FEBS Letters 581 (2007) 815–820

Substrate specificity and products of side-reactions catalyzed by jasmonate:amino acid synthetase (JAR1)

Andrzej Guranowski[a,1], Otto Miersch[a], Paul E. Staswick[b], Walter Suza[b,2], Claus Wasternack[a,*]

[a] *Leibniz-Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany*
[b] *Department of Agronomy and Horticulture, University of Nebraska, Lincoln, NE 68583, USA*

Received 1 December 2006; revised 17 January 2007; accepted 22 January 2007

Available online 2 February 2007

Edited by Ulf-Ingo Flügge

Abstract **Jasmonate:amino acid synthetase (JAR1) is involved in the function of jasmonic acid (JA) as a plant hormone. It catalyzes the synthesis of several JA-amido conjugates, the most important of which appears to be JA-Ile. Structurally, JAR1 is a member of the firefly luciferase superfamily that comprises enzymes that adenylate various organic acids. This study analyzed the substrate specificity of recombinant JAR1 and determined whether it catalyzes the synthesis of mono- and dinucleoside polyphosphates, which are side-reaction products of many enzymes forming acyl ~ adenylates. Among different oxylipins tested as mixed stereoisomers for substrate activity with JAR1, the highest rate of conversion to Ile-conjugates was observed for (±)-JA and 9,10-dihydro-JA, while the rate of conjugation with 12-hydroxy-JA and OPC-4 (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-butyric acid) was only about 1–2% that for (±)-JA. Of the two stereoisomers of JA, (−)-JA and (+)-JA, rate of synthesis of the former was about 100-fold faster than for (+)-JA. Finally, we have demonstrated that (1) in the presence of ATP, Mg^{2+}, (−)-JA and tripolyphosphate the ligase produces adenosine 5′-tetraphosphate (p_4A); (2) addition of isoleucine to that mixture halts the p_4A synthesis; (3) the enzyme produces neither diadenosine triphosphate (Ap_3A) nor diadenosine tetraphosphate (Ap_4A) and (4) Ap_4A cannot substitute ATP as a source of adenylate in the complete reaction that yields JA-Ile.**

*Corresponding author. Fax: +49 345 5582 1219.
E-mail address: cwastern@ipb-halle.de (C. Wasternack).

[1]Permanent address: Department of Biochemistry and Biotechnology, Agricultural University, Poznań, Poland.
[2]Present address: Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA.

Abbreviations: 12-AOC-JA-Ile-Me, 12-hydroxy-acetoyl jasmonic acid isoleucine methyl ester; 4-CL, 4-coumarate:CoA ligase; Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; DH-JA, 9,10-dihydro-JA; Dinor-OPDA, 3-oxo-2-(2*Z*-pentenyl)cyclopent-4-ene-1-hexanoic acid; GC/MS, gas chromatography/mass spectrometry; HPLC, high pressure liquid chromatography; IAA, indole-3-acetic acid; JA, jasmonic acid; JA-Ile, JA isoleucine conjugate; JAR, jasmonate amino acid synthetase; NTP, nucleoside triphosphate; OPC-4, 3-oxo-2-(2*Z*-pentenyl)-cyclopentane-1-butyric acid; OPC-4-Ile-Me, 3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-butanoyl-isoleucine methyl ester; OPC-6-Ile-Me, 3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-hexhexanoyl-isoleucine methyl ester; OPC-8-Ile-Me, 3-oxo-2-(2*Z*-pentenyl)-cyclopentane-1-octanoyl-isoleucine methyl ester; OPDA, 12-oxo-phytodienoic acid; p_3, tripolyphosphate; p_4, tetrapolyphosphate; p_4A, adenosine 5′-tetraphosphate; TLC, thin layer chromatography

Keywords: Jasmonate:amino acid synthetase; Substrate specificity; Firefly luciferase super family; Adenosine 5′-tetraphosphate synthesis; Ap_3A, Ap_4A

1. Introduction

The effect of plant hormones such as indole-3-acetic acid (IAA) and jasmonic acid (JA) may be regulated by enzymatic reactions that yield amino acid conjugates [1,2]. The hormone conjugating enzymes convert a carboxylic acid to an amide-linked conjugate in a two-step reaction that utilizes ATP. For example, the *Arabidopsis thaliana* jasmonate:amino acid synthetase JAR1 [3], which is the object of this study, catalyzes the activation of jasmonate:

$$\mathrm{E} + \text{jasmonate} + \mathrm{ATP} \leftrightarrow \mathrm{E} : \text{jasmonoyl} \sim \mathrm{pA} + \mathrm{PP_i} \quad (1)$$

and the transfer of the acyl moiety onto the α-amino group of an amino acid such as isoleucine:

$$\mathrm{E} : \text{jasmonoyl} \sim \mathrm{pA} + \mathrm{Ile} \rightarrow \text{jasmonoyl-Ile} + \mathrm{AMP} + \mathrm{E}. \quad (2)$$

Plants contain many different JA related compounds [4]. Among those found linked to amino acids are JA-Ile, JA-Leu and JA-Val, all of which can be synthesized by recombinant JAR1 [3]. Plants contain many lipid-derived oxygenation products collectively called 'oxylipins'. Among them are a variety of jasmonates other than JA, including the JA biosynthetic intermediates 12-oxo-phytodienoic acid (OPDA) and dinor-OPDA, and JA metabolites such as 9,10-dihydrojasmonic acid, and 12-hydroxy-JA. Some of these are known to have biological activities. This raises the question of whether any of these compounds are also substrates of JAR1. Isotope exchange assays with labeled PP_i indicated that OPDA and other JA biosynthetic intermediates were not adenylated by JAR1 (step 1) [3]. But it is conceivable that JAR1 might conjugate OPDA with an amino acid even though it does not support isotope exchange in the absence of an amino acid. Several other potential JAR1 substrates have not yet been evaluated. Another open question is the substrate specificity of JAR1 with respect to the JA stereoisomers, (−)-JA and (+)-JA.

