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Structure–activity relations of substituted, deleted or stereospecifically altered jasmonic acid in gene expression of barley leaves

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Abstract

Jasmonic acid and 66 structurally related compounds were tested to find the structural requirements which induce the expression of jasmonate-responsive genes in barley. An intact cyclopentanone ring as well as a pentenyl side chain exhibiting only minor alterations are necessary for this activity. The (−)-enantiomeric and the (+)-7-*iso*-enantiomeric structure increase activity of jasmonoyl compounds.

Keywords: Hordeum vulgare; jasmonic acid; structurally related compounds; structure-activity relationship; altered gene expression

1. Introduction

(−)-Jasmonic acid (**1**) is the basic structure of a naturally occurring family of compounds named jasmonates which inhibit or stimulate several events in plant growth and development (for reviews see Sembdner & Parthier (1993); Creelman & Mullet (1997)). Also numerous plant responses to biotic and abiotic stress are mediated by jasmonates. Wounding of leaves (Bergey, Hoi, & Ryan, 1996), osmotic stress or desiccation (Lehmann, Atzorn, Brückner, Reinbothe, Leopold, Wasternack, & Parthier, 1995), touch (Weiler, Albrecht, Groth, Xia, Luxem, Liß, Andert, & Spengler, 1993), elicitation (Gundlach, Müller, Kutchan, & Zenk, 1992) or pathogen attack (Penninckx, Eggermont, Terras, Thomma, Desamblanx, Buchala, Metraux, Manners, & Broekaert, 1996) are accompanied with elevated levels of jasmonic acid followed by an altered gene expression (Wasternack & Parthier, 1997). Among the numerous jasmonate-inducible genes, some were identified to code for proteinase inhibitors, seed and vegetative storage proteins, leaf thionins, enzymes such as lipoxygenases and phenylalanine ammonia lyases or of phytoalexine, alkaloid and protein metabolism [cf. Creelman & Mullet (1997); Wasternack & Parthier (1997)].

Jasmonate-responsive events follow perception of jasmonate at the site of a receptor or a binding protein. However, nothing is known so far on putative jasmonate receptors or proteins which bind jasmonates specifically. At this stage of knowledge, indications on structural requirements of a distinct response to jasmonates may help to elucidate any perception mechanism.

Jasmonic acid (**1**) and its methyl ester **1**-Me are ubiquitously occurring constituents of different plant tissues (Creelman & Mullet, 1997; Meyer, Miersch, Büttner, Dathe, & Sembdner, 1984). In addition, the native isomer (+)-7-*iso*-jasmonic acid (**5**), amino acid conjugates, amides (**26**), hydroxylated and dehydrated jasmonates were found [cf. Sembdner, Atzorn, & Schneider (1994); Kramell, Atzorn, Schneider, Miersch, Brückner, Schmidt, Sembdner, & Parthier (1995); Miersch, Knöfel, Schmidt, Kramell, & Parthier (1998)]. All of these derivatives are chiral compounds biosynthesized in distinct enantiomeric forms. In order to analyze jasmonate-responsive events, the (±)-jasmonic acid (**6**) or its methyl ester **6**-Me were frequently used for applications. However, due to the simultaneous occurrence of different stereoisomeric forms and various metabolites in plant tissues, the question arises on stereospecificity and on the most active compound.

First, structure-activity relationships in jasmonate-induced events were analyzed by bioassays (Yamane, Sugawara, Suzuki, Shimamura, & Takahashi, 1980; Meyer, Miersch, Vorkefeld, & Sembdner, 1985; Miersch & Sembdner, 1985; Koda, 1992; Koda, Takahashi, Kikuta, Greulich, Toshima, & Ichihara, 1996; Ward & Beale, 1993; Ueda, Kato, Yamane, & Takahashi, 1981;

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Ravid, Kahn, & Sachs, 1975). They revealed that the configurational requirements were different for the effects of jasmonates on germination, senescence, plant growth, levels of carotenoids or chlorophyll, tuber formation, and coleoptile growth. Thus, different receptors for these signal compounds cannot be excluded (Koda, 1992, Koda, 1997). In the last years, several octadecanoids and other precursors of jasmonic acid (**1**) and structural related substances were tested for their ability to induce coiling of tendrils (Weiler, Kutchan, Gorba, Brodschelm, Niesel, & Bublitz, 1994; Weiler, 1997), alkaloid formation (Blechert, Brodschelm, Holder, Kammerer, Kutchan, Müller, Xia, & Zenk, 1995), odor formation (Krumm, Bandemer, & Boland, 1995), and tuber induction (Koda et al., 1996). Most interestingly, potato plants and *Arabidopsis* accumulate upon wounding a different pattern of octadecanoids and jasmonates which led to the suggestion that plants may have an 'oxylipin signature' (Weber, Vick, & Farmer, 1997). Furthermore, jasmonates and their amino acid conjugates can alter gene expression in barley leaves without interconversion to each other (Kramell, Miersch, Hause, Ortel, Parthier, & Wasternack, 1997). Activity was preferentially found for JA amino acid conjugates and their molecular mimics, the indanoyl amino acid conjugates, when tested by odor formation (Krumm et al., 1995), whereas octadecanoids such as 12-*oxo*-phyto-dienoic acid (PDA, **47**) were most active in tendril coiling (Weiler et al., 1994).

