Applied jasmonates accumulate extracellularly in tomato, but intracellularly in barley

Heike Bücking[a], Hella Förster[b], Irene Stenzel[b], Otto Miersch[b], Bettina Hause[b,*]

[a] *Department of Plant Biology, Michigan State University, East Lansing, MI 48824-1312, USA*
[b] *Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle, Germany*

Received 9 January 2004; revised 11 February 2004; accepted 11 February 2004

First published online 3 March 2004

Edited by Ulf-Ingo Flügge

Abstract Jasmonic acid (JA) and its derivatives are well-characterized signaling molecules in plant defense and development, but the site of their localization within plant tissue is entirely unknown. To address the question whether applied JA accumulates extracellularly or intracellularly, leaves of tomato and barley were fed with ^{14}C-labeled JA and the label was localized in cryofixed and lyophilized leaf tissues by microautoradiography. In tomato the radioactivity was detectable within the apoplast, but no label was found within the mesophyll cells. By contrast, in barley leaf tissues, radioactivity was detected within the mesophyll cells suggesting a cellular uptake of exogenously applied JA. JA, applied to leaves of both plants as in the labeling experiments, led in all leaf cells to the expression of JA-inducible genes indicating that the perception is completed by JA signal transduction.

Key words: ^{14}C-labeled jasmonic acid; Barley; In situ hybridization; Microautoradiography; Tomato

1. Introduction

Jasmonic acid (JA) and its derivatives, commonly known as jasmonates, are lipid-derived cyclopentanones which are signals in plant defense and in distinct developmental stages [1]. Jasmonates are synthesized from α-linolenic acid via 12-oxo-phytodienoic acid (OPDA). Initial steps in JA biosynthesis catalyzed by a 13-lipoxygenase, an allene oxide synthase (AOS), and an allene oxide cyclase (AOC), are localized within the chloroplast, whereas the final steps including reduction of OPDA and β-oxidation of the carboxylic acid side chain occur in peroxisomes [2,3]. JA, exogenously applied or generated intracellularly following stresses such as wounding or osmotic stress, induces the expression of numerous genes. Among them are those encoding proteinase inhibitors (PINs) of tomato (*Lycopersicon esculentum*) [4], defensins or thionins of *Arabidopsis* [5,6], and a 23 kDa protein (jasmonate-induced protein, JIP-23) of barley (*Hordeum vulgare*) whose function is so far unknown [7,8].

In the last decade several jasmonate signaling pathways were described. Among them the wound-response pathway is studied in detail, particularly in tomato. Here, JA as well as the 18 amino acid peptide systemin were shown to be essential signals at least of a local wound response. Systemin and its precursor protein are upstream-located components of a wound-induced, intercellular signaling pathway that requires both the biosynthesis and action of JA. Systemin is processed from the 200 amino acid peptide prosystemin which is constitutively expressed in vascular bundles [9]. The binding of systemin to its membrane-located receptor [10] is followed by rapid signaling events including altered levels of cytosolic Ca^{2+} or the activation of mitogen-activated protein kinases (for review, see [1,11]), and leads to the expression of 'early genes'. Concomitantly, a systemin-dependent activation of AOC occurring upon substrate generation may result in a rapid increase in JA, which is known to induce the expression of prosystemin [4,12]. Consequently, an amplification in wound signaling by a systemin-dependent AOC activation and by JA-inducible prosystemin gene expression may occur in vascular bundles, where both processes are located [9,13,14]. However, the expression of PIN genes takes place in spongy and palisade parenchyma [15] suggesting that an active transfer or a diffusion of a signal compound from phloem to the neighboring mesophyll cells should occur. Despite these indications for a role of JA in local wound signaling, it is unknown how and where JA accumulation and perception takes place [16]. Although the existence of a JA receptor is reasonable, no such receptor has so far been identified and it is even unknown whether the JA perception and/or the accumulation of jasmonates occur extracellularly or intracellularly.