JAR1 and the many related plant enzymes belong to the acyl ~ adenylate-forming firefly luciferase superfamily [1]. This superfamily is composed of a variety of different enzymes including firefly luciferase, fatty acyl-CoA synthetases,

0014-5793/$32.00 © 2007 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2007.01.049

acetyl-CoA synthetases, non-ribosomal peptide synthetases and 4-coumarate-CoA synthetases (4-CLs) [5]. All of these can also catalyze the formation of uncommon mononucleoside polyphosphates, such as adenosine 5′-tetraphosphate (p_4A) and adenosine 5′-pentaphosphate (p_5A), and some can produce dinucleoside polyphosphates, such as diadenosine 5′,5‴-P^1, P^3-triphosphate (Ap_3A) and/or diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) (for reviews see [6–8]). In those reactions, which are analogous to the reverse of reaction (1), PP_i is substituted with tripolyphosphate (PPP_i or p_3), tetrapolyphosphate ($PPPP_i$ or p_4), ADP (ppA) or ATP (pppA), respectively. The first demonstration of the synthesis of uncommon mono- and dinucleoside polyphosphates by a similar mechanism was demonstrated for certain aminoacyl-tRNA synthetases [9–12], but those ligases do not belong to the firefly luciferase superfamily. Usually, the (di)nucleoside polyphosphate synthesis is prompted by the absence of the respective acyl-acceptor (e.g. CoA, 4′-phosphopantetheine, and tRNAs) and it has been noticed that the (di)adenosine polyphosphate product spectra are specific for each enzyme. For example the aminoacyl-tRNA synthetases specific for lysine or phenylalanine can produce both p_4A, Ap_3A and Ap_4A but the firefly luciferase [13,14] and *Arabidopsis* 4-CL [15] were unable to synthesize Ap_3A, but do produce p_4A and Ap_4A. Finally, the yeast acetyl-CoA synthetase was able to produce p_4A but not dinucleoside polyphosphates [16]. The same enzyme highly purified from *Escherichia coli* (kindly donated by Dr. Alan J. Wolfe) synthesized none of the aforementioned (di)nucleoside polyphosphates, although it was fully active to carry out acetylation of CoA (Guranowski, unpublished data). These examples show that the capacity of an enzyme to synthesize (di)nucleoside polyphosphates is unpredictable and therefore should be verified experimentally in each case.

In this study, in addition to elucidating the aforementioned questions on oxylipin specificity, we have tested the recombinant (GST)-JAR1 as a potential p_4A and/or Ap_4A 'synthase'. The rationale was that this plant enzyme might affect the pool of uncommon (di)nucleoside polyphosphates, which have been shown in other organisms to play roles as intracellular [17] and extracellular [18] signal molecules.

2. Materials and methods

2.1. Materials

All nucleotides including adenosine 5′-tetraphosphate (p_4A), diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A) as well as tripolyphosphate and glutathione–agarose were from Sigma (St. Louis, MO, USA). Thin layer chromatography (TLC) aluminum plates precoated with silica gel containing fluorescent indicator were from Merck (Darmstadt, Germany).

Jasmonic acid isomers were prepared according to [19]. Other oxylipins were prepared according to [20] and they were 99.5% pure as demonstrated by HPLC analysis. [$^{13}C_6$] JA-L-isoleucine was prepared from (±)-JA and [$^{13}C_6$] L-isoleucine (Cambridge Isotope Laboratories, Andover, MA, USA) as described previously [3].

Recombinant jasmonate:amino acid synthetase (GST)-JAR1 was prepared from the cells of *Escherichia coli* (strain BL21) essentially as described earlier [1]. For the reactions involving analysis of products by TLC, the enzyme was eluted with 50 mM Tris/HCl (pH 9.5) containing 10 mM reduced glutathione. To prolong stability of the enzyme, these preparations were brought to 2% glycerol and supplied with bovine serum albumin (0.5 mg/ml) and kept frozen at −20 °C in 0.1 ml aliquots. Enzyme prepared for quantitative assays was stored in aliquots at −80 °C in 7% glycerol. Protein concentration of the latter was determined with a BioRad DC protein analysis kit (Hercules, CA, USA) after dialysis of the enzyme to remove glutathione. Concentrations ranged from 1.3 to 2.6 mg per ml.

2.2. Enzyme assays

2.2.1. Synthesis of JA-Ile and other oxylipin-isoleucines. For the analysis of products by TLC the reaction mixture (50 µl) contained 50 mM HEPES/NaOH (pH 8.3), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM ATP, 2 mM ammonium salt of (−) or (+) stereoisomer of jasmonate or other oxylipin, 2 mM L-isoleucine and 10 µl of the (GST)-JAR1 preparation. The incubation was carried out at 30 °C. At indicated times, 3 µl aliquots were spotted onto two parallel silica gel plates. One chromatogram was developed for 60 min in dioxane:ammonium hydroxide:water (6:1:4, by volume), dried and the nucleotides were visualized under short-wave ultraviolet lamp. The other TLC plate was developed for 50 min in chloroform:ethyl acetate:acetic acid (5:4:1, by volume), dried, sprayed with anisaldehyde reagent and heated to develop spots of JA (pink), JA-Ile (yellow) and other oxylipins and oxylipin-Ile conjugates if present.