More indicative than application of these compounds is the determination of their endogenous rise in correlation with the response under study. Among the few results available so far, a transient rise of PDA (**47**), followed by a transient rise of JA (**1**), was detected in elicitated cell cultures (Parchmann, Gundlach, & Mueller, 1997), which finally form phytoalexines (Gundlach et al., 1992; Müller, Brodschelm, Spannagel, & Zenk, 1993). Also tendril coiling is preceded by an endogenous rise of PDA (**47**) (Stelmach, Müller, Hennig, Laudert, Andert, & Weiler, 1990). Contrasting, in barley leaves stressed by sorbitol treatment JA (**1**) accumulates earlier and to 10-fold higher level than PDA (**47**) and their corresponding methyl esters (**1**-Me, **47**-Me) inducing altered gene expression (Kramell et al., in prep.). A transient rise of JA upon wounding of tomato leaves was also observed repeatedly and preceded proteinase inhibitor 2 gene expression (Conconi, Miquel, Browse, & Ryan, 1996). All these data support the hypothesis that different plants may have a preferential signal among octadecanoids, jasmonates and JA amino acid conjugates, all of them originate from α-linolenic acid.

The preferential activity of JA in altered gene expression of barley leaves prompted us to study the structural requirements of this type of identified signals in more detail. We used the very sensitive jasmonate-responsive gene expression of barley leaves, the mRNA accumulation of genes coding for a 6 kDa and 23 kDa protein, respectively, as a reporter system for the response to the various compounds applied. Comparison of qualitative and quantitative data concern the following structural requirements: (i) a pentenyl side chain or only minor modifications; (ii) an intact pentanone ring; (iii) an acetic acid side chain; and (iv) an (−)-enantiomeric or (+)-7-*iso*-enantiomeric structure.

2. Results and discussion

2.1. Synthesis of jasmonate-related compounds, and analysis of biological activity of jasmonates by gene expression studies

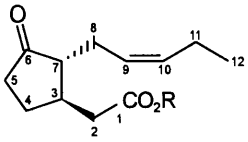
In order to test structural requirements for activity, we synthesized jasmonate compounds carrying subtle alterations in (i) the pentenyl side chain, (ii) the stereochemistry, (iii) the cyclopentanone ring or (iv) the carboxylic side chain (Fig. 1). The biological activity of these compounds, we recorded upon treatment of barley leaf segments for 24 h, by mRNA accumulation of the most sensitive JA-responsive genes which code for a 6 kDa protein, identified as a thionin (Andresen, Becker, Schlüter, Burges, Parthier, & Apel, 1992), and for a 23 kDa protein designated as JIP-23 (jasmonate-induced protein of 23 kDa) which is of unknown function (Hause, Demus, Teichmann, Parthier, & Wasternack, 1996).

Fig. 2 shows concentration dependencies for some of these compounds. As exemplified for (−)- and (+)-forms of JA-Me (**1**-Me, **2**-Me), and JA-Leu (**12**, **13**), respectively, the transcript accumulation for JIP-6 and JIP-23 differed at least one order of magnitude between both stereoisomeric forms. By comparing other pairs of stereoisomeric forms, finally 5×10^{-5} M was chosen to be a representative concentration for the test (Fig. 3).

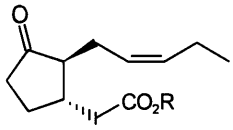
Previous studies revealed maximum accumulation for JIP-23 mRNA at 24 h upon treatment of leaf segments with JA or its methyl ester (Lehmann et al., 1995; Kramell et al., 1997). At this time, various jasmonates were sufficiently taken up (Dathe, Kramell, Daeter, Kramell, Slovik, & Hartung, 1993). Except PDA (**47**), the biogenetic precursor of JA, which is converted into 7-*iso*-JA (**5**) up to 2 nmol/g fresh weight during 24 h, other applied compounds seem to be little metabolized during this time. In addition, the major metabolites formed from JA in barley leaves were hydroxylated jasmonates (Sembdner, Meyer, & Miersch, 1988) which do not effect mRNA induction as shown here (Fig. 1). Amino acid conjugates, being active metabolites of JA, do not appear after JA application (Kramell et al., 1997).

2.2. Altered pentenyl side chain

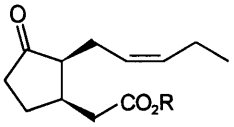
Preliminary studies with jasmonates carrying an altered pentenyl side chain revealed that this part of the



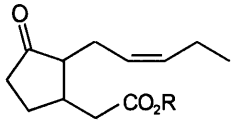
		%JIP-mRNA	
		23	6
R = H (3*R*,7*R*)-JA [(-)-JA]	1	50	25
R = CH_3	1 -Me	120	110
R = (*R*)-mandelyl	3	8	10



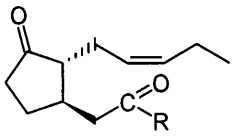
		%JIP-mRNA	
		23	6
R = H (3*S*,7*S*)-JA [(+)-JA]	2	5	2
R = CH_3	2-Me	20	10
R = (*S*)-mandelyl	4	5	3



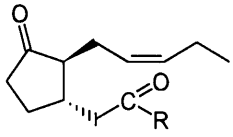
		23	6
R = H (3*R*,7*S*)-JA [(+)-7-iso-JA]	5	50	50
R = CH_3	5-Me	120	105



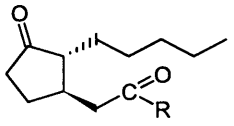
		23	6
R = H (+/-)-JA	6	30	50
R = CH_3	6-Me	100	100
R = C_4H_9	7	100	80



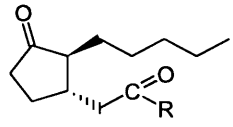
		23	6
R = (*S*)-Ile [(3*R*,7*R*)-*N*-Jasmonyl-(*S*)-isoleucine]	8	40	40
R = (*S*)-Ile-Me	8-Me	90	100
R = (*S*)-Val	10	25	20
R = (*S*)-Leu	12	40	30
R = (*S*)-Trp	14	0	4
R = (*S*)-Phe	16	35	50
R = (*S*)-Met	18	5	25
R = (*S*)-Tyr	20	30	60
R = (*S*)-Ala-Me	21	90	100
R = (*R*)-Val	22	1	1
R = (*R*)-Phe	24	0	0
R = tyramine	26	0	0