Here, the site of JA accumulation is addressed in tomato and barley leaf tissues. The monocotyledonous plant barley shows obvious differences to dicotyledonous plants with respect to JA biosynthesis [17,44] and the function of JA during development of seedlings [7]. In particular, wounding of barley plants does not result in JA biosynthesis and a concomitant gene expression (Wasternack, personal communication). Moreover, for barley different JA-signaling pathways have been proposed, since exogenously applied JA and levels of endogenously formed JA led to the expression of different genes possibly due to independent perception sites [18,19]. The data presented here suggest that following application of JA jasmonates accumulate extracellularly in tomato, but intracellularly in barley, accompanied in both plants by a complete JA signaling as indicated by the expression of respective marker genes.

*Corresponding author. Fax: (49)-345-5582 1509.
E-mail address: bhause@ipb-halle.de (B. Hause).

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; DIG, digoxigenin; JA, jasmonic acid; JIP, jasmonate-induced protein; OPDA, 12-oxo-phytodienoic acid; PBS, phosphate-buffered saline; PIN, proteinase inhibitor

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00178-4

2. Materials and methods

2.1. Plant materials, treatments and determination of radioactivity

L. esculentum Mill. cv. Lukullus or plants of the transgenic line *35S::AOCantisense* were grown as described [20]. *H. vulgare* cv. Salome was grown in soil for 7 days as described previously [7]. (±)-[2-^{14}C]JA was synthesized according to [21]. For treatments, tomato leaves cut at the petiole and 5 cm long segments of primary barley leaves were floated on 50 μM JA, or 50 μM (±)-[2-^{14}C]JA (specific radioactivity 1 mCi/mmol), respectively, and incubated at 25°C under continuous white light (120 μmol/m^2/s, provided by fluorescent lamps, Narva, Berlin, Germany, NC 250/01) for 4 or 24 h, respectively. Incubated leaves were used either for cryofixation and embedding or were extracted by methanol. JA metabolites were separated according to [22]. After adding scintillant to the high performance liquid chromatography (HPLC) eluents, radioactivity was directly measured in a Tri-Carb Liquid Scintillation Analyzer (Beckman, Germany).

2.2. Sample preparation for microautoradiography

Small pieces of leaves were cryofixed by plunging in supercooled propane and freeze-dried under low temperature and high vacuum conditions (CFD, Leica, Bensheim, Germany). To prevent a re-crystallization of water in the plant tissues, the freeze-drying was started at −100°C for 7 days and continued for 2 days each at −90°C, −80°C and −60°C. The samples were then slowly warmed up to room temperature, followed by a pressure infiltration according to the method described by [23] directly in 100% epoxy resin [24]. After polymerization dry sections of 1 μm thickness were made with glass knives and mounted on poly-L-lysine-coated slides.

2.3. Microautoradiography

The dry sections were overlaid with a thin layer of the nuclear research emulsion L4 (Ilford, Dreieich, Germany) with a crystal size of 0.11 μm. The thickness of the dried layer was approximately 5 μm. After exposure at 4°C for at least 6 months, the film was developed with the fine grain developer D19 A/S [25], rinsed in water and then fixed with a commercial b/w fixer (Tetenal, Norderstedt, Germany). As controls, slides without sections and with unlabeled leaf sections were processed in the same way as described for the labeled samples. On these slides almost no silver grains were visible. Sections were counterstained with toluidine blue and examined with a microscope Axioskop (Zeiss, Jena, Germany). Micrographs were taken with a CCD camera (Sony, Tokyo, Japan) and processed by Photoshop (Adobe, Seattle, WA, USA).

2.4. In situ hybridization

Small pieces of tomato leaves (freshly harvested or floated on 45 μM JA for 24 h) were fixed with 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, 135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2), dehydrated in a graded ethanol series and embedded in Paraplast (Sigma, Taufkirchen, Germany). Cross-sections of 8 μm were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated. In situ hybridization was carried out as described recently [17] using digoxigenin (DIG)-labeled antisense and sense RNA probes obtained from PIN2 cDNA by in vitro transcription.