For quantitative assays reactions were done at 25 °C in 25 mM Tris–HCl (pH 8.6) with 0.5 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM L-Ile and 0.5 mM oxylipin. Measurement of ADP synthesis was done in the absence of oxylipin and amino acid. Enzyme concentration for these reactions was at 0.36 or 0.44 mg/ml. Quantitative data were determined for reactions run for 10 min. Initial trial experiments were done with (±)-JA over time points ranging from 2 to 60 min to compare the release of AMP with the synthesis of JA-Ile. Enzyme reactions were stopped by removing 20 µl aliquots and adding them to 80 µl of KH_2PO_4 at pH 4.8. For quantitation of JA-Ile an internal standard consisting of 0.75 nmol of [$^{13}C_6$]-JA-Ile was added to each aliquot, and the jasmonates were extracted twice with an equal volume of chloroform containing 10% acetic acid. After drying in a stream of nitrogen the jasmonates were dissolved in 50 µl methanol and derivatized for 30 min at room temperature with the addition of 5 µl of (trimethylsilyl)diazomethane (Aldrich, St. Louis, MO, USA). Solvent was removed by evaporation under nitrogen and the residue was suspended in isooctane for analysis by GC/MS. A Finnigan Trace GC with a DB-5ht column (15 m × 0.25 mm, 0.1µ) from J.W. Scientific (Folsom, CA) coupled to a Finnigan DSQ mass spectrometer was used for the analysis. The injector port was at 280 °C, and the column gradient was 60–300 °C in 25 min. The amount of JA-Ile was obtained from the molecular ion integrated peak areas using the isotope dilution method described previously [21].

AMP was quantified by HPLC using a S200 series pump, UV/Vis detector and autosampler (Perkin Elmer). The column was a 150 mm × 4.6 mm Ultra IBD (Restek Corp. Bellefonte, PA, USA) run isocratically with 1% methanol in 50 mM KH_2PO_4 at pH 5.5. Stopped reactions (50 µl) were directly injected to the column at a flow rate of 1 ml/min. Integrated peak areas were compared to AMP standards run in a solution containing the same components as the stopped enzyme reactions minus the amino acid. A minor background AMP contaminant in the ATP was subtracted from each reaction for data analysis.

2.2.2. Synthesis of adenosine 5′-tetraphosphate. The reaction mixture (50 µl) contained 50 mM HEPES/NaOH (pH 8.3), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM ATP, 2 mM ammonium salt of (−)-JA, 10 or 20 mM tripolyphosphate and the (GST)-JAR1 (approx. 20 µg). After prolonged (3–16 h) incubation at 30 °C, aliquots (3 µl) were spotted onto silica gel plates and the chromatogram developed for 60 min in dioxane:ammonium hydroxide:water (6:1:5, by volume). After drying the nucleotides were visualized under short-wave ultraviolet lamp.

2.2.3. Trials aimed to demonstrate synthesis of Ap_4A, Ap_3A or other Ap_4Ns. The reaction mixture contained 50 mM HEPES/NaOH (pH 8.3), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ammonium salt of (−)-JA, 5 mM ATP (alone to check synthesis of Ap_4A), 5 mM ADP (added in order to test the synthesis of Ap_3A) or 5 mM other canonical NTP (added to test possible synthesis of Ap_4N), and the (GST)-JAR1 preparation. After 16 h incubation the reaction mixtures were analyzed as described above.

2.3. Identification of the oxylipin-Ile conjugates by GC-MS

The reaction mixtures described in Section 2.2.1 were evaporated and supplied with 20 µl methanol and the samples were methylated with 100 µl ethereal diazomethane for 15 min. After evaporation of solvents 100 µl acetonitrile was added and the precipitated salts were