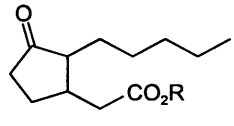
		23	6
R = (*S*)-Ile [(3*S*,7*S*)-*N*-Jasmonyl-(*S*)-isoleucine]	9	20	10
R = (*S*)-Val	11	8	6
R = (*S*)-Leu	13	+	1
R = (*S*)-Trp	15	0	3
R = (*S*)-Phe	17	2	10
R = (*S*)-Met	19	+	1
R = (*R*)-Val	23	1	1
R = (*R*)-Phe	25	0	0



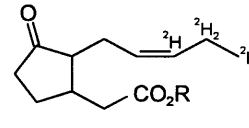
		%JIP-mRNA	
		23	6
R = (*S*)-Ile [(3*R*,7*R*)-*N*-9,10-Dihydro-jasmonoyl-(*S*)-isoleucine]	27	2	1
R = (*S*)-Phe	29	0	0



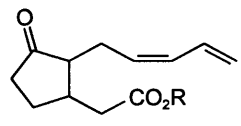
		%JIP-mRNA	
		23	6
R = (*S*)-Ile [(3*S*,7*S*)-*N*-9,10-Dihydro-jasmonoyl-(*S*)-isoleucine]	28	1	0
R = (*S*)-Phe	30	0	0



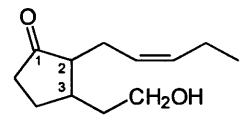
		23	6
R = H 9,10-Dihydro-JA	31	1	2
R = CH_3	31- Me	20	25



		23	6
R = H (10-^{2}H,11-$^2H_2$,12-$^2H_3$)JA	32	20	2
R = CH_3	32- Me	100	25

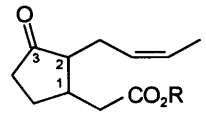


		23	6
R = H 11,12-Didehydro-JA	33	0	0
R = CH_3	33- Me	0	+

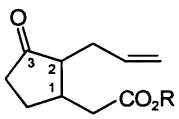


		23	6
2-(2*Z*-pentenyl)-3-(2-hydroxy-ethyl)cyclopentanone	34	1	1

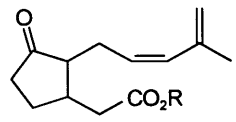
Fig. 1. Structures of jasmonates and related compounds which were tested on their ability to induce *jip23* and *jip6* expression. Upon Northern blot analysis (5 μg total RNA per lane), transcript accumulation was recorded quantitatively as described in 'Experimental' and is given in % of (±)-JA-Me (100%).



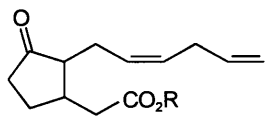
		%JIP-mRNA	
		23	6
R = H	3-Oxo-2-(2Z-butenyl) cyclopentane-1-acetic acid	20	30
R = CH_3	**35**- Me	100	100



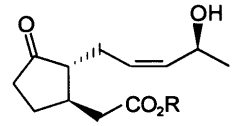
		%JIP-mRNA	
		23	6
R = H	3-Oxo-2-(2-propenyl) cyclopentane-1-acetic acid	0	0
R = CH_3	**36**- Me	0	+



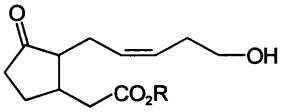
		23	6
R = H	11-Methylene-JA	0	+
R = CH_3	**37**-Me	0	+



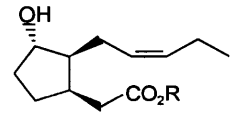
		23	6
R = H	12-Methylene-JA	5	5
R = CH_3	**38**-Me	25	50



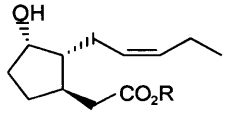
		23	6
R = H	(11*S*)-Hydroxy-JA	0	0
R = CH_3	**39**- Me	0	0



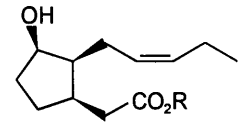
		23	6
R = H	12-Hydroxy-JA	0	0
R = CH_3	**40**- Me	0	0



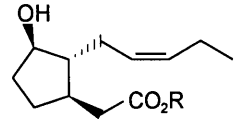
		%JIP-mRNA	
		23	6
R = H	(3*R*,6*S*,7*S*)-Cucurbic acid [(+)-CA]	+	0
R = CH_3	**41**- Me	+	1



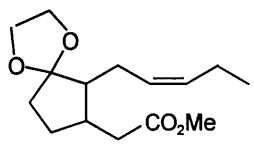
		%JIP-mRNA	
		23	6
R = H	(3*R*,6*S*,7*R*)-CA	0	0
R = CH_3	**42**- Me	0	0



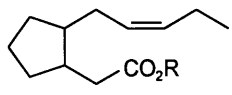
		23	6
R = H	(3*R*,6*R*,7*S*)-CA	5	10
R = CH_3	**43**- Me	30	50



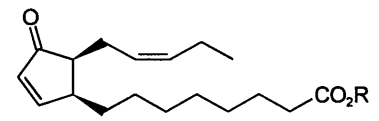
		23	6
R = H	(3*R*,6*R*,7*R*)-CA	0	1
R = CH_3	**44**- Me	0	1



