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QUANTITATION OF THE OCTADECANOID 12-OXO-PHYTODIENOIC ACID, A SIGNALLING COMPOUND IN PLANT MECHANOTRANSDUCTION*

BOGUSLAWA A. STELMACH, AXEL MÜLLER, PETER HENNIG, DIETMAR LAUDERT, LEO ANDERT and ELMAR W. WEILER†

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität, D-44780 Bochum, Germany

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Key Word Index—*Bryonia dioica*; *Phaseolus vulgaris*; Cucurbitaceae; Fabaceae; signalling molecules; octadecanoids; 12-oxo-phytodienoic acid; coronatine; mechanotransduction.

Abstract—The octadecanoid 12-oxo-phytodienoic acid (OPDA) is an intermediate in biosynthesis of jasmonic acid in plants. A technique for the quantitation of this compound is described which has a limit of detection of 20 pg *cis*-OPDA corresponding to 4 ng g^{-1} tissue for the overall procedure and which uses high isotopic abundance [$^2H_5$]*cis*-(±)-*OPDA*, synthesized enzymatically by recombinant allene oxide synthase, as internal standard. The levels of *cis*-OPDA have been determined in a wide variety of monocotyledonous and dicotyledonous angiosperms and were found to vary considerably among different species. In mechanically stimulated tendrils of *Bryonia dioica*, the level of *cis*-OPDA increases several-fold, correlating with the initiation and progression of the free coiling response. In *Phaseolus vulgaris* internodes undergoing a thigmomorphogenic response, the levels of *cis*-OPDA were also found to increase several-fold well before the development of thigmomorphogenic symptoms. The thigmomorphogenic reaction could also be triggered by application of the octadecanoid structural analog, coronatine. Coronatine did not induce OPDA accumulation in treated tissues and is thus active *per se*. In both species, *Bryonia dioica* and *Phaseolus vulgaris*, the (+)-enantiomer of *cis*-OPDA is found and accumulates after mechanical stimulation. Our results establish 12-oxo-phytodienoic acid as a signalling compound in higher plant mechanotransduction.

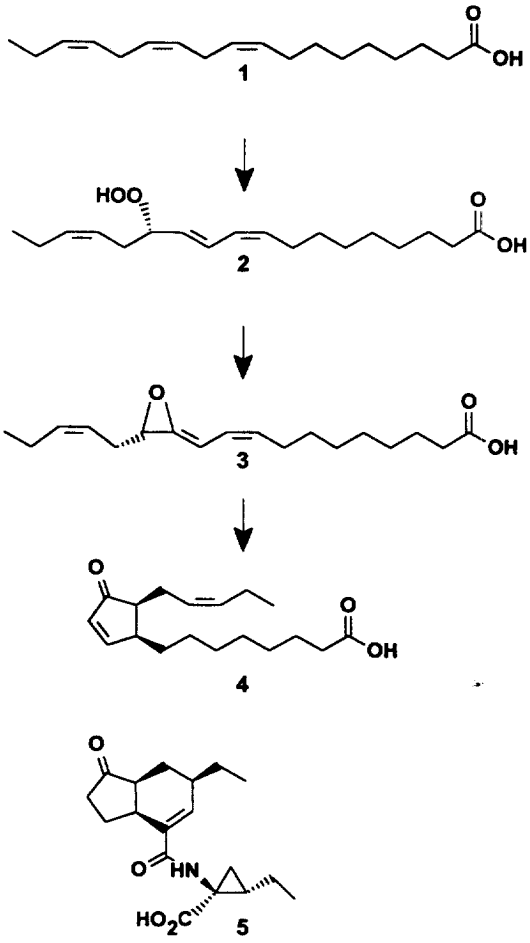
INTRODUCTION

The biosynthesis of jasmonic acid from α-linolenic (**1**) acid involves the cyclic C18-metabolite 12-oxo-phytodienoic acid (OPDA) (Scheme 1) [1]. OPDA is derived from 13-hydroperoxylinolenic acid (**2**) via an unstable allene oxide, 12,13-epoxylinolenic acid (**3**). The first reaction is catalysed by the enzyme allene oxide synthase, while cyclization of 12,13-epoxylinolenic acid is catalysed by allene oxide cyclase [2, 3]. Stereocontrol is exerted at the level of the cyclase, and OPDA formed by this enzyme is the cis-(+)-enantiomer (**4**) (having a 9*S*,13*S*-configuration [4]). Most recent results have shown that OPDA is not simply an intermediate in jasmonate biosynthesis, but that it may be a biologically relevant metabolite in its own right. Blechert *et al.* [5] have proven that β-oxidation is not obligatory for an octadecanoid to be active. In their work, the β-oxa compound methyl trihomojasmonate proved as effective as methyl jasmonate in elicitation of secondary metabolites in tissue cultures. OPDA methyl ester has been found to be considerably more active than methyl jasmonate in inducing *Bryonia dioica* tendril coiling [6, 7]. Moreover, OPDA methyl ester acted much faster than methyl jasmonate, which was very difficult to reconcile with a precursor function of this compound [6]. Proof that OPDA was active *per se* came from analysis of its structural analogue, coronatine (Scheme 1, **5**), which elicits tendril coiling at nanomolar levels without inducing jasmonate accumulation [7].