The leaf segments of barley were fixed in 3% (w/v) paraformaldehyde in PBS, dehydrated, embedded in polyethylene glycol, and cut into 5 μm sections according to [7]. To visualize JIP23 transcript accumulation in barley epidermal cells, the leaves were fixed and the abaxial epidermal strips were isolated and treated with cell wall-digesting enzymes to facilitate penetration of the cDNA probe into the cells [26]. Both sections and epidermal strips were processed for in situ hybridization with the DIG-labeled cDNA of JIP23 as described in [7].

2.5. Extraction of RNA and Northern blot analysis

Total RNA was purified from frozen tissues by treatments with buffered phenol:chloroform:isoamyl alcohol 25:24:1 (v/v/v) and 20 μg per lane was subjected to RNA gel blot analysis according to [27]. Blots were hybridized at 60°C for 16 h with ^{32}P-labeled cDNAs of tomato *PIN2* (800 bp fragment) according to [12]. The loading control was performed by ethidium bromide staining of rRNA.

3. Results

3.1. After application of radioactive JA, the radioactivity is restricted to the apoplast in a tomato leaf, but occurs intracellularly in a barley leaf

To analyze where JA is located in tomato and in barley leaf tissues following treatments with JA, leaves of both species were floated on [^{14}C]JA and processed for microautoradiography. By commonly used preparation techniques of light and electron microscopy any diffusible compounds such as phytohormones cannot be localized within tissues, since aqueous chemical specimen preparation does not preserve the in vivo distribution of these compounds. Therefore, we used a combination of cryofixation, freeze-drying and pressure infiltration in an epoxy resin to prevent losses and dislocations of water-soluble compounds during specimen preparation. This 'water-free' method does not significantly affect the distribution of highly diffusible compounds or ions [28]. Moreover, the structural preservation of the tissue was sufficient, and only mild shrinking artifacts were detectable in the palisade parenchyma of tomato (Fig. 1A).

The distribution of the radioactively labeled JA is indicated in the micrographs by the occurrence of black silver grains in the film layer covering the sections. In tomato leaves treated for 24 h with [^{14}C]JA, the label was restricted to the apoplastic compartment (Fig. 1A–C). After 4 h of incubation the pattern of labeling was identical, but the labeling was weaker (not shown). The enlargements of the spongy (Fig. 1B) and the palisade parenchyma (Fig. 1C) show clearly that most of the radioactivity has accumulated in the intercellular space and in the cell wall area. Only a few silver grains can be seen within the cells. This might be due to some naturally occurring radioactivity during the exposure time of about 6 months.

By contrast, in barley leaf segments the radioactivity was homogeneously distributed throughout the tissue (Fig. 1D,E). Outside the leaf tissue almost no silver grains were found (not shown). The label occurred evenly distributed over the complete cell area mostly occupied by the central vacuole. An accumulation of radioactivity in cytoplasm or organelles like nuclei and chloroplasts could not be detected. This points to a localization of radioactivity mainly in the vacuole of leaf cells. Whether there is a specific transport of jasmonates into the vacuole and how such a transport could be facilitated is completely unknown to date.

In the experimental set-up used for microautoradiography a metabolic conversion of JA could not be excluded. Therefore, parallel samples were used to extract neutral and acidic metabolites followed by separation by radio-HPLC. In both plant tissues, a main part of the radioactivity was due to free [^{14}C]JA, whereas the rest consisted of a mixture of acidic JA metabolites.