		23	6
JA-Me-ethylene-ketal	**45**	20	20



		23	6
R = H	6-Deoxy-JA	0	0
R = CH_3	**46**- Me	0	0



		23	6
R = H	12-Oxo-PDA	40	15
R = CH_3	**47**- Me	100	30

Fig. 1—*continued.*

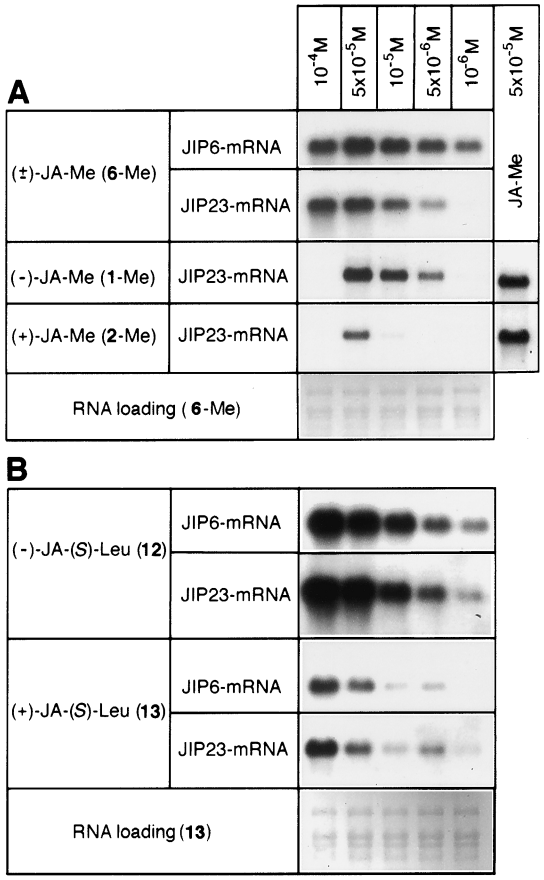


Fig. 2. Concentration dependency of **1**-Me, **2**-Me and **6**-Me (A) and **12** and **13** (B) with respect to accumulation of JIP-6-mRNA and JIP-23-mRNA, respectively. For A 5 μg and for B 20 μg total RNA were subjected for Northern blot analysis. Constant RNA loading is exemplified given for **6**-Me (A) and **13** (B). In the case of Northern blot analysis for treatments with **1**-Me and **2**-Me, RNAs of **6**-Me were added for comparison. All other RNAs were probed on identical filters.

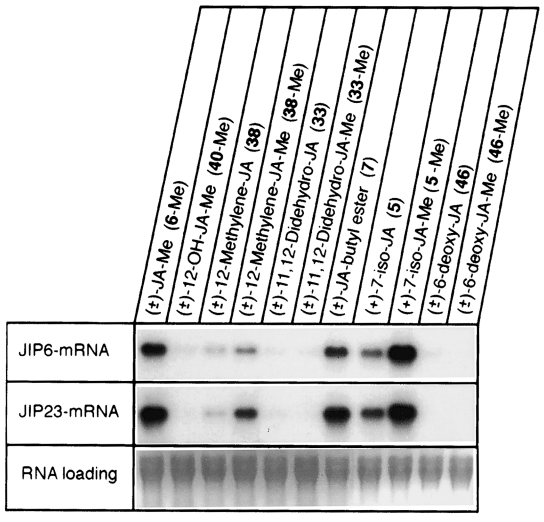


Fig. 3. Northern blot analysis of accumulation of mRNAs coding for JIP-6 or JIP-23. 5 μg total RNA of leaf segments treated with 5×10^{-5} M solution of compounds indicated were used. RNA loading is given for **6**-Me.

jasmonate molecule represents an important structural determinant for bioactivity (Yamane et al., 1980; Miersch & Sembdner, 1985; Ueda et al., 1981). Here, we have tested jasmonates that were altered in each position of the pentenyl side chain. Hydroxylation at C-11 (**39**) or at C-12 (**40**) led to completely inactive jasmonates. This contrasts with proteinase inhibitor gene expression in potato leaves where tuberonic acid and its methyl ester (**40**, **40**-Me) are active (Ishikawa, Yoshihara, & Nakamura, 1994). A double bond between carbon 11 and 12, as given in compounds (**33**) and (**33**-Me), reduces biological activity down to zero. Introduction of a methylene residue at C-11 (**37**) lowers the biological activity dramatically, as well as elongation by a methylene group at C-12 (**38**) leads to reduced activity. From comparison of compounds **33**, **37** and **38**, all of them possessing an additional double bond, we can assume that the conjugated π-electron system occurring in **33**, **33**-Me, **37** and **37**-Me led to complete inactivity, whereas little activity was observed for **38** and **38**-Me which lack a conjugated π-electron system. Shortening of the pentenyl side chain by omission of C-12 (**35**) did not reduce biological activity, whereas a lack of two carbon atoms, C-12 and C-11 (**36**), eliminated all biological activity. A deuterated pentenyl side chain, which leads to a more bulky structure, did not change the biological activity of the methyl ester (**32**-Me), but reduced significantly that of the free acid (**32**).

The (±)-9,10-dihydroderivative of JA (**31**) or its methyl ester (**31**-Me) behave clearly less biological activity than (±)-JA (**6**) or its methyl ester (**6**-Me), respectively. For (−)- or (+)-derivatives of the JA isoleucine conjugate (**8** and **9**, respectively), the alteration of positions 9 and 10 to the corresponding dihydroderivatives (**27** and **28**) reduces biological activity much more dramatically than that occurring after identical changes of (±)-JA (**6**) to 9,10-dihydro-JA (**31**). Similar results were found for the (−)-enantiomer (**29**) and the (+)-form (**30**) of 9,10-dihydrojasmonoyl-(*S*)-conjugate containing bulky phenylalanine residues, each compared with corresponding jasmonoyl conjugates **16** and **17**, respectively. It remains to be elucidated whether the pentenyl side chain is less important in jasmonic acids than in its amino acid conjugates for the overall bioactivity.