Analysis of the biological functions of OPDA are hampered by the unavailability of straightforward analytical tools for its quantitation at physiological levels, and only one report deals with OPDA levels in plants undergoing a physiological response [8]. The present study was, therefore, carried out with several goals in mind: (i) to synthesize a heavy isotope-labelled internal standard for OPDA, (ii) to use it to

* Dedicated to Prof. Dr. C. A. Ryan, on the occasion of his 65th birthday.

† Author to whom correspondence should be addressed.



Scheme 1.

determine OPDA levels in a range of taxonomically distant higher plants using GC-MS, (iii) to quantitate OPDA levels in mechanoreacting tendrils of *B. dioica* and (iv) to analyse if OPDA has a more general role in plant mechanotransduction. For this latter purpose, the thigmomorphogenic response of *Phaseolus vulgaris* internodes was used. Collectively, our data prove that OPDA, rather than jasmonic acid, is a signal transducer in higher plant mechanotransduction.

RESULTS

Quantitative determination of cis-*OPDA by capillary gas chromatography-mass spectrometry*

Extraction and pre-purification of OPDA from diverse plant tissues has been achieved based on a protocol reported for jasmonic acid [9]. Since an internal standard for OPDA was unavailable, a protocol for its production based on the readily available 17[$^2H_2$], 18[$^2H_3$]α-linolenic acid was devised. This involves the enzymatic production of 13-hydroperoxy-17[$^2H_2$], 18[$^2H_3$]linolenic acid by a soybean lipoxygenase [10] and further conversion of the hydroperoxide by recombinant *Arabidopsis thaliana* allene oxide synthase [10] overexpressed in *Escherichia coli* [4]. Recovery of the internal standard was tested for *B. dioica* tendril and *P. vulgaris* internode tissue and found to be, on average, 79% (*B. dioica*) or 84% (*P. vulgaris*). The reproducibility of the analysis was determined for repetitive parallel processings of the same batch of tissue and found to be (% coefficients of variation, $n = 9$): 23.9 (*B. dioica*) or 12.9 (*P. vulgaris*) at a level of 200–300 ng [$^2H_5$]OPDA g^{-1} fresh weight, which is in the range of normal tissue levels of this metabolite (see Table 1). A linear detector response using the GC-MS setup described in the experimental section was obtained between 0 and 3 ng of injected OPDA. The analytical limit of detection for *cis*-OPDA was determined for *cis*-OPDA in the extract matrix (standard added at the beginning of extraction) and, against this matrix background, was found to be 20 pg of compound injected into the instrument. This allowed to analyse small aliquots (1/100) of the initial extracts, representing only 5 or 10 mg of tissue and corresponds to an overall limit of detectability for *cis*-OPDA of 4 ng g^{-1} tissue extracted. The technique is thus well suited to quantitate physiological levels of OPDA from even small tissue samples and should consequently be of considerable use in analysing the topology of OPDA accumulation under conditions where both local and systemic responses are to be expected. As an example of the performance of the devised technique, Fig. 1 gives the total ion traces of GC-mass spectrometry scans for a typical plant extract and single-ion monitoring traces of endogenous OPDA as well as of the internal standard. It can be seen that there is isotopic fractionation on the GC column, with the pentadeuterated internal standard eluting slightly earlier than the endogenous compound. It can further be seen that there is only little (approximately 7%) conversion of both, the internal standard and endogenous *cis*-OPDA, to the *trans*-isomer, mainly due to thermal isomerization in the GC injection port. Since both, the internal standard and endogenous OPDA, isomerize to the same extent (cf. Fig. 1), and the HPLC-step used for extract pre-purification separates the *cis*-isomer from the *trans*-isomer with baseline separation, all values reported herein reflect *cis*-OPDA initially present in the tissue at the beginning of the workup procedure. We have not analysed tissue *trans*-OPDA, because it is not biosynthetically formed initially, but subsequently converted via enolization of *cis*-OPDA and thus will represent OPDA which is stored or sequestered in the tissue, but not part of a dynamic pool.

Levels of cis-*OPDA in monocotyledonous and dicotyledonous species*

Using this method, it has been possible to quantitate the levels of *cis*-OPDA in a wide variety of plant species (Table 1). In only a few cases, levels were too

Table 1. Levels of *cis*-OPDA in various species of mono- and dicotyledonous angiosperms

