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The jasmonate precursor, 12-oxo-phytodienoic acid, induces phytoalexin synthesis in *Petroselinum crispum* cell cultures

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The pentacyclic biosynthetic precursor of jasmonic acid, 12-oxo-phytodienoic acid, was found to induce synthesis of the major flavonoid, apiin, in cell suspension cultures of *Petroselinum crispum*. The accumulation of apiin was preceded by an increase in the relative levels of poly (A)$^+$ RNAs that code for the flavonoid biosynthetic enzymes phenylalanine ammonia lyase, 4-coumarate:CoA ligase and chalcone synthase. Poly (A)$^+$ RNAs reached maximal levels at approximately 4–6 h after the addition of elicitor while flavonoids continued to accumulate in the cultures for at least 6 days. 12-Oxo-phytodienoic acid is the first pentacyclic precursor in the jasmonic acid biosynthetic chain which functions as a signal transducer for phytoalexin induction.

Jasmonate; 12-Oxo-phytodienoic acid; Phytoalexin induction; Phenylalanine ammonia lyase; 4-Coumarate:CoA ligase; Chalcone synthase

1. INTRODUCTION

Phytoalexins are low molecular weight compounds which are induced in higher plants upon potential pathogen or herbivore attack [1]. These compounds serve as antibiotics in the defense systems of plants and can be provoked even in plant suspension cultures by the addition, for instance, of microbial cell wall preparations, so called elicitors [2]. An intracellular signal transduction system between the elicitor–receptor complex [3] and the gene activation process that leads to the formation of phytoalexin biosynthetic enzymes [4] must be present. Recently, we have demonstrated that jasmonic acid and methyl jasmonate (Fig. 1) are involved in the signal cascade that leads to the formation of secondary plant products [5]. Jasmonic acid is potentially an integral component of a general signal transduction system that regulates inducible defense genes in plants as has been previously suggested in an impressive series of experiments involving the induction of protease inhibitor proteins by mechanical wounding of differentiated tomato plants [6]. Without knowledge of its function in plants, jasmonic acid has been compared to the prostaglandins, the chemically similar mammalian hormones [7]. The metabolic cascade involved in the biosynthesis of jasmonic acid [7] begins with α-linolenic acid and proceeds through several established intermediates such as 12-oxo-phytodienoic acid (12-oxo-PDA) (Fig. 1) on to jasmonate. All of these octadecanoic precursors of jasmonate activate the synthesis of wound inducible, high molecular weight proteinase inhibitors [8] and trigger *Bryonia* tendril coiling upon mechanical stimulation [9] The first pentacyclic intermediate in the jasmonate cascade is 12-oxo-PDA. This compound, as the methyl ester (Fig. 1), is the most active inducer of benzophenanthridine alkaloid biosynthesis in *Eschscholtzia californica* cell cultures thus far tested (Z.-Q. Xia, E. Spannagl and M.H. Zenk, unpublished). This compound also provokes the strongest effect in the *Bryonia* tendril coiling response [9], while the free acid is less active than jasmonic acid in proteinase inhibitor I induction in tomatoes [8]. In order to analyze the physiological effect of 12-oxo-PDA on phytoalexin synthesis and on specific gene activation, cell cultures of *Petroselinum crispum* were exposed to this member of the jasmonate signal chain. *P. crispum* was chosen as the experimental plant material, because by far the most physiological, biochemical and molecular genetic data are available for the elicitation response of this species [10].

Here we show that 12-oxo-PDA induces changes in the rate of synthesis of the major flavonoid, apiin, in parsley cell suspension cultures. The increased accumulation of apiin is preceded by relative increases in the poly (A)$^+$ RNAs that encode the flavonoid biosynthetic enzymes, phenylalanine ammonia lyase (PAL), 4-coumarate:CoA ligase (4CL) and chalcone synthase (CHS). Clearly not only jasmonate [5], but also the first pentacyclic octadecanoic acid along the jasmonate biosynthetic pathway can act as a signal transducer in the elicitation process. Whether 12-oxo-PDA occupies a critical position in the signal transduction chain or sim-

Abbreviations: PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; 12-oxo-PDA, 12-oxo-phytodienoic acid

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ply serves as precursor to jasmonate remains to be resolved.