2.3. The cyclopentanone ring and stereospecific aspects

Jasmonates are characterized by a cyclopentanone ring exhibiting chiralic centers at C-3 and C-7 which lead to two diastereoisomers, the naturally occurring (−)-jasmonic acid (**1**, *trans*-membered side chains, 3*R*,7*R*) and (+)-7-*iso*-jasmonic acid (**5**, *cis*-derivative, 3*R*,7*S*). Due to the keto-enol tautomerism a stable mixture of them contains about 10% of compound **5**. Enantiomeric forms are (+)-jasmonic acid (**2**) and (−)-7-*iso*-jasmonic acid.

When comparing biological activities of stereoisomeric forms of jasmonic acids (**1**, **2**, **5**), their methyl esters (**1**-Me, **2**-Me, **5**-Me), amino acid conjugates (**8**–**25, 27**–**30**), as well as of compounds altered in the cyclopentanone structure (**41**–**47**), our studies support the following suggestions:

1. The free acids, their methyl esters or their amino acid conjugates, each of them in the (3*R*,7*R*)-form (**1**, **3**, **8**, **10**, **12**, **16**, **18**), are more active than the corresponding (3*S*,7*S*)-forms (**2**, **4**, **9**, **11**, **13**, **17**, **19**), whereas similar activity was found for the (3*R*,7*R*)- and (3*R*,7*S*)-configuration when **1** and **5** or **1**-Me and **5**-Me were considered.
2. An intact cyclopentanone structure is necessary for biological activity. 6-deoxy-jasmonic acid (**46**) and its methyl ester (**46**-Me) are inactive. Among the various cucurbic acid derivatives (**41**-**44**), only compounds with *cis*-membered side chains (**41, 41**-Me, **43** and **43**-Me) show activity. The presence of a hydroxyl group at C-6 with (6*R*)-configuration as in **43**-Me led to a compound with significant higher activity than **41** or **41**-Me. If **6**-Me is blocked in the keto-function as given by its methylene ketal (**45**), a remarkable decrease of activity was observed. Cucurbic acid isomers and their methyl esters showed similar effects on root growth of rice seedlings (Seto, Kamuro, Quian, & Shimizu, 1992). In the system **43**-Me was more active than **41**-Me, whereas **42**-Me or **44**-Me, possessing *trans*-membered side chains are inactive. It is interesting to note that also in the methyl 3-methyl-jasmonate (3-methyl-**1**-Me) a *cis*-orientation of the side chains is favoured (Ward, Gaskin, Sessions, Koda, Wasternack, & Beale, 1997). However, this compound is completely inactive in several bioassays, presumably due to the bulky structure of the methyl group (Ward et al., 1997). Also at C-7, bulky structures led to completely inactive derivatives (Ward et al., 1997). Introduction of a double bond in the cyclopentanone-moiety as in compounds **47** or **47**-Me did not alter biological activity.

2.4. Conjugation with amino acids and modification of the carboxylic acid side chain

Amino acid conjugates of (−)-JA (**1**) or (+)-7-*iso*-JA (**5**) are naturally occurring derivatives (Sembdner et al., 1994). They accumulate upon stress caused by treatment of barley leaves with sorbitol solution (Kramell et al., 1995). In wounded tomato leaves (−)-JA-(*S*)-Ile (**8**) accumulates concomitantly with (−)-JA (**1**), but quantitatively only 10% of **1** (Dorans, Boland, Krumm, Atzorn, Kramell, Wasternack, & Bowles, in prep.). Biological activities differ remarkably due to the type of the naturally occurring and structurally altered amino acid conjugates:

1. Most of the aromatic amino acid conjugates were inactive. In the case of (−)-JA-L-Phe (**16**), some activity was detectable, which was less in the (+)-configuration (3*S*, 7*S*) (**17**).
2. Decarboxylation of the tyrosine conjugate (**20**), leads to the tyramine conjugate, which is inactive.
3. The activity was dependent on the stereospecificity of the compounds not only with respect to the chiral centers in the jasmonic acid moiety (3*R*,7*R* vs. 3*S*,7*S*, cf. above), but also with respect to the amino acid moiety. Each amino acid conjugate in the (*R*)-configuration was inactive, whereas its (*S*)-configurated counterpart was found to be active (**22**, **24**, **23**, **25** vs. **10**, **16**, **11**, **17**, respectively).

Methylation of the carboxyl group increased activity in most of the jasmonic acid derivatives, whereas esterification with mandelic acid (**3**, **4**) or reduction to the alcohol (**34**) led to lowering or inactivation of the biological activity. Interestingly, the n-butyl ester of jasmonic acid (**7**) showed methyl jasmonate-like activity.

Compounds elongated at the carboxylic side chain as shown for the biosynthetic precursor of **5**, the 12-oxophytodienoic acid (**47**) keep their ability to accumulate JIP transcripts, however, odd numbered side chains lead to inactive substances (Wasternack, Ortel, Miersch, Kramell, Beale, Greulich, Feussner, Hause, Krumm, Boland, & Parthier, 1998; Beale et al., in prep.). Similar results were found for the release of volatiles by the Lima bean (Boland, Hopke, & Piel, 1998).

To summarize the intact carbonyl group, pentenyl side chain, and acetic acid side chain are essential prerequisites for the expression of *jip23* and *jip6*. Methylation of the carboxyl group led to higher activity of the respective compound. Stereoisomeric forms with the (−)-(3*R*,7*R*)-structure showed always highest activities. All of these biologically active compounds occur in plants and suggest an optimization of the jasmonate structure during evolution.