Family	Species	*cis*-OPDA level [ng g^{-1} fr. wt]
Apiaceae	*Anethum graveolens* ssp. *hortorum*	4.09 ± 0.04
	Anthriscus cerefolium ssp. *cerefolium*	5.57 ± 1.20
	Coriandrum sativum	3.42 ± 0.17
Asteraceae	*Lactuca sativa* ssp. *capitata*	7.12 ± 2.82
	Taraxacum officinale	27.4 ± 8.4
Boraginaceae	*Borago officinalis*	< 4*
Brassicaceae	*Arabidopsis thaliana*	1006 ± 21
	Brassica oleracea ssp. *rapifera*	36.7 ± 2.8
	Brassica oleracea ssp. *sabauda*	16.5 ± 0.6
	Brassica oleracea ssp. *viridis*	22.1 ± 2.1
	Lepidium sativum	11.7 ± 1.6
	Raphanus sativus ssp. *sativus*	32.0 ± 2.8
Chenopodiaceae	*Beta vulgaris* ssp. *vulgaris*	7.80 ± 1.32
	Spinacia oleracea	< 4*
Cucurbitaceae	*Bryonia dioica*	42.1 ± 9.0
Fabaceae	*Phaseolus vulgaris* ssp. *vulgaris*	306.8 ± 8.7
	Phaseolus vulgaris ssp. *nanus*	198.9 ± 32.7
Lamiaceae	*Thymus vulgaris*	12.5 ± 1.0
Liliaceae	*Allium cepa*	< 4*
Poaceae	*Avena sativa*	3.19 ± 0.92
	Hordeum vulgare	622.6 ± 85.0
	Triticum aestivum	338.0 ± 65.6
	Zea mays	487.3 ± 37.0
Rosaceae	*Sanguisorba minor*	6.71 ± 0.31
Solanaceae	*Lycopersicon esculentum*	9.29 ± 2.35
	Nicotiana tabacum	< 4*

* limit of detection.

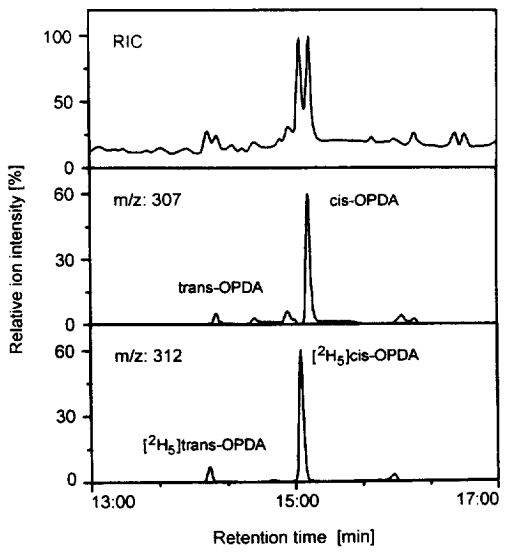


Fig. 1. Analysis of OPDA as its methyl ester in plant extracts. Shown is a typical extract from shoots (first internode plus apex) of *P. vulgaris* ssp. *vulgaris* cv. Neckargold, which was prepared as detailed in the experimental section. The HPLC fraction corresponding to *cis*-OPDA was collected and analysed by GC-MS. Shown are (top) the reconstructed total ion current (RIC) trace as well as the two single ion traces of *m/z* 307 and 312 which are used for quantitation of OPDA and detect $[M+H]^+$ of OPDA methyl ester (*m/z* 307) (middle panel) or $[^2H_5]$OPDA methyl ester (*m/z* 312) (lower panel).

low to be detectable by our technique. It is striking that *cis*-OPDA levels varied considerably among species, from less than 4 ng g^{-1} fr. wt to over 1 $\mu g\ g^{-1}$ fr. wt (*A. thaliana*). Since all experiments were based on $n \geqslant 3$ independent extractions on different occasions and since levels were grouped within limits of method reproducibility in each case, the differences reflect true species characteristics, at least for the developmental state analysed (leaves of juvenile plants, *B. dioica* tendrils).

Levels of cis-*OPDA during the process of tendril coiling in Bryonia dioica*

The involvement of octadecanoids in the process of mechanotransduction in *B. dioica* tendrils is documented [11]. Evidence points to a role of the cyclic C18-precursors of jasmonic acid rather than to jasmonic acid itself [6, 7]. While it has been shown that the level of jasmonic acid in coiled tendrils is by a factor of three higher than that in straight control organs (60 compared to 20 ng g^{-1} fresh weight [6]), no further data on octadecanoid levels during this process became available, due to the difficulties of analysing the trace levels of these lipophilic compounds in tendril tissue. Using the technique described above, it has now been possible to obtain complete kinetic data on *cis*-OPDA levels during the coiling

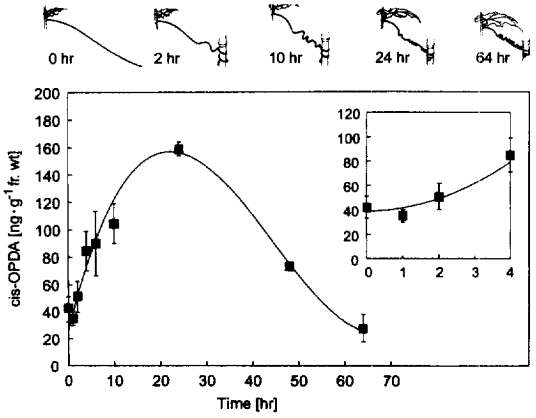


Fig. 2. Kinetic analysis of *cis*-OPDA levels in tendrils of *B. dioica* during the contact-induced coiling reaction (permanent contact stimulus for the time indicated). Shown are mean values ± s.d. from three or more independent experiments with triplicate analysis of each sample (except for the data point at 48 hr which represents one sample analysed in triplicate). The insert shows a close-up of the data from stimulations over a period from 0 to 4 hr (axes as in main graph). The sketches depict tendrils showing the progress of the coiling reaction typical for the times indicated.