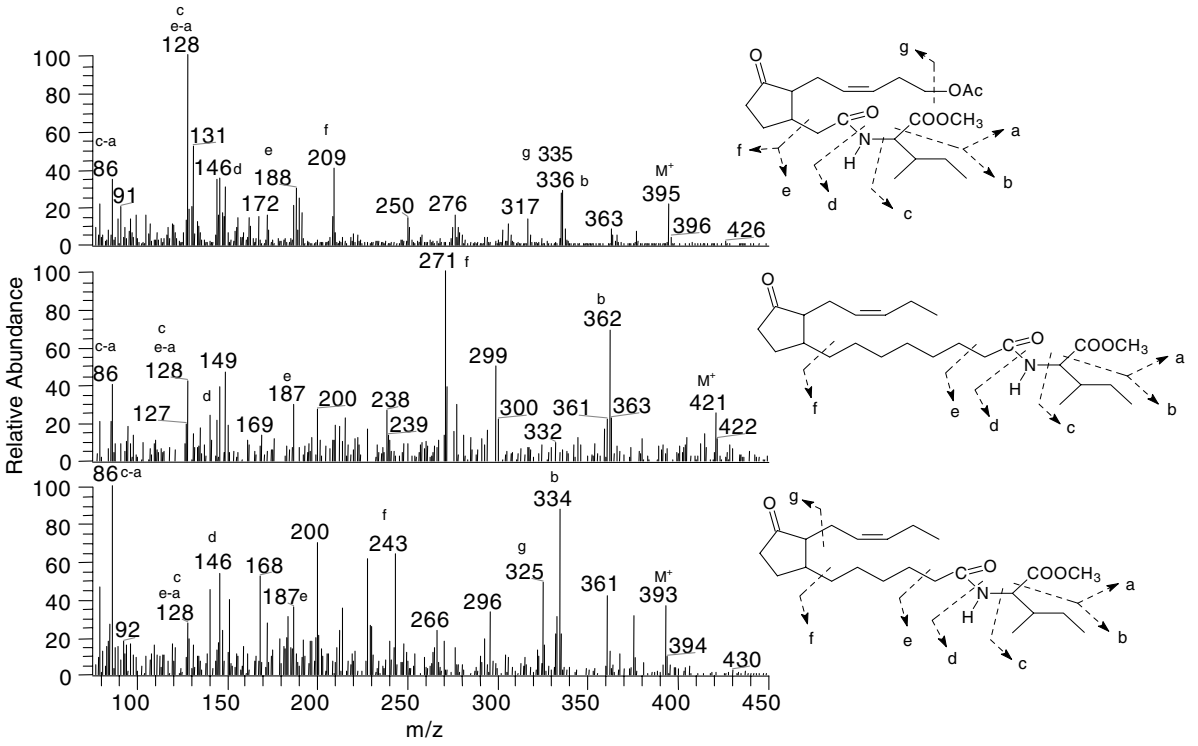

Fig. 1. Mass spectral fragmentation pattern of methyl 12-acetoxyjasmonoyl-isoleucine (top), isolated from in vitro enzyme assay described in Section 2.2.1 and isolated from tomato leaves as shown in 2.4, OPC-8-Ile-Me (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-octanoyl-isoleucine methyl ester) isolated from in vitro enzyme assay described in Section 2.2.1 (middle) and OPC-6-Ile-Me (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-hexanoyl-isoleucine methyl ester) (bottom) isolated from in vitro enzyme assay described in Section 2.2.1. The reaction product of the substrate 12-hydroxy-JA was derivatized by methylation and acetylation. In case of the substrates OPC-8 and OPC-6 the reaction products were methylated. All compounds were analyzed with a Finnigan PolarisQ GC/MS under EI conditions.

centrifuged. Supernatants were analysed by GC/MS. In the case of 12-hydroxy-JA acetylation was necessary. Therefore, the reaction was treated with a mixture of 5 μl pyridine and 25 μl acetic acid anhydride for 1 h at 50 °C, and these solvents were subsequently evaporated before GC/MS analysis.

GC/MS-analysis was performed with Polaris Q, Thermo-Finnigan, using positive EI at 70 eV, an ion source temperature of 200 °C and the column Rtx-5w/Integra Guard (Restek, Bad Homburg, Germany) (5 m inert precolumn connected with column 15 m × 0.25 mm, 0.25 mm film thickness, cross bond with 5% diphenyl–95% dimethyl polysiloxane). The injection temperature of 220 °C, and the interface temperature of 250 °C was used at a helium flow of 1 ml min^{-1}. A splitless injection of 1 μl samples was done and the following column temperature program was used: 1 min 60 °C, 25 °C min^{-1} to 180 °C, 5 °C min^{-1} to 270 °C, 10 °C min^{-1} to 300 °C, 10 min 300 °C. For identification, test substances of OPC-8-Ile, OPC-6-Ile and 12-OAc-JA-Ile were synthesized as described [22] and they exhibited GC/MS spectra identical to that shown in Fig. 1. The oxylipin-Ile conjugates were identified as follows:

9,10-dihydro-JA-Ile-Me: Rt 14.48 min; for MS-data see [23].

12-OAc-JA-Ile-Me: Rt 19.54 min; for fragmentation pattern see Fig. 1(top).

OPC-8-Ile-Me (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-octanoyl-isoleucine methyl ester): Rt 21.95 min; *m/z* (%) 421 $[M^+]$ (26), 362 $[M-COOMe]^+$ (26), 299 $[(CH_2)_5COIleMe]^+$ (51), 271 $[(CH_2)_7COIleMe]^+$ (100), 200 (28), 187 $[CH_2COIleMe]^+$ (29), 146 $[IleMe]^+$ (38), 128 $[CH_2COIleMe - COOMe]^+$ and $[IleMe-NH]^+$ (41), 86 $[IleMe-COOMe]^+$ (36), for fragmentation pattern see Fig. 1(middle).

OPC-6-Ile-Me (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-hexanoyl-isoleucine methyl ester): Rt 20.61 min;*m/z* (%) 393 $[M^+]$ (36), 361 $[M-MeOH]^+$ (41), 334 $[M-COOMe]^+$ (87), 325 $[M-CH_2-CHCHC_2H_5]^+$ (51), 296 (33), 243$[(CH_2)_5COIleMe]^+$ (64), 200 (69) 187 $[CH_2COIleMe]^+$ (28), 146 $[IleMe]^+$ (51), 128 $[CH_2COIleMe-COOMe]^+$ and $[IleMe-NH]^+$ (26), 86 $[IleMe-COOMe]^+$ (100), for fragmentation pattern see Fig. 1(bottom).

OPC-4-Ile-Me (3-oxo-2-(2*Z*-pentenyl)cyclopentane-1-butanoyl-isoleucine methyl ester): Rt 17.78 min; MS-data see [24].

2.4. Isolation of 12-OH-JA-Ile from tomato leaves

Leaves of intact tomato plants (*Lycopersicon esculentum* cv. Lukullus) grown in a greenhouse were wounded by crushing with a forcep and harvested after 6 h. Leaves (30 g) were homogenized in methanol, the extract filtered, evaporated and dissolved in chloroform. Acidic compounds were extracted by a saturated bicarbonate solution, that was acidified with 5 M acetic acid and evaporated to dryness. Three extractions with methanol gave a crude extract, which was separated on DEAE-Sephadex A25 (Ac^--form in methanol). Eluates with 3 M acetic acid in methanol were further separated by HPLC on Eurospher 100-C18 (5 μm, 250 × 4 mm) (Knauer, Berlin, Germany) with a gradient of 40–100% methanol (0.2% acetic acid) in 25 min. Fractions between R_t 10 and 11 min were collected, evaporated to dryness, methylated with ethereal diazomethane, acetylated by a mixture of pyridine/acetic acid anhydride (2:1) and further separation by HPLC using the same conditions as previously. Fractions between 16 and 16.30 min were evaporated, analysed by GC-MS as described in Section 2.3 and compared with the authentic compound.

