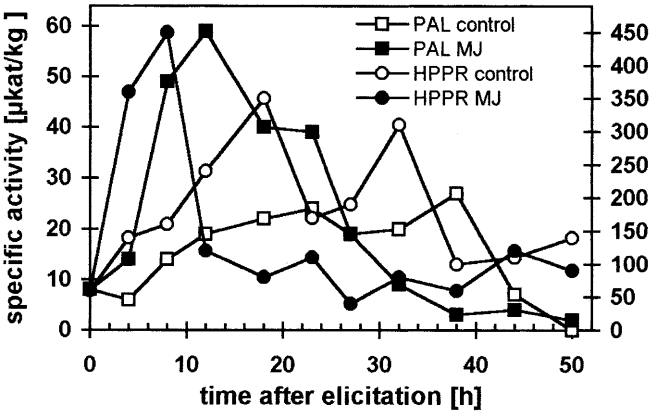

Fig. 4 Time course of specific activities of PAL and HPPR in protein extracts from suspension cultures of *C. blumei* after addition of 100 µM MJ at day 3 of the culture period, in comparison to control cultures

Addition of MJ

After addition of 100 µM MJ to *C. blumei* suspension cultures at day 3 of the culture period, RA accumulation in the suspension cells was enhanced (Fig. 3). Higher RA levels could be observed from 16 h after elicitation, and they stayed higher than the control levels until day 5 after elicitation (when measurements were discontinued). Highest RA contents with 3.3% of the cell dry weight were measured 56 h after elicitation in comparison to the control levels at 1.0%. This shows that RA levels are increased for several days after addition of MJ as also monitored for cell cultures of *L. erythrorhizon* (Mizukami et al. 1993). These authors, however, reported a much higher effect of MJ on the RA concentrations of the *Lithospermum* cells than for fungal elicitors (yeast extract). In suspension cultures of *C. blumei*, the effects of the *Pythium* elicitor and of MJ were comparable. As already stated by Mizukami et al. (1993) for their experiments with *L. erythrorhizon*, the *C. blumei* cultures also responded more slowly to the treatment with MJ than to the addition of a fungal elicitor.

In MJ-treated cultures, the specific activity of PAL was transiently increased about threefold compared to control levels (Fig. 4). Highest PAL activities were observed 12 h after the treatment; afterwards, PAL activity decreased rapidly even below the control levels. The specific activity of RAS was generally not increased by the MJ treatment, in contrast to the observations after addition of *Pythium* elicitor. In contrast, RAS activities were even lower than the activities in control cultures which increased from 140 µkat kg^{-1} at the onset of the elicitation period to 350 µkat kg^{-1} after 46 h and slowly decreased again. In extracts from elicited cells, RAS activities around 170 µkat kg^{-1} were measured during the entire observation period. The activity of TAT was constantly higher at levels of 20–50% over control levels (35–65 µkat kg^{-1}) in MJ-treated cell cultures, but no discrete activity peak could be determined. The activity of HPPR showed a sharp and transient increase with a maximal activity 8 h after elicitation (Fig. 4). As also observed for PAL, the specific activity of HPPR increased about threefold compared to control levels. These observations also parallel the report of Mizukami et al. (1993), who observed only a slight increase in TAT activity and a high and transient increase in HPPR activity. These findings are in concordance with our hypothesis that not TAT, but HPPR activity may be crucial for the enhancement of RA biosynthesis.

Effect of MS applied via the gas phase

MJ is a constituent of the fragrant essential oils of *Jasminum* (Sembdner and Parthier 1993) and is more volatile than jasmonic acid itself. It should therefore be possible to treat suspension cells with MJ via the gas phase. MJ is distributed in the gas phase and has to dissolve in the culture medium again in order to access the suspension cells. This process leads to a continuous application of MJ to the cells over a longer time period. Suspension cultures of *C. blumei* were cultured in CB2 medium in the upper compartment of two-tier flasks and MJ (0–2 mM) was added at day

3 of the culture period to the lower compartment which contained 0.1 M potassium phosphate buffer pH 5.6, corresponding to the pH of the CB2 culture medium. The RA content of the cells determined 24 and 48 h after the treatment was enhanced in a concentration-dependent manner (Fig. 5). Whereas the enhancement of RA accumulation after 24 h was very low and only detectable at higher MJ concentrations, the stimulating effect was clearly visible after 48 h. At the highest MJ concentration applied (2 mM in the buffer), the RA content was more than tripled in comparison to untreated cell cultures.

Conclusion

The accumulation of RA in suspension-cultured cells of *C. blumei* can be stimulated not only by a higher sucrose content in the medium as reported (Zenk et al. 1977; Petersen and Alfermann 1988; Gertlowski und Petersen 1993), but also by treatments simulating a pathogen attack, i.e., the addition of sterile fungal elicitor preparations or of methyl jasmonate, a compound putatively involved in signal transduction reactions leading to defense reactions (Gundlach et al. 1992; Müller et al. 1993). This supports the hypothesis that RA is a defense compound. The constitutive concentration of RA in the plant cells can be further increased by enhanced biosynthesis under simulated pathogen stress. This is not only true for suspension cultures of *C. blumei*. In shoot cultures of *C. blumei*, a slightly increased RA content could be observed after application of MJ via the gas phase (data not shown). Our experiments as well as the findings that phytoalexins can also be detected in low concentrations in uninfected plants (Knogge et al. 1987) show that the borderline between constitutively accumulated and induced defense compounds is not a strict one.

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