reaction (Fig. 2). These were obtained from experiments carried out over the course of a year, using different plants each time and large numbers of tendrils per data point (≥30 specimens per data point and replicate). The organs were brought in contact with the support structure at t_0 and were allowed to react according to their normal behaviour (i.e., were left undisturbed after the establishment of contact until the time of harvest). It can be seen from Fig. 2 that a uniform time course of *cis*-OPDA accumulation with little variation from experiment to experiment is established in the organ. Peak levels are about five times higher than levels of *cis*-OPDA in straight, unstimulated tendrils. OPDA levels begin to rise after a lag-period of approximately 1 hr (see Fig. 2, insert), i.e. after the completion of the osmotically driven contact coiling response and before the beginning of the irreversible free coiling reaction, a process involving differential growth and supportive tissue differentiation [12]. The enantiomeric composition of cis-OPDA was analysed by chiral GC-MS [4]. Only the *cis*-(+)-enantiomer was detected. The time course of *cis*-OPDA increase is in agreement with a role of this octadecanoid as a signalling compound in tendril mechanotransduction.

Octadecanoids are signal transducers in Phaseolus vulgaris *thigmomorphogenesis*

An important, as yet unanswered, question is whether signalling through octadecanoids is a particular feature of mechanotransduction in *B. dioica* or whether octadecanoids have a more general role in plant mechanosignalling. It is well known that bending/shear forces exerted by wind have a profound impact on stature development of plants and that in places where such forces play no role, as in a greenhouse, plant development differs considerably from that in an open field or natural habitat [13]. A model system to analyse these processes is *Phaseolus vulgaris* thigmomorphogenesis, where wind force is substituted for by gentle internode rubbing for a few seconds every other hour [14]. It becomes evident immediately that this treatment results in a dramatic growth response. Mechanical stimulation of the first internode (that develops between the nodes bearing the cotyledons and the primary leaves) for 10–12 sec every other hour during the photoperiod (i.e. 7 times during a day from 8–20 hr) inhibits elongation of all subsequently forming internodes by up to 85% (2 days, i.e. 14 times stimulated), while the length of the first internode was less affected simply because this had grown to almost final length at the beginning of the treatment (data not shown). Treatment on a single day over a period of only 6 h (i.e. 4 times 10–12 sec mechanical stimulation) is sufficient to inhibit subsequent elongation of the second internode by 70% over a period of at least 6 days. Thus, we are dealing with a phenomenon of induction, but also stimulus-response coupling that allows a graded response depending on the intensity of stimulation.

In order to test the involvement of octadecanoids in this process, the first internodes (i.e., those which received the mechanical stimulus) plus the apical parts of the shoot were analysed for their levels of *cis*-OPDA. In each out of 9 independent experiments, *cis*-OPDA levels were found to increase (Table 2), on average three-fold. Within the first 6 hr of stimulation (i.e. 4 times 10–12 sec stimulus delivery), levels of *cis*-OPDA had almost doubled (*vide infra*, Fig. 6). Chiral GC-MS revealed that, as in all other species analysed so far [4], *cis*-OPDA extracted from control tissue (first internodes) is the (+)-enantiomer (Fig. 3, left

Table 2. Increase in levels of *cis*-OPDA following mechanical stimulation of *P. vulgaris* first internodes*

Experiment	Increase factor†
1	1.8
2	2.7
3	1.7
4	2.5
5	5.0
6	5.9
7	2.5
8	1.6
9	3.7
average ($n = 9$)	3.0‡

* mechanical stimulation 7 times per 12 hr photoperiod as detailed in the experimental section.

† 100% (1×) = *cis*-OPDA level of untreated controls at 0 hr; increase factor calculated for peak level of *cis*-OPDA.

‡ average absolute level: 305 ng g^{-1} fr. wt.

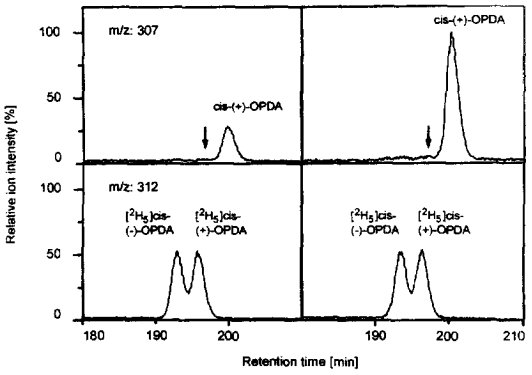


Fig. 3. Analysis of enantiomeric composition of *cis*-OPDA, extracted from control shoots (first internode plus apex) of *P. vulgaris* (upper left panel) or shoots mechanostimulated over a period of 48 hr (stimulation every other hour during photoperiod, i.e. 14 times for 10–12 sec over the course of the experiment, upper right panel), as the methyl ester. The lower panels show the location and enantiomer separation of the internal standard of [$^2H_5$]*cis*-(±)-OPDA methyl ester in the same chromatograms. The arrows indicate the position of *cis*-(−)-OPDA methyl ester (determined from independently performed separations of cis-(±)-OPDA methyl ester).