3. Results and discussion

3.1. Synthesis of different oxylipin-Ile conjugates by (GST)-JAR1

In addition to JA-Ile, which is the major oxylipin-amino acid conjugate identified in plant tissues [4,25], other conjugates such as 9,10-dihydro-JA-Ile have also been identified [23] Thus, we wondered whether JAR1, which effectively catalyzes synthesis of JA-Ile, could be responsible for the synthesis of

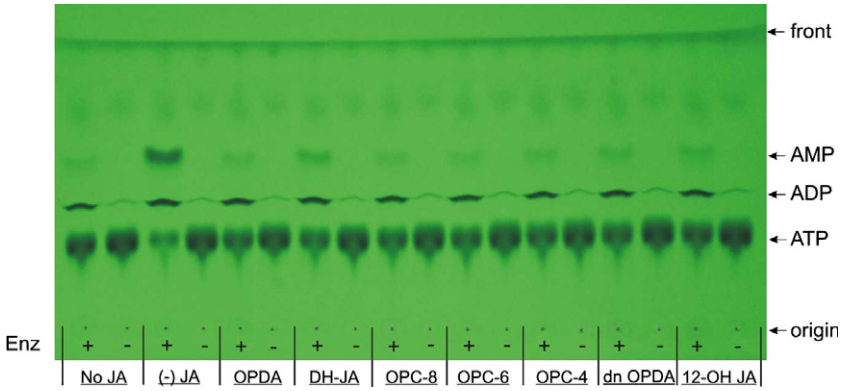

Fig. 2. Enzyme (JAR1)- and substrate (oxylipin)-dependent appearance of AMP demonstrated by thin-layer chromatography. The reaction mixture contained: 50 mM HEPES/NaOH (pH 8.3), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM ATP, 2 mM isoleucine, 2% glycerol, BSA (0.5 mg/ml), 2 mM oxylipin substrate, as indicated (except first two reaction mixtures) and GST-JAR1 fraction (approx. 20 μg) where indicated. After 6 h incubation at 30 °C, 3 μl aliquots were spotted onto origin of TLC-silica gel aluminum plates containing fluorescent indicator (from Merck), and developed for 60 min in dioxane:concentrated ammonia:water (6:1:4, by volume). The chromatogram was photographed under short-wave UV light.

amino acid conjugates with acid moieties other than JA. TLC analysis of reaction mixtures that contained a given oxylipin, isoleucine and (GST)-JAR1 demonstrated the enzyme-dependent accumulation of the following conjugates: 9,10-dihydro-JA-Ile, 12-OH-JA-Ile, OPC-8-Ile, OPC-6-Ile, OPC-4-Ile and as a control (±)-JA-Ile (data not shown). The product formation could be clearly seen by characteristic but unstable color complexes. Therefore, the products produced in these reactions were verified by GC/MS following their acetylation and methylation. The mass spectral identification of 12-OAc-JA-Ile-Me, the methylated derivatives of OPC-8-Ile, and OPC-6-Ile synthesized by JAR1 are shown in Fig. 1. All of these compounds have not previously been reported, whereas the 9,10-dihydro-JA-Ile-Me and OPC-4-Ile-Me mass spectra were identical to published data [23,24].

AMP, another product of the complete reaction catalyzed by JAR1, could be monitored by its enzyme- and oxylipin-dependent appearance by TLC (Fig. 2) or HPLC. Although several oxylipins yielded AMP during prolonged incubations there appeared to be a strong preference for JA by JAR1. In order to quantify the activity of JAR1 with various jasmonates we developed an HPLC assay for the rate of AMP synthesis in the presence of isoleucine and individual jasmonates. To first validate the assay the synthesis of JA-Ile was monitored by GC/MS in parallel with the assay for AMP production. In three independent tests the rate of the reaction determined by the two methods differed by no more than 13.4% over a series of reaction times ranging from 2 to 60 min. This established that monitoring AMP production was a reliable means to determine the activity of JAR1 in conjugate synthesis.

Table 1
Activity of JAR1 on several jasmonates

	Activity (nmol/min/mg protein)	(SE) (nmol/min/mg protein)
(±)-JA	10.05	(0.40)
(±)-DH-JA	7.56	(0.50)
12-OH-JA	0.12	(0.02)
(±)-OPDA	ND	
(±)-OPC-4	0.17	(0.02)
(±)-OPC-6	ND	
(±)-OPC-8	ND	
(−)-JA	29.80	(3.73)
(+)-JA	0.24	(0.07)

Values are means of three assays, with S.E.
ND, not detected at detection limit of 0.06 nmol/min/mg.

The assay results for (±)-JA, four of its biosynthetic intermediates and two other jasmonates are shown in Table 1. The rate of AMP production was monitored over the first 10 min of the reaction in each case. Highest activity was found with (±)-JA at 10 nmol/min/mg protein, while the rate for (±)-dihydro-JA was about 25% lower. The rates for 12-OH-JA and the JA precursor (±)-OPC-4 were only 1–2% that of JA, while the other JA precursors yielded no detectable activity. This confirms that JAR1 has a strong preference for (±)-JA and (±)-dihydro-JA, although the much lower activity with other substrates may still be sufficient to result in accumulation of their conjugated forms in vivo.