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]dCTP and the hybridization transfer membrane, Hybond-N, were from Amersham-Buchler, Braunschweig, Germany. 12-Oxo-PDA was synthesized using flax seedling enzymes according to [11]. The free acid was converted to the methyl ester by treatment with an etheral diazomethane solution. Apiin was obtained from Roth, Karlsruhe, Germany.

2.2. *Cell cultures*

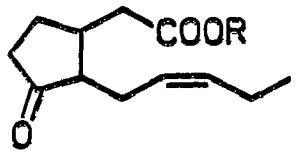
P. crispum callus was induced from seeds on Linsmaier and Skoog [12] medium. Suspension cultures were established from callus using the specific *Petroselinum* medium I [13]. The cultures were grown in 1-liter Erlenmeyer flasks containing 500 ml medium at 23°C and 650 lx incandescent light on a gyratory shaker at 100 rpm. To 200 ml cell suspension containing 64 g fwt of cells was added 2 μmol 12-oxo-PDA methyl ester dissolved in 70 μl ethanol. The suspension was mixed well and was equally distributed into 10 × 20 ml portions in 100-ml Erlenmeyer flasks. For control samples, 70 μl ethanol was added to an additional 200 ml of cell suspension culture. The suspension was mixed and aliquoted as above. Cells were harvested at the indicated time intervals by centrifugation (10 min, 2000 × g, 4°C) and were stored at −80°C until extraction.

2.3. *Flavonoid isolation*

2 g cells (fwt) were suspended in 5 ml 80% ethanol and the mixture heated for 5 min in a boiling water bath. The cell debris was removed by centrifugation (10 min, 2000 × g, 4°C), re-extracted as above and the combined supernatants were reduced *in vacuo* to 3 ml. 40 μl of the clear extract was subjected to HPLC analysis [14]. Apiin was identified and quantitated by comparison to the commercially available standard compound.

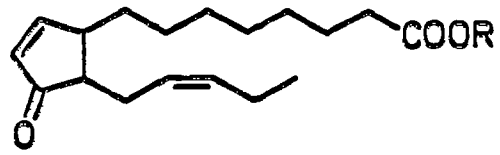
2.4. *Northern blot analysis*

Total RNA was isolated and RNA gels were run and blotted exactly according to [15]. Each individual parsley cDNA clone was labeled by nick translation with [α-^{32}P]dCTP and hybridization and washing were carried out according to [16]. One blot was used for all three clones in each elicitation experiment. The blots were regenerated inbetween each hybridization according to the manufacturer's instructions.



R = H Jasmonic acid

R = CH_3 Methyl jasmonate



R = H 12-Oxo-phytodienoic acid

R = CH_3 12-Oxo-phytodienoic acid methyl ester

Fig. 1. Structure of jasmonic acid and of 12-oxo-phytodienoic acid and the corresponding methyl esters. Stereochemistry not denoted.

3. RESULTS

3.1. *Flavonoid induction*

Illumination of dark grown *P. crispum* cell suspension cultures results in a well documented increase in flavonoid biosynthesis [17]. The major flavone glycoside was found to be apiin (7-*O*-[β-D-apiofuranosyl (1→2) β-D-glucosyl]-apigenin) along with thirteen other flavonoids in amounts sufficient to be isolated and identified [18]. Our newly established suspension culture of *P. crispum*, when grown under standard conditions (for technical reasons under non-saturating, weak incandescent light) were found upon HPLC analysis to produce a major flavonoid together with at least ten other flavonoids. Upon comparison with an authentic sample, this major flavonoid was identified as apiin (Fig. 2). As presented in Fig. 3, this flavonoid showed a constant rate of accumulation during an eight day cultivation period. If, however, 12-oxo-PDA, which is either a signal substance or is metabolized to jasmonate, is added to the culture, an increase in the basal rate of flavonoid synthesis would be expected. 12-Oxo-PDA methyl ester, supplied to parsley suspension cultures to a final concentration of 10 μM, indeed elicited the rate of synthesis of apiin. The increase in the accumulation of apiin was observable within 12 h after the addition of elicitor and continued over the basal rate for an additional 6 days after which the apiin concentration remained constant. The substance, 12-oxo-PDA clearly lead to a rapid and transient stimulation of flavonoid biosynthesis during the first 24 h after addition to the cell culture after which the rate slowly leveled. This latter effect could be explained either by isomerization of the molecule to an inactive structure or by metabolic inactivation by glucosidation or by amino acid conjugation. In the *Petroselinum* system, 12-oxo-PDA, as was found with jamonate in numerous plant cell culture systems [5], induces complex, multistep secondary pathways in much the same manner as a fungal elicitor.