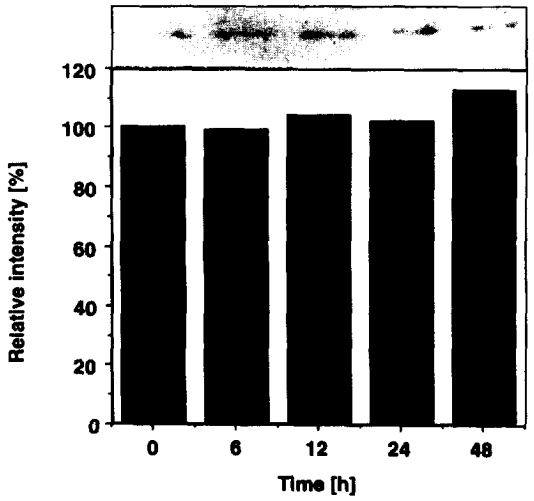


Fig. 4. Immunodetection of *P. vulgaris* allene oxide synthase on nitrocellulose blots of whole cell protein extracted from shoots (first internode plus apex) of control (0 hr) or mechanically stimulated *P. vulgaris*. Stimulation was for 10–12 sec every other hour over a period of 6–48 hr during the photoperiod beginning at $t = 0$ hr. The upper panel shows a photograph of the allene oxide synthase band from a typical experiment (10 μg of protein applied per lane), and the bars give the relative intensities of the bands (0 hr = 100%) determined densitometrically.

upper panel). The same holds true when the analysis was carried out with extracts from stimulated plants (Fig. 3, upper right panel). The experiment again demonstrates the clear increase in *cis*-OPDA during mechanical stimulation of the internodes and, further, that the same stereoisomer is formed in the inductive situation and in the control tissue.

The rate limiting enzyme in biosynthesis of *cis*-OPDA is allene oxide synthase. It has been shown that this enzyme is rapidly and strongly induced upon tissue wounding in *Arabidopsis thaliana* [10]. To test whether this was also the case during internode mechanostimulation, protein extracts from internodes were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and allene oxide synthase levels determined by a specific antibody raised against the recombinant enzyme from *A. thaliana* [10] (Fig. 4). It is clear that no detectable changes in the amount of enzyme present in the tissue occurs during stimulation. Thus, it is likely that mechanostimulation increases substrate availability for OPDA biosynthesis rather than pathway capacity.

Next, the ability of coronatine, a *cis*-OPDA analogue in *B. dioica* tendril coiling [7], in substituting for the mechanical stimulus in the bean internode system was analysed. Coronatine proved extremely active in this biological system, too (Fig. 5). A morphological effect comparable to mechanical stimulation over the course of a single day was obtained treating the first internodes with a 10 μM solution of coronatine over the same time interval and using the same timing of treatments (7 treatments, corresponding to a total amount of 1 nmol (0.35 μg) of coronatine applied per internode). Next, repetitive treatments with coronatine or repetitive mechanical stimulation were

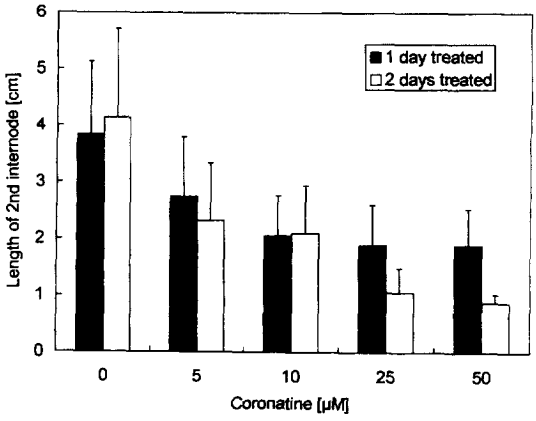


Fig. 5. Inhibition of elongation of the second internode of *P. vulgaris* plants following treatment of the first internode with coronatine at the concentrations indicated (0 μM = solvent controls). Shown are mean ± s.d. for $n \geq 30$ specimens (representing $n \geq 3$ independent experiments). Coronatine solution was brushed onto the first internode over the course of one or two photoperiods (1 day treated and 2 days treated, respectively) every second hour starting at 0 hr (i.e., coronatine was applied 7 or 14 times, respectively). This timing followed the one selected for mechanical stimulation in other experiments. The lengths of the internodes were in each case determined 6 days after the beginning of the treatments.

directly compared for their effects on levels of *cis*-OPDA in the treated internode plus apex (Fig. 6). Mechanostimulation led to a stimulus-dependent increase in *cis*-OPDA while coronatine treatment did not. Thus, as in the case of *B. dioica* tendril coiling and the induction of benzophenanthridine alkaloid

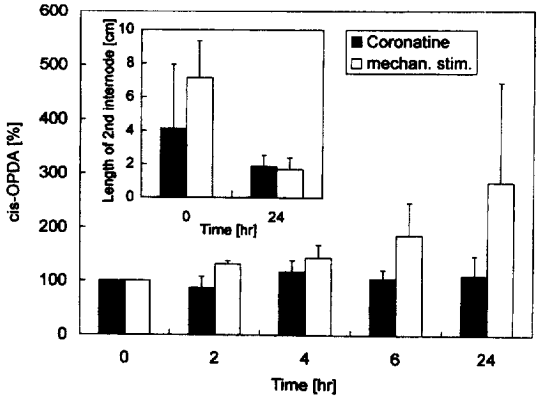


Fig. 6. Levels of *cis*-OPDA in shoots (first internode plus apex) of mechanically stimulated or coronatine treated *P. vulgaris* plants (mean ± s.d. of $n = 4$ independent experiments in each series, except for coronatine, 24 hr, representing two independent experiments with quadruplicate replication each time). The insert shows the length of the second internodes from controls (0 hr) and from treated plants (24 hr), mean ± s.d. of $n \geqslant 30$ specimens representing $n = 4$ independent experiments. Controls were as follows: unstimulated (open bar, 0 hr) or treated with solvent only (closed bar, 0 hr). The lengths of the internodes were in each case determined 6 days after the beginning of the treatments.