The formation of Ile conjugates carrying an acid moiety other than JA raises the question of whether these compounds occur in plant tissues. As described in Section 2 we have isolated for the first time 12-OH-JA-Ile from Arabidopsis and tomato leaves. The fragmentation pattern of 12-OH-JA-Ile isolated from tomato leaves was identical to that of synthesized 12-OH-JA-Ile as shown by GC/MS analysis presented in Fig. 1. Another example for naturally occurring 12-OH-JA amino acid conjugates is *N*-(acetoxyjasmonoyl)-phenyl alanine methyl ester identified in *Praxelia clematida* [26] All of these findings are consistent with the enzymatic properties of recombinant Arabidopsis JAR1 protein. It will be interesting to see, whether the increasing number of JA-metabolites detected in plant tissues (cf. [27]) have single or unique functions potentially related to signaling or storage. Initially, the term "oxylipin signature" was introduced to describe the pattern of distinct octadecanoids and jasmonates [28], which differs between leaves and flowers [29,30]. Meanwhile, the initially suggested JA-independent signaling properties of JA-Ile [25] have been supported by the characterization of the *jar1* mutant, which is defective in a subset of jasmonate-dependent responses [3]. The picture that has emerged is that plants, or at least distinct plant tissues, utilize various jasmonate signals to fine-tune the jasmonate regulatory network.

3.1.1. Comparison of the rates of the synthesis of (−)-JA-Ile and (+)-JA-Ile. We next examined the ability of JAR1 to uti-

lize two stereoisomers of JA, (−)-JA and (+)-JA. JA-Ile was detected by GC/MS after two-minute reactions with Ile and either of the stereoisomers. The reaction with (−)-JA yielded a rate of 29.8 nmol/min/mg protein (Table 1). In contrast, the rate of synthesis of the conjugate with (+)-JA was over 100-fold lower. These rate measurements were preceded by analysis performed by TLC and spraying of the chromatogram with anisaldehyde following prolonged incubation of (GST)-JAR1 with racemic (±)-JA. These conditions led to the complete disappearance of both JA isomers and the appearance of equal amounts of (−)-JA-Ile and (+)-JA-Ile. Thus, despite its much lower rate of conjugate synthesis, JAR1 is capable of using (+)-JA as a substrate. Among the stereoisomers (−)-JA, (+)-JA, (−)-JA-Ile, and (+)-JA-Ile the corresponding (−)-forms are biologically more active in application experiments than the (+)-forms [25,31]. Although (+)-*7-iso*-JA is the initial biosynthetic form, it is generally believed to equilibrate primarily to (−)-JA in vivo. The strong activity of recombinant JAR1 in the synthesis of (−)-JA-Ile is consistent both with (−)-JA-Ile being the most active in vivo and with its preferential accumulation in plant tissue compared to other JA amino acid conjugates [4,25].

3.2. Syntheses of adenosine 5′-tetraphosphate (p_4A) and diadenosine polyphosphates

Knowing that JAR1 is a member of the family of adenylate-forming enzymes, we examined whether the enzyme catalyzed the synthesis of p_4A, as many of these types of enzymes do.

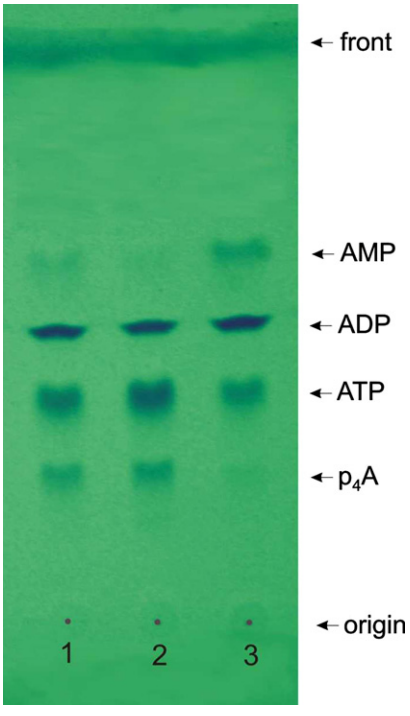

Fig. 3. Synthesis of adenosine tetraphosphate catalyzed by the recombinant GST-JAR1. The reaction mixture contained: 50 mM HEPES/NaOH (pH 8.3), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM ATP, 2% glycerol, BSA (0.5 mg/ml), 2 mM (−)-jasmonate (ammonium salt), GST-JAR1 and 10 mM (lane 1), 20 mM tripolyphosphate (lane 2), or 10 mM tripolyphosphate and 2 mM leucine (lane 3). The reaction was carried out for 16 h at 30 °C. Aliquots (3 μl) were spotted onto the origin and the chromatogram was developed in dioxane:ammonia:water (6:1:5, by volume).

Incubation of (GST)-JAR1 in the mixture that contained ATP, Mg^{2+}, (−)-JA and tripolyphosphate led to accumulation of a compound that migrated in the TLC system as authentic p_4A (Fig. 3). Lane 3 shows that the addition of isoleucine to the mixture containing tripolyphosphate halted the p_4A synthesis and led to the appearance of AMP, which reflects the completion of the synthesis of (−)-JA-Ile (see above). This result also indicates that if JAR1 synthesizes p_4A *in planta* it would only occur when sufficient JA is available and Ile or other amino acids suitable for JA conjugate synthesis are limiting.

The capacity of recombinant JAR1 to support the synthesis of diadenosine polyphosphates such as Ap_4A, Ap_3A and Ap_4Ns was tested in the following mixtures: with ATP as the only nucleotide, which served as both adenylate donor and potential acceptor, with ATP and with ADP, the latter potentially serving as an adenylate acceptor, and with ATP and either CTP, GTP or UTP, each of which could be potential adenylate acceptors. None of these dinucleoside polyphosphates accumulated to detectable levels based on TLC analysis (not shown). So, with respect to the catalytic capacity to synthesize (di)nucleoside polyphosphates JAR1 most closely resembles the acetyl-CoA ligase from yeast [16]. The latter enzyme was able to produce p_4A and p_5A but not diadenosine polyphosphates. In contrast with other ligases that were able to produce and reuse (di)adenosine polyphosphates, JAR1 was not able to use Ap_4A as a source of adenylate in the synthesis of JA-Ile.