We have shown recently, that the JA-(*S*)-isoleucine conjugates (**8**, **9**) are highly active in *jip* expression of barley without being cleaved in the cells (Kramell et al., 1997). Now, we show that also methyl esters of conjugates (**8**-Me, **21**) exhibit high activity. Removal of the carboxyl group in the tyrosine conjugate **20**, turned to an inactive tyramine conjugate (**26**), indicating that this part of the conjugate is essential, and demonstrating that the amide structure was not saponified to active (−)-JA (**1**).

The structure–activity relationship demonstrates that jasmonate-induced gene expression in barley is suited to evaluate the structures of the most active jasmonate-like signals. However, we have to keep in mind that the activity of the naturally acting signal is determined by its local concentration at a distinct time, or can result from a concerted action of several signals. Both scenarios can be observed. The latter case occurs for proteinase inhibi-

tor 2 gene expression in tomato leaves which is dependent on jasmonate and ethylene (O'Donnell, Calvert, Atzorn, Wasternack, Leyser, & Bowles, 1996). It remains to be elucidated wether the 'oxylipin signature', a distinct ratio of octadecanoids and jasmonates in different plants, exhibits specific signalling qualities (Weber et al., 1997). In addition it is known that JA-like responses such as tuber formation, exhibit preferential activity with a JA metabolite, the 12-β-D-glucopyranosyloxy-jasmonic acid (*O*-glycoside of **40**) (Koda, 1992).

3. Experimental

3.1. Plant material and incubation conditions

Primary leaves of 7 day-old seedlings of barley (*Hordeum vulgare* L. cv. Salome) grown under greenhouse conditions with continuous light ($130\,\mu mol\,m^{-2}\,s^{-1}$) at 20–25° were used in all experiments. Segments of 5 cm, cut 1 cm below the tip were floated on freshly prepared aqueous solutions of the respective compound. If not indicated otherwise, 50×10^{-5} M concentration was used of each compound. Prior to preparation of the solutions each of the compounds were tested for purity by HPLC (see below). Floating was performed in Petri dishes containing 50 ml solution per 25 segments at 25° under continuous white light ($120\,\mu mol\,m^{-2}\,s^{-1}$) provided by fluorescent lamps (Narva, Berlin, NC 250/01). After incubation leaves were frozen with liquid nitrogen and kept at −70° until RNA isolation.

3.2. Source and synthesis of compounds tested

(±)-Methyl jasmonate (**6**-Me) and methyl 9,10-dihydrojasmonate (**31**-Me) were from Firmenich (Geneva, Switzerland) and used for the preparation of other jasmonates.

All compounds were purified by HPLC and checked for purity by GC-MS. Free acids were obtained by alkaline hydrolysis of the corresponding methyl esters according to Miersch, Preiss, Sembdner, and Schreiber, 1987. Jasmonic acid (**6**) was separated by chiral phase-HPLC into (−)-jasmonic acid (**1**) and (+)-jasmonic acid (**2**) (Kramell, Schneider, Miersch, & Parthier, 1996). Methyl esters were obtained by treatment of free acids with ethereal diazomethane, **1**-butyl ester (**7**) by esterification of **1** with *n*-butanol in the presence of sulfuric acid. Amino acid conjugates (**8**–**25**, **27**–**30**) including the tyramine conjugate (**26**) were synthesized, and the enantiomers were separated as described in Kramell, Schmidt, Schneider, Sembdner, and Schreiber (1988) and Schneider, Kramell, and Brückner (1989). Compounds **3** and **4** were synthesized according to Kramell, Schneider, Schmidt, Sembdner, and Schreiber (1990). (10-^{2}H,11-^{2}H$_2$, 12-^{2}H$_3$)JA (**32**) was synthesized according to Miersch (1991), and 12-hydroxy-JA (**40**) was prepared according to Kitahara, Iwamoto, Takagi, Mori, and Matsui (1984). 11,12-didehydro-JA (**33**), 3-*oxo*-2-(2*Z*-butenyl)cyclopentane-1-acetic acid (**35**), 3-*oxo*-2-(2-propenyl)cyclopentane-1-acetic acid, 11-methylene-JA (**37**) and 12-methylene-JA (**38**) were synthesized *via* the Wittig reaction as described in Miersch (1991) starting from methyl 3-*oxo*-2-(formylmethyl)cyclopentane-1-acetate with subsequent use of allylidene-triphenylphosphoran, ethylidene-triphenylphosphoran, methylidene-triphenylphosphoran, 2-methylallylidene-triphenylphosphoran and 3-butenylidene-triphenylphosphoran. (−)-(11*S*)-hydroxy-JA (**39**) was prepared by microbiological hydroxylation of JA with *Aspergillus niger* (Miersch, Porzel, & Wasternck, in press). (+)-7-*iso*-JA (**5**) and cucurbic acid (**41**) were isolated from the culture filtrate of *Botryodiplodia theobromae* (Miersch et al., 1987). Cucurbic acid isomers (3*R*,6*S*,7*R*)-CA (**42**), (3*R*,6*R*,7*S*)-CA (**43**) and (3*R*,6*R*,7*R*)-CA (**44**) could be obtained from a mixture of (+)-7-*iso*-JA/(−)-JA by reduction with $NaBH_4$ according to Dathe, Schindler, Schneider, Schmidt, Porzel, Jensen, and Yamaguchi (1991). 2-(2*Z*-pentenyl)-3-(2-hydroxyethyl)-cyclopentanone (**34**) was synthesized *via* reduction of the ethylene-ketal (**45**) of JA-Me with $LiAlH_4$. JA-Me-ethylene-ketal (**45**) was synthesized from **6**-Me and ethylene-glycol, and 6-deoxy-JA (**46**) by Clemmensen reduction of **6**-Me. 12-*oxo*-phytodienoic acid (**47**) was prepared enzymatically from α-linolenic acid by a flax seed extract according to Zimmerman and Feng (1978) and was further purified by TLC and HPLC.