induction in *Eschscholtzia californica* cell cultures [7], coronatine is not an inducer of octadecanoid accumulation in the bean system either and, consequently, must be considered active *per se*.

Anatomical effects of mechanostimulation or coronatine application on the internodes of treated plants were comparable and mainly involve the peripheral parenchymatous tissues of the stem, where cells swell in radical direction. Cell walls of these cells appear reinforced, with the final result that a prolific collenchymatous supportive tissue developed (not shown). This process bears close resemblance to that occurring during tendril free coiling [12]. In addition only narrow diameter xylem vessels formed in internodes of mechanostimulated or coronatine treated plants, whereas in internodes from control plants, wide diameter vessels were regularly found besides narrow ones. Thus, reinforcement of the stem of treated plants also involved the xylem tissue.

DISCUSSION

While efficient and sensitive analytical tools have been developed for the analysis of jasmonic acid from plant extracts [9], no such techniques were available for the quantitation of its precursor, OPDA. The present study and a complementing procedure allowing analysis of enantiomeric composition of this important plant signalling molecule also developed in our laboratory [4] now fill this gap. Since extraction and workup procedures were based on the earlier method by Mueller and Brodschelm [9], and a comparable sensitivity of the techniques has been achieved, concise procedures for the quantitative analysis of the two most important octadecanoids are now at hand (this study and [9]). Furthermore, chemical derivatization of *cis*-OPDA to 1-oxo-2-(2′-pentenyl)-cyclopentane-3-octanoic acid (OPC-8:0), the next metabolite in biosynthesis of jasmonic acid from *cis*-OPDA [1] is readily achieved, yielding access to a deuterated internal standard for this octadecanoid, as well (P. Hennig, unpublished results). It is thus conceivable that analytical tools for a broader range of octadecanoids will soon be available.

While the biological significance of the wide spectrum of *cis*-OPDA levels observed in different angiosperms (cf. Table 1) remains to be shown, this survey has revealed species particularly well suited for further research on the physiology and biosynthesis of C18-octadecanoids, such as *A. thaliana* and *P. vulgaris* among the dicotyledonous angiosperms and *Hordeum vulgare* and *Zea mays* among the monocotyledonous angiosperms.

Among the biological functions of *cis*-OPDA, a role in mechanotransduction has now been established. It is still too early to allow generalizations, but the fact that in two only distantly related species and morphologically non-homologous organs, *cis*-OPDA is a signal transducer in mechanotransduction, proves that its role must be a more general one. While exogenously applied methyl jasmonate will elicit tendril coiling [11], it does so only at an order of magnitude higher levels compared to methyl-OPDA and acts much slower [7, 11]. Levels of jasmonic acid in mechanoreacting tendrils remain lower than those of *cis*-OPDA and rise very late in the process [6]. In the case of *P. vulgaris* thigmomorphogenesis, levels of JA have remained below limits of detection throughout the experiment (data not shown). Collectively, available data discourage a role of endogenous jasmonate in the process of mechanotransduction in the two species, but strongly suggest that instead the octadecanoid *cis*-OPDA is a key signal transducer in both systems. Consequently, information about the mechanisms triggering OPDA production following mechanical stimulation, about OPDA release from plastids and its distribution within and between (?) cells and about its primary mechanism(s) of action is now urgently required to further our understanding of the molecular events underlying the processes of mechanotransduction in higher plants.

EXPERIMENTAL

Growth and treatments of plants. The plants used for the taxonomic survey of OPDA levels were grown from seeds obtained from local suppliers and raised in a temperate greenhouse with supplementary light from fluorescent tubes (16 hr photoperiod). The following species and cultivars were used (ages in days after sowing at time of harvest in brackets following species names): *Anethum graveolens* ssp. *hortorum* L. cv. Blattreicher (19); *Anthriscus cerefolium* ssp. *cerefolium* Hoffm. (16); *Coriandrum sativum* L. (16); *Lac-*