3.3. How to explain an apparent ATPase activity of JAR1?

The thin-layer chromatogram shown in Fig. 2 reveals the enzyme-dependent but acyl-independent conversion of ATP to ADP. The initial rate of ADP production in the absence of jasmonate or amino acid was determined by HPLC and found to be 1.32 nmol/min/mg protein (SE 0.46), roughly 8-fold lower than the rate of JA-Ile synthesis. It was previously demonstrated that some aminoacyl-tRNA synthetases [32,33] converted ATP to ADP by phosphorolysis of enzyme-bound aminoacyl-adenylate. In the case of JAR1, however, the ADP appeared also in the absence of the acid. This ATPase reaction is beyond the scope of this study but we tentatively propose that there occurred first (auto)adenylation of enzyme and then phosphorolysis of the enzyme-adenylate complex. Autoadenylation of the enzymes involved in the synthesis of (di)adenosine polyphosphates have been reported [34–37] and phosphorolysis of the enzyme-adenylate complex has been demonstrated experimentally for the diadenosine polyphosphate phosphorylase [34,35].

In summary, we have established that although the JAR1 enzyme shows a strong preference for conjugating (−)-JA, it is also capable of synthesizing the Ile conjugate of certain other jasmonates in vitro, albeit at lower rates. Whether these alternate jasmonate conjugates have important signaling roles in plants is currently unknown, but our results suggest that a closer examination of the occurrence and distribution of additional oxylipin conjugates is warranted. The *Arabidopsis jar1* mutant,which is known to be deficient in its accumulation of JA-Ile, may provide additional clues about the role of the other jasmonate conjugates in plant biology. Whether the enzyme's capacity to synthesize adenosine 5′-tetraphosphate has an in vivo function is also unclear. The relatively high cellular concentration of amino acids under most conditions

suggests this reaction would generally be inhibited in the plant.

Acknowledgments: We gratefully acknowledge the financial support given by the Deutsche Forschungsgemeinschaft within the framework of the SFB 648 as a guest visiting support to A.G. and by the National Science Foundation (Award MCB-0130868) to P.S.

References

[1] Staswick, P.E., Tiryaki, I. and Rowe, M.L. (2002) Jasmonate response locus *JAR1* and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole 3-acetic acids in an assay for adenylation. Plant Cell 14, 1405–1415.

[2] Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C. and Suza, W. (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17, 616–627.

[3] Staswick, P.E. and Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell 16, 2117–2127.

[4] Kramell, R., Atzorn, R., Schneider, G., Miersch, O., Brückner, C., Schmidt, J., Sembdner, G. and Parthier, B. (1995) Occurrence and identification of jasmonic acid and its amino acid conjugates induced by osmotic stress in barley leaf tissue. J. Plant Growth Reg. 14, 29–36.

[5] Kleinkauf, H. and von Döhren, H. (1996) A nonribosomal system of peptide biosynthesis. Eur. J. Biochem. 236, 335–351.

[6] McLennan, A.G. (2000) Dinucleoside polyphosphates – friends or foe? Pharmacol. Ther. 87, 73–89.

[7] Sillero, A. and Günther Sillero, M.A. (2000) Synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase and several ligases. Pharmacol. Ther. 87, 91–102.

[8] Guranowski, A. (2004) Metabolism of diadenosine tetraphosphate (Ap_4A) and related nucleotides in plants. Review with historical and general perspective. Front. Biosci. 9, 1398–1411.

[9] Zamecnik, P.C., Stephenson, M.L., Janeway, C.M. and Randerath, K. (1966) Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with purified lysyl sRNA synthetase. Res. Commun. 24, 91–97.

[10] Plateau, P., Mayaux, J.F. and Blanquet, S. (1981) Zinc(II)-dependent synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphopshate by *Escherichia coli* and yeast phenylalanyl transfer ribonucleic acid synthetases. Biochemistry 20, 4654–4662.

[11] Goerlich, O., Foeckler, R. and Holler, E. (1982) Mechanism of synthesis of adenosine(5′)tetraphospo(5′)adenosine (AppppA) by aminoacyl-tRNA synthetases. Eur. J. Biochem. 126, 135–142.

[12] Jakubowski, H. (1983) Synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate and related compounds by plant (*Lupinus luteus*) seryl-tRNA and phenylalanyl-tRNA synthetases. Acta Biochim. Pol. 30, 51–69.

[13] Guranowski, A., Günther Sillero, M.A. and Sillero, A. (1990) Firefly luciferase synthesizes P^1,P^4-bis(5′-adenosyl)tetraphosphate (Ap_4A) and other dinucleoside polyphosphates. FEBS Lett. 271, 215–218.

[14] Ortiz, B.A., Sillero, A. and Günther Sillero, M.A. (1993) Specific synthesis of adenosine(5′)tetraphospho(5′)nucleoside and adenosine(5′)oligophospho(5′)adenosine ($n > 4$) catalyzed by firefly luciferase. Eur. J. Biochem. 212, 263–270.

[15] Pietrowska-Borek, M., Stuible, H-P., Kombrink, E. and Guranowski, A. (2003) 4-Coumarate: coenzyme A ligase has the catalytic capacity to synthesize and reuse various (di)adenosine polyphosphates. Plant Physiol. 131, 1401–1410.