All jasmonates (except **5**, **5**-Me and **47**) carrying a steric centre near the keto-group possess, the *cis*-membered side chains and the *trans*-membered side chains in a ratio of about 1:9 (Miersch, 1991).

3.3. Isolation of RNA and Northern blot analysis

Using a modification of the procedure described in Andresen et al. (1992), total RNA of leaves was extracted by phenol-$CHCl_3$-isoamyl alcohol treatment as described in Chirgwin, Przybyla, MacDonald, and Rutter (1979). RNA electrophoresis (5 µg total RNA per lane if not otherwise indicated), and Northern blot analysis with JIP-specific cDNA probes were performed according to Sambrook, Fritsch, and Maniatis (1989). For hybridization the plasmids pHvJ256 and pHvJ3015 were used. They contain cDNAs coding for barley JIP-6 mRNA and JIP-23 mRNA, respectively (Andresen et al., 1992). After transfer of RNA onto nitrocellulose BA 85 (Schleicher & Schüll, Darmstadt, Germany) filters were hybridized with cDNA inserts prepared from the plasmids pHvJ256 and pHvJ3015 and labelled with DIG-labelled α-dATP (Boehringer, Mannheim, Germany) according to the manufactures instructions. Hybridization was performed in 50% (v/v) formamide, 6 × SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, $100\,\mu g\,ml^{-1}$ sheared salmon sperm

DNA at 65° for 20 h each followed by 0.1 × SSC, 0.1% (w/v) SDS at 50° for 20 min. Finally, filters were exposed to AGFA X-ray film with an intensifying screen at −70° for various times. RNA size was estimated using the RNA ladder of 0.24–9.5 kD.

3.4. Quantitation of transcript accumulation

For quantitative comparison transcript accumulation was measured by densitometric tracing (Sharp JX 325) in two different approaches. (i) After Northern blot transfer, the filters were exposed for at least three different times onto the X-ray film to give a linear range of intensity of the signal for **6**-Me. mRNA accumulated in response to **6**-Me was used as an internal standard at two different concentrations of total RNA (2.5 and 5 μg per lane) per each filter. After scanning of each signal area subtracted by background intensity for a distinct area compound, mean values from the different filters and the different exposures were related to the corresponding value for **6**-Me (100%). (ii) Due to the fact that only one size of transcript is detectable by the above mentioned cDNA probes [cf. Andresen et al. (1992); Leopold, Hause, Lehmann, Graner, Parthier, & Wasternack (1996)] dot blot analysis could be performed in addition to the above mentioned approach. Hence, five different concentrations of total RNA of each sample were applied in identical volumes onto the NC filter to give an approximate identical size of the signal. After hybridization and film exposure signal intensity per each peak area were detected by densitometric tracing. From each concentration dependence the mean value was calculated in the linear range of densitometry. Subsequently, these mean values of each compound were correlated to that of **6**-Me (100%) determined with the same filter.

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References

Andresen, I., Becker, W., Schlüter, K., Burges, J., Parthier, B., & Apel, K. (1992). *Plant Molecular Biology*, *19*, 193.

Beale et al. (1998). (in prep).

Bergey, D. R., Hoi, G.-A., & Ryan, C. A. (1996). *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 12953.

Blechert, S., Brodschelm, W., Holder, S., Kammerer, L., Kutchan, T. M., Müller, M. J., Xia, Z. Q., & Zenk, M. H. (1995). *Proceedings of the National Academy of Sciences of the United States of America*, *92*, 4099.

Boland, W., Hopke, J., & Piel, J. (1998). In: P. Schreier, M. Herderich, H.-U. Humpf, & W. Schwab (Eds.), *Natural product analysis* (p. 255). Braunschweig/Wiesbaden: Friedr. Vieweg & Sohn Verlagsgesellschaft mbH.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979). *Biochemistry*, *18*, 5294.

Creelman, R. A., & Mullet, J. E. (1997). *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 355.

Conconi, A., Miquel, M., Browse, J. A., & Ryan, C. A. (1996). *Plant Physiology*, *111*, 797.

Dathe, W., Schindler, C., Schneider, G., Schmidt, J., Porzel, A., Jensen, E., & Yamaguchi, I. (1991). *Phytochemistry*, *30*, 1909.

Dathe, W., Kramell, H.-M., Daeter, R., Kramell, R., Slovik, S., & Hartung, W. (1993). *Journal of Plant Growth Regulation*, *12*, 133.

Dorans, A. M. I., Boland, W., Krumm, T., Atzorn, R., Kramell, R., Wasternack, C., & Bowles, D. J. (1998). *Journal of Biological Chemistry* (in prep.).

Gundlach, H., Müller, M. J., Kutchan, T. M., & Zenk, M. H. (1992). *Proceedings of the National Academy of Sciences of the United States of America*, *89*, 2389.

Hause, B., Demus, U., Teichmann, C., Parthier, B., & Wasternack, C. (1996). *Plant & Cell Physiology*, *37*, 641.

Ishikawa, A., Yoshihara, T., & Nakamura, K. (1994). *Biosciences and Biotechnological Biochemistry*, *58*, 544.

Kitahara, T., Iwamoto, M., Takagi, Y., Mori, K., & Matsui, M. (1984). *Agricultural and Biological Chemistry*, *48*, 1731.

Koda, Y. (1992). *International Review of Cytology*, *135*, 155.

Koda, Y. (1997). *Physiologia Plantarum*, *100*, 639.

Koda, Y., Takahashi, K., Kikuta, Y., Greulich, F., Toshima, H., & Ishihara, A. (1996). *Phytochemistry*, *41*, 93.