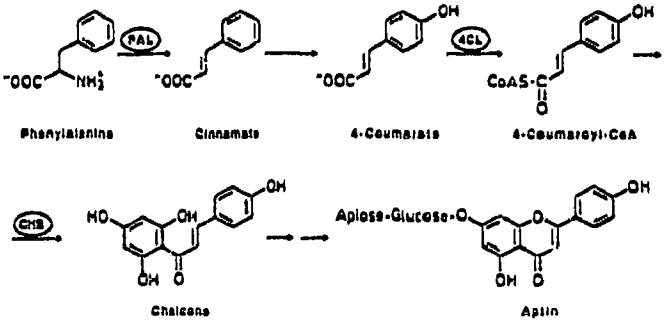


Fig. 2. Partial biosynthetic sequence from phenylalanine to apiin.

3.2. *Poly (A)⁺ RNA induction*

It has been previously shown that several metabolically related enzymes of the flavonoid pathway are induced via transient gene activation in elicitor-treated parsley cells [19,20]. PAL and 4CL belong to the general phenylpropanoid pathway. Both enzymes are induced by either UV light or elicitors [21]. CHS is induced only by UV light with an induction pattern that is slightly delayed when compared to those of PAL and 4CL at the transcriptional level [21]. While the rate of transcription of the genes encoding the enzymes of the general phenylpropanoid pathway increased after stimulation of the cells to a sharp maximum around 4 h, maximal CHS gene transcription was achieved 2 h later.

Since it was demonstrated that the pentacyclic octadecanoic acid, 12-oxo-PDA, induced flavonoid biosynthesis, it was expected that the induction proceeded via de novo transcription. As shown in Fig. 4, the addition of 12-oxo-PDA methyl ester to a parsley cell suspension culture resulted, after a one hour lag, in the transient increase in the rate of transcription of the genes for the first (PAL) and for the last (4CL) enzyme of the general phenylpropanoid pathway. The rates of PAL and 4CL transcription were coordinate, displaying the same kinetics of induction. In both cases, poly (A)⁺ RNA levels peaked at approximately 4 h and slowly decreased thereafter. The transcript encoding CHS, an enzyme specifically of the flavonoid pathway, showed a distinct difference in the kinetics of induction. Although the increase in the rate of transcription was comparable to that of PAL and 4CL, the induction initiated only after a 3 h lag phase. The transcript level then increased for at least 3 h. This observation is in agreement with the kinetics of poly (A)⁺ RNA accumulation in response to UV light as reported previously in much detail [21]. The coordinated induction of transcription of the genes of the flavonoid pathway upon addition of the phytodienoic acid to this cell suspension culture suggests that either 12-oxo-PDA or a metabolic product such as jasmonate are functioning as endogenous signal transmitters in the elicitation process.

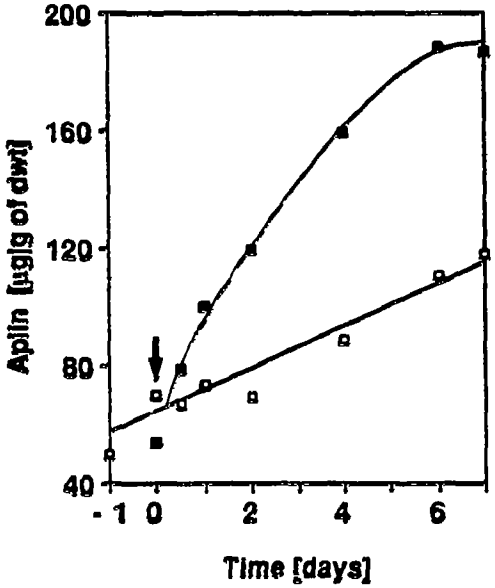


Fig. 3. Induction of accumulation of the flavonoid, apiin, in parsley cell cultures in response to the addition of 12-oxo-PDA methyl ester. ↓, point of 12-oxo-PDA addition; ■, cells treated with 12-oxo-PDA; □, untreated cells.