tuca sativa ssp. *capitata* L. cv. Grand Rapid (10); *Taraxacum officinale* Web. cv. Treibriese (16); *Borago officinalis* L. (16); *Arabidopsis thaliana* (L.) Heynh, race Columbia (35); *Brassica oleracea* ssp. rapifera L. cv. Merrick (16); *B. oleracea* ssp. *sabauda* L. cv. Goldgelber (16); *B. oleracea* ssp. *viridis* L. cv. Grüner Ring (16); *Lepidium sativum* L. cv. Krause (6); *Raphanus sativus* ssp. *sativus* L. cv. Eiszapfen (16); *Beta vulgaris* ssp. *vulgaris* L. cv. Rote Kugel 2 (16); *Spinacia oleracea* L. (10); *Phaseolus vulgaris* ssp. *vulgaris* L. cv. Neckargold (14); *P. vulgaris* ssp. *nanus* L. cv. Facta (18); *Thymus vulgaris* L. (19); *Allium cepa* L. cv. Zittauer Gelbe (21); *Avena sativa* L. (10); *Hordeum vulgare* L. cv. Baronesse (10); *Triticum aestivum* L. (10); *Zea mays* L. (10); *Sanguisorba minor* Scop. (16); *Lycopersicon esculentum* Mill. cv. First-in-the-Field (60); *Nicotiana tabacum* L. (60). In all cases, leaves were harvested and immediately frozen in liquid N_2.

Bryonia dioica Jacq. was grown in phytotron chambers exactly as described [6, 15]. Tendrils were harvested when 12–18 cm long and completely unstimulated (for controls) or were subjected to continuous mechanical stimulation as detailed in [15] prior to harvest. Immediately following harvest, tendrils were immersed in liquid N_2. Per data point and replicate experiment, a minimum of 30 organs were harvested (approximately 2.5 to 3 g fr. wt). Experiments were repeated as indicated in the text.

Phaseolus vulgaris L. cv. Neckargold was grown in a growth cabinet at 24° (day) and 20° (night), photoperiod 16 hr, light intensity 90 W m^{-2} at ambient relative humidity (60–90%). Experiments were started when the first internode had reached 2 cm in length (8–10 days after sowing). This internode was stimulated every other hour during the photoperiod (from 8:00 hr to 20:00 hr) by rubbing between thumb and forefinger ($\times 12$, duration 10–12 sec per treatment) and plants were otherwise left undisturbed. Controls were grown in the same plot and left undisturbed. The application of coronatine (in 40% aq. Me_2CO containing 0.1% Tween-20) was achieved by spreading the soln over the whole surface of the first internodes (approximately 15 μl per treatment per organ) every other hr (from 8:00 hr to 20:00 hr). Controls were brushed with solvent only (40% aq. Me_2CO containing 0.1% Tween-20). At the times indicated, the primary leaves were removed and the shoot above the cotyledons (i.e. the first internode and apex) was harvested and immersed immediately in liquid N_2. Per data point and replicate experiment, a minimum of 30 shoots (approximately 1.5 g fr. wt) were harvested. Experiments were repeated $\times 9$ (mechanostimulation) and $\times 4$ (coronatine application *vs* mechanostimulation). The lengths of internodes of control and treated plants were always determined 6 days after the beginning of the treatments.

Determination of cis-12-*oxo-phytodienoic acid.* The frozen tissue (usually, a sample of 5 g fr. wt or as indicated above) was ground to a fine powder in a mortar with pestle in liquid N_2. Three representative samples of the ground tissue (1.0 g fr. wt each except for *P. vulgaris* and *B. dioica*: 0.5 g fr. wt each) were then drawn and extracted in parallel with peroxide-free Et_2O (3×25 ml, 3 hr each time, 20°) with intermittent mixing. At the start of the first extraction, an int. standard of [$^2H_5$]*cis*-OPDA was added (amounts varying between 200 and 300 ng). Extracts were filtered and then, following the protocol of [9], subjected to solid phase pre-purification using aminopropyl-columns (Chromabond NH_2, 0.5 g, Macherey–Nagel, Düren). Following application of the extracts, the columns were pre-eluted with 5 ml $CHCl_3$–*iso*-PrOH (2:1). OPDA was then eluted with 10 ml Et_2O–HOAc (49:1). These eluates were reduced to dryness on a rotary film evaporator at 30°, and the residues were re-dissolved in 0.2 ml HPLC—solvent [*n*-hexane–*iso*-PrOH–HOAc (98:1.61:0.11)]. Particles were removed by centrifugation. Aliquots of 0.1 ml (representing half the initial extract) were subject to HPLC (column: Nucleosil 100, 10 μm particle size, 250 mm $\times$ 4 mm i.d., flow rate 1 ml min^{-1}; R_t(*cis*-OPDA) = 15 min; solvent as above). The *cis*-OPDA fr. was evapd to dryness at 30°, redissolved in 0.1 ml MeOH and treated with ethereal CH_2N_2 (precautions as in [2]). The samples, dried in a gentle stream of N_2, were finally re-dissolved in 50 μl $CHCl_3$, and aliquots of 1 μl (representing 1/100 of the initial extract, i.e. 5 or 10 mg of extracted tissue) were subjected to GC-MS.