Kramell, R., Schmidt, J., Schneider, G., Sembdner, G., & Schreiber, K. (1988). *Tetrahedron*, *44*, 57.

Kramell, R., Schneider, G., Schmidt, J., Sembdner, G., & Schreiber, K. (1990). *Zeitschrift für Naturforschung*, *45b*, 377.

Kramell, R., Atzorn, R., Schneider, G., Miersch, O., Brückner, C., Schmidt, J., Sembdner, G., & Parthier, B. (1995). *Journal of Plant Growth Regulation*, *14*, 29.

Kramell, R., Schneider, G., & Miersch, O. (1996). *Phytochemical Analysis*, *7*, 209.

Kramell, R., Miersch, O., Hause, B., Ortel, B., Parthier, P., & Wasternack, C. (1997). *FEBS Letters*, *414*, 197.

Kramell et al. (1998). *Journal of Biological Chemistry* (in prep.)

Krumm, T., Bandemer, K., & Boland, W. (1995). *FEBS Letters*, *377*, 523.

Lehmann, J., Atzorn, R., Brückner, C., Reinbothe, S., Leopold, J., Wasternack, C., & Parthier, B. (1995). *Planta*, *197*, 156.

Leopold, J., Hause, B., Lehmann, J., Graner, A., Parthier, B., & Wasternack, C. (1996). *Plant Cell & Environment*, *19*, 675.

Miersch, O., Preiss, A., Sembdner, G., & Schreiber, K. (1987). *Phytochemistry*, *26*, 1037.

Miersch, O., & Sembdner, G. (1985). *Acta Universitatis Agriculturae*, *33*, 101.

Miersch, O. (1991). *Zeitschrift für Naturforschung*, *46b*, 1724.

Miersch, O., Knöfel, H.-D., Schmidt, J., Kramell, R., & Parthier, B. (1998). *Phytochemistry*, *47*, 327.

Miersch, O., Porzel, A., & Wasternack, C. (1998). *Phytochemistry* (in press).

Meyer, A., Miersch, O., Büttner, C., Dathe, W., & Sembdner, G. (1984). *Journal of Plant Growth Regulation*, *3*, 1.

Meyer, A., Miersch, O., Vorkefeld, S., & Sembdner, G. (1985). *Acta Universitatis Agriculture*, *33*, 471.

Müller, M., Brodschelm, W. Spannagel, E., & Zenk, M. H. (1993). *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 7490.

O'Donnell, P. J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H. M. O., & Bowles, D. J. (1996). *Science*, *274*, 1914.

Parchmann, S., Gundlach, H., & Mueller, M. J. (1997). *Plant Physiology*, *115*, 1057.

Penninckx, I. A. M. A., Eggermont, K., Terras, F. R. G., Thomma, B. P. H. J., Desamblanx, G. W., Buchala, A., Metraux, J. P., Manners, J. M., & Broekaert, W. F. (1996). *Plant Cell*, *8*, 2309.

Ravid, U., Kahn, R., & Sachs, R. M. (1975). *Journal of Agriculture and Food Chemistry*, *23*, 835.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning. A laboratory manual* (2nd ed.) NY: Cold Spring Harbor Laboratory Press.

Schneider, G., Kramell, R., & Brückner, C. (1989). *Journal of Chromatography*, *483*, 459.

Sembdner, G., & Parthier, B. (1993). *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*, 569.

Sembdner, G., Atzorn, R., & Schneider, G. (1994). *Plant Molecular Biology*, *26*, 1459.

Sembdner, G., Meyer, A. & Miersch, O. (1988). In: *Abstract of the 13th International Conference on Plant Growth Substances*, Calgary, Canada, July 17–22 (p. 173).

Seto, H., Kamuro, Y., Quian, Z. H., & Shimizu, T. (1992). *Journal of Pesticide Science*, *17*, 61.

Stelmach, B. A., Müller, A., Hennig, P., Laudert, D., Andert, L., & Weiler, E. W. (1990). *Phytochemistry*, *47*, 539.

Ueda, J., Kato, J., Yamane, H., & Takahashi, N. (1981). *Plant Physiology*, *52*, 305.

Ward, J. L., & Beale, M. H. (1993). *Journal of Chemistry Society Perkin Transactions I*, 379.

Ward, J. L., Gaskin, P., Sessions, R., Koda, Y., Wasternack, C., & Beale, M. H. (1997). *Tetrahedron*, *53*, 8181.

Wasternack, C., Ortel, B., Miersch, O., Kramell, R., Beale, M. H., Greulich, F., Feussner, I., Hause, B., Krumm, T., Boland, W., & Parthier, B. (1998). *Journal of Plant Physiology*, *152*, 345.

Wasternack, C., & Parthier, B. (1997). *Trends in Plant Science*, *2*, 302.

Weber, H., Vick, B. A., & Farmer, E. E. (1997). *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 10473.

Weiler, E. W. (1990). *Phytochemistry*, *47*, 539.

Weiler, E. W., Albrecht, T., Groth, B., Xia, Z.-Q., Luxem, M., Liß, H., Andert, L., & Spengler, P. (1993). *Phytochemistry*, *32*, 591.

Weiler, E. W., Kutchan, T. M., Gorba, T., Brodschelm, W., Niesel, U., & Bublitz, F. (1994). *FEBS Letters*, *345*, 9.

Weiler, E. (1997). *Naturwissenschaften*, *84*, 340.

Yamane, H., Sugawara, J., Suzuki, I., Shimamura, E., & Takahashi, N. (1980). *Agricultural Biology and Chemistry*, *44*, 2857.

Zimmerman, D. C., & Feng, P. (1978). *Lipids*, *13*, 313.