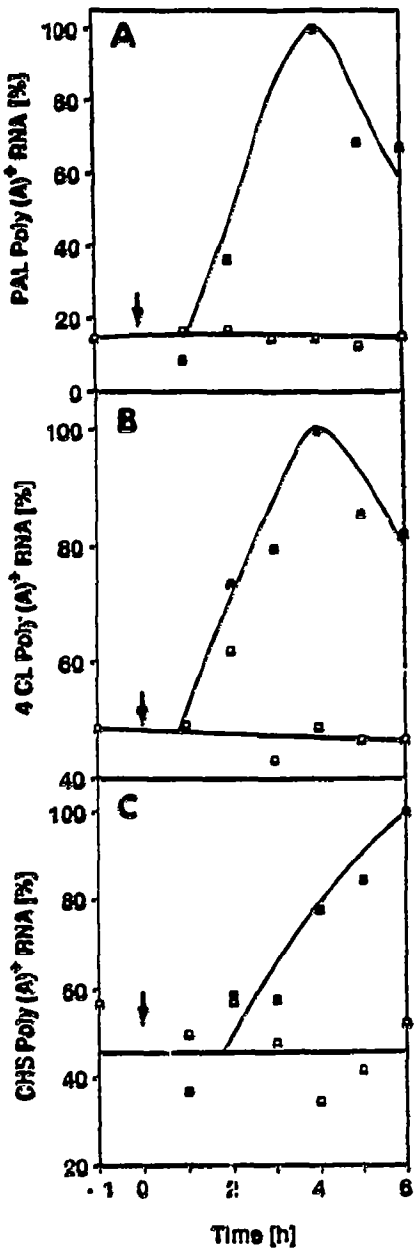


Fig. 4. Induction of poly (A)⁺ RNA in parsley cell cultures in response to the addition of 12-oxo-PDA methyl ester. Changes in the abundance of transcript encoding (A) PAL, (B) 4CL and (C) CHS. All three determinations were done from one cell culture harvested at each time point. ↓, point of 12-oxo-PDA addition; ■, cells treated with 12-oxo-PDA; □, untreated cells.

4. DISCUSSION

We have previously shown that jasmonate, when added to cell suspension cultures of diverse plant species, mimicked the action of elicitors in that it provoked in a qualitatively and quantitatively comparable manner the induction of low molecular weight defense compounds such as flavonoids, alkaloids, terpenoids, etc. [5]. It was, furthermore, demonstrates that concommitant to the induction of flavonoids in *Glycine max* cell cultures, the addition of jasmonate lead to an increase in expression of the gene for the central enzyme of the phenylpropanoid pathway, PAL [5]. This gene activation could be monitored by an increase in PAL poly $(A)^+$ RNA and enzyme activity levels [5]. Here, we have shown that the established [7] jasmonate precursor, 12-oxo-PDA, either in itself or as a result of reduction and β-oxidation to jasmonic acid, induces the well-documented defense response in parsley cell cultures. This defense response induced by 12-oxo-PDA proceeded with transcriptional activation of at least three genes involved in the biosynthesis of apiin and related flavonoids. PAL and 4CL are known to be induced both by UV light and by elicitors in the parsley system in a coordinate pattern [4]. Surprisingly however, the CHS gene, normally activated only by UV light [21], was also transcribed by the addition of this member of the jasmonate cascade. If there is a clear-cut separation in *P. crispum* cell cultures between the solely UV-containing white light-inducible flavonoids and the solely fungal elicitor-inducible furanocoumarins [10], then we have to assume that, as a result of convergent signal transduction, both groups of genes (light- and elicitor-activated) respond to the same intracellular signal compounds of the jasmonate family. Both the light and the elicitor response would thus use the same 'second messenger' (jamonate) which would be produced either by a light perception mechanism or by an elicitor–receptor complex. As shown in this paper, there is now evidence that the members of the pentacyclic jasmonate family transmit the elicitor signal intracellularly. Whether 12-oxo-PDA acts in itself or after further metabolism must be experimentally determined. The concept developed by Farmer and Ryan [8] has been proven correct. Jasmonic acid (and derivatives) is an integral part of the general signal transduction system that regulates the inducible defense genes in plants.

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