GC-MS. Enantiomeric composition of *cis*-OPDA was determined using a γ-Dex 120 capillary column (Supelco, Bellefonte) under conditions exactly as described [4]. The mass spectrometer (TSQ 7000, Finnigan, Bremen) was operated in chemical ionization mode using methane as the reactant gas and single ion monitoring of the following ions (*m/z*: 275, 280, 307 and 312). Quantitation of OPDA was performed on a Finnigan Magnum ion trap mass spectrometer coupled to a Varian GC 3400 gas chromatograh. Injections (1 μl) were made with an A200S autoinjector (Finnigan, Bremen). GC conditions: splitless injection (260°), transfer-line temp. 260°; column: DB-17, 30 m $\times$ 0.25 m $\times$ 0.25 μm film thickness (J and W, Folsom); helium carrier gas; temperature programme: 1 min isotherm 80°, linear increase (30° min^{-1}) to 200°, then (5° min^{-1}) to 250°, 10 min isotherm 250°. The mass-spectrometer was operated in full-scan mode (*m/z* 50–400) using chemical ionization and methanol as reactant gas. Quantitations were based on ion traces for *m/z* = 307 ([M+H]$^+$, OPDA) *vs* *m/z* = 312 ([M+H]$^+$, [$^2H_5$]OPDA). A linear detector response was obtained between 0 and 3 ng of OPDA injected. Aliquot sizes of internally standardized plant extracts were predetermined to fall within this linear range of the dose-response curve.

Synthesis of [$^2H_5$]cis-12-*oxo-phytodienoic acid.* 17[$^2H_2$], 18[$^2H_3$]α-linolenic acid, prepd from its ethyl ester (DLM-2351, Cambridge Isotope Laboratories, Andover, isotopic enrichment 98%, chemical purity > 98%), was converted to the 13-hydroperoxy-derivative as described [10]. The pentadeuterated 13-

hydroperoxylinolenic acid was then converted enzymatically with recombinant *A. thaliana* allene oxide synthase [10] expressed in *E. coli* under conditions detailed in [4]. Isotope labelled *cis*-(±)-OPDA was obtained in 8% overall yield and >98% purity after HPLC (*vide supra*, for conditions, columm: Zorbax Sil, 5 μm particle size, 250 mm × 4 mm i.d., precolumn 5 mm × 4 mm i.d., R_t (*cis*-OPDA) = 19.5 min; R_t (*trans*-OPDA) = 16.0 min). Purity, isotopic abundance and enantiomeric composition were checked by GC-MS using the methods described above. There was no alteration in isotopic abundance during the course of prepn of [$^2H_5$]*cis*-OPDA from α-linolenic acid. GC-CIMS (MeOH), *m/z* (rel. int): 313 (16.9), 312 $[M+H]^+$ (79.9), 311(2.9), 310 (0.3), 309 (0).

Biochemical analysis. Protein extractions [16], SDS-polyacrylamide gel electrophoresis [17] and protein blotting to nitrocellulose membranes [18] followed standard procedures. The allene oxide synthase antiserum was described in [10]. Immunodetection of antibody decorated bands [19] was achieved with the ECL chemiluminescence system (Amersham, Braunschweig).

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REFERENCES

1. Vick, B. A. and Zimmerman, D. C., *Plant Physiology*, 1984, **75**, 458.
2. Hamberg, M. and Fahlstadius, P., *Archives of Biochemistry and Biophysics*, 1990, **276**, 518.
3. Brash, A. R. and Song, W. C., in *Methods in Enzymology*, 1996, **272**, 250.
4. Laudert, D., Hennig, P., Stelmach, B. A., Müller, A., Andert, L. and Weiler, E. W., *Analytical Biochemistry*, 1997, **246**, 211.
5. Blechert, S., Brodschelm, W., Hölder, S., Kammerer, L., Kutchan, T. M., Mueller, M. J., Xia, Z.-Q. and Zenk, M. H., *Proceedings of the National Academy of Science USA*, 1995, **92**, 4099.
6. Weiler, E. W., Albrecht, T., Groth, B., Xia, Z.-Q., Luxem, M., Liß, H., Andert, L. and Spengler, P., *Phytochemistry*, 1993, **32**, 591.
7. Weiler, E. W., Kutchan, T. M., Gorba, T., Brodschelm, W., Niesel, U. and Bublitz, F., *FEBS*, 1994, **345**, 9.
8. Mueller, M. J., Brodschelm, W., Spannagl, E. and Zenk, M. H., *Proceedings of the National Academy of Sciences, U.S.A.*, 1993, **90**, 7490.
9. Mueller, M. J. and Brodschelm, W., *Analytical Biochemistry*, 1994, **218**, 425.
10. Laudert, D., Pfannschmidt, U., Lottspeich, F., Holländer-Czytko, H. and Weiler, E. W., *Plant Molecular Biology*, 1996, **31**, 323.
11. Falkenstein, E., Groth, B., Mithöfer, A. and Weiler, E. W., *Planta*, 1991, **185**, 316.
12. Kaiser, I., Engelberth, J., Groth, B. and Weiler, E. W., *Botanica Acta*, 1994, **107**, 24.
13. Mitchell, C. A., *Horticultural Science*, 1996, **31**, 31.
14. Jaffe, M. J., *BioScience*, 1980, **30**, 239.
15. Liß, H. and Weiler, E. W., *Planta*, 1994, **194**, 169.
16. Palmgren, M. G. and Sommarin, M., *Plant Physiology*, 1989, **90**, 1009.
17. Laemmli, U. K., *Nature*, 1970, **227**, 680.
18. Towbin, H., Staehelin, T. and Gordon, J., *Proceedings of the National Academy of Sciences, U.S.A.*, 1979, **76**, 4350.
19. Knecht, D. A. and Dimond, R. L., *Analytical Biochemistry*, 1984, **136**, 180.