[16] Guranowski, A., Günther Sillero, M.A. and Sillero, A. (1994) Adenosine 5′-tetraphopshate and adenosine 5′-pentaphosphate are synthesized by yeast acetyl coenzyme A synthetase. J. Bacteriol. 176, 2986–2990.

[17] McLennan, A.G., Barnes, L.D., Blackburn, G.M., Brenner, Ch., Guranowski, A., Miller, A.D., Rotllán, P., Soria, B., Tanner, J.A. and Sillero, A. (2001) Recent progress in the study of the intracellular function of diadenosine polyphosphates. Drug Dev. Res. 52, 249–259.

[18] Hoyle, C.H.V., Hilderman, R.H., Pintor, J.J., Schlüter, H. and King, B.F. (2001) Diadenosine polyphosphates as extracellular signal molecules. Drug Dev. Res. 52, 260–273.

[19] Kramell, R., Schneider, G. and Miersch, O. (1996) Resolution of racemic jasmonic acid and abscisic acid by chiral high performance liquid chromatography. Phytochem. Anal. 7, 209–212.

[20] Miersch, O. and Wasternack, C. (2000) Octadecanoid and jasmonate signaling in tomato (*Lycopersicon esculentum* Mill.) leaves: endogenous jasmonates do not induce jasmonate biosynthesis. Biol. Chem. 381, 715–722.

[21] Cohen, J.D., Baldi, B.G. and Slovin, J.P. (1986) $^{13}C_6$-[benzene ring]-indole-3-acetic acid. Plant Physiol. 80, 14–19.

[22] Kramell, R., Schmidt, J., Schneider, G., Sembdner, G. and Schreiber, K. (1988) Synthesis of *N*-(jasmonoyl) amino acid conjugates. Tetrahedron 44, 5791–5807.

[23] Cross, B.E. and Webster, G.R.B. (1970) New metabolites of *Gibberella fujikuroi.* Part XV. *N*-Jasmonoyl- and *N*-dihydrojasmonoyl-isoleucine. J. Chem. Soc. (C), 1839–1842.

[24] Schmidt, H.-J., Kramell, R., Brückner, C., Schneider, G., Sembdner, G., Schreiber, K., Stach, J. and Jensen, E. (1990) Gas chromatographic/mass spectrometric and tandem mass spectrometric investigations of synthetic amino acid conjugates of jasmonic acid and endogenously occurring related compounds from *Vicia faba* L.. Biomed. Environ. Mass Spectrom. 19, 327–338.

[25] Kramell, R., Miersch, O., Hause, B., Ortel, B., Parthier, B. and Wasternack, C. (1997) Amino acid conjugates of jasmonic acid induce jasmonate-responsive gene expression in barley (*Hordeum vulgare* L.). FEBS Lett. 414, 197–202.

[26] Bohlmann, F., Wegner, P., Jakupovic, J. and King, R.M. (1984) Struktur und synthese von *N*-(acetoxyjasmonoyl)-phenylalaninmethylester aus *Praxelis clematidea*. Tetrahedron 40, 2537–2540.

[27] Wasternack, C. (2006) Biosynthesis, signal transduction and action in: Plant Hormone Signaling (Hedden, P. and Thomas, S., Eds.), Annals of Plant Review, Vol. 24, pp. 185–228, Blackwell, Oxford, UK.

[28] Weber, H., Vick, B.A. and Farmer, E.E. (1997) Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. Proc. Natl. Acad. Sci. USA 16, 10473–10478.

[29] Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J. and Wasternack, C. (2000) Tissue-specific oxylipin signature of tomato flowers – allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J. 24, 113–126.

[30] Miersch, O., Weichert, H., Stenzel, I., Hause, B., Maucher, H., Feussner, I. and Wasternack, C. (2004) Constitutive overexpression of allene oxide cyclase in tomato (*Lycopersicon esculentum* cv. Lukullus) elevates levels of jasmonates and octadecanoids in flower organs but not in leaves. Phytochemistry 65, 847–856.

[31] Miersch, O., Kramell, R., Parthier, B. and Wasternack, C. (1999) Structure–activity relations of substituted, deleted or stereospecifically altered jasmonic acid in gene expression of barley leaves. Phytochemistry 50, 353–361.

[32] Led, J.J., Switon, W.K. and Jensen, K.F. (1983) Phosphorolytic activity of *Escherichia coli* glycyl-tRNA synthetase towards its cognate aminoacyl adenylate detected by ^{31}P-NMR spectroscopy and thin layer chromatography. Eur. J. Biochem. 136, 469–479.

[33] Guédon, G., Ebel, J.-P. and Remy, P. (1987) Yeast phenylalanyl-tRNA synthetase: evidence for the formation of ADP by phosphorolysis of enzyme-bound aminoacyl adenylate. Biochimie 69, 1175–1181.

[34] Guranowski, A. and Blanquet, S. (1986) Diadenosine-5′,5‴-P^1,P^4-tetraphosphate α,β-phosporylase from yeast supports nucleoside diphosphate-phosphate exchange. J. Biol. Chem. 261, 5943–5946.

[35] Lazewska, D. and Guranowski, A. (1990) P^{α}-chiral phosphorothioate analogues of bis(5′-adenosyl)tetraphosphate (Ap_4A); their enzymatic synthesis and degradation. Nucleic Acids Res. 18, 6083–6088.

[36] Booth, J.W. and Guidotti, G. (1995) Analleged yeast polyphosphate kinase is actually diadenosine-5′,5‴-P^1,P^4-tetraphosphate-α,β-phosphorylase. J. Biol. Chem. 270, 19377–19382.

[37] Madrid, O., Martín, D., Atencia, E.A., Sillero, A. and Günther Sillero, M.A. (1998) T4-DNA ligase synthesizes dinucleoside polyphosphates. FEBS Lett. 433, 283–286.