3.2. Accumulation of mRNA of JA-inducible genes occurs in all cells of leaves after JA treatment

To test whether the accumulation of jasmonates in tomato and barley was accompanied by a JA response in all cells which accumulate jasmonates at their outside and inside, respectively, the expression of corresponding JA-inducible genes such as *PIN2* for tomato and *JIP23* for barley was examined by in situ hybridization (Figs. 2 and 3). Hybridizations with the DIG-labeled PIN2 antisense probe revealed an occurrence of PIN2 mRNA in all mesophyll cells of tomato after treat-

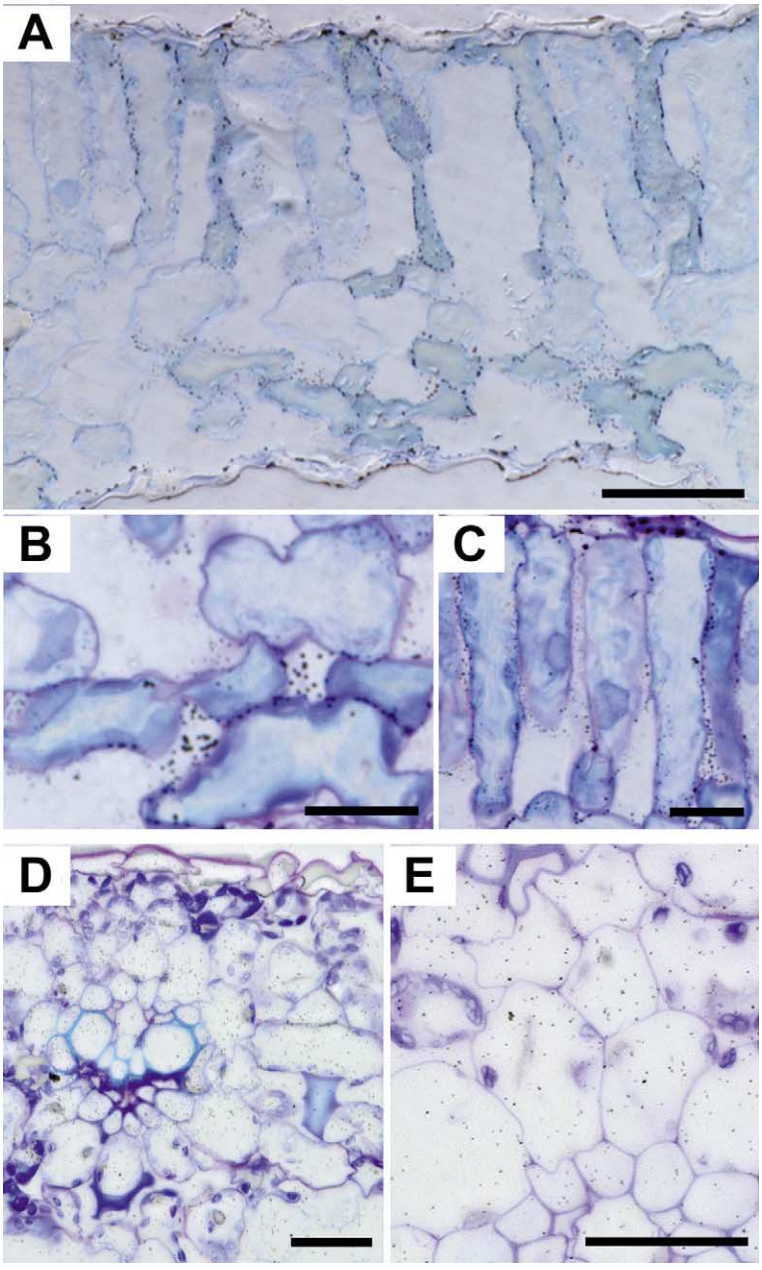

Fig. 1. Subcellular localization of radioactivity in leaves of tomato (A–C) and barley (D,E) after application of [^{14}C]JA. Tomato leaflets (A–C) and barley leaf segments (D,E) were floated on a 50 μM solution of (±)-[2-^{14}C]JA for 24 h and processed for microautoradiography followed by staining with toluidine blue. The presence of ^{14}C can be seen as black dots. Bars represent 25 μm in A,D,E and 10 μm in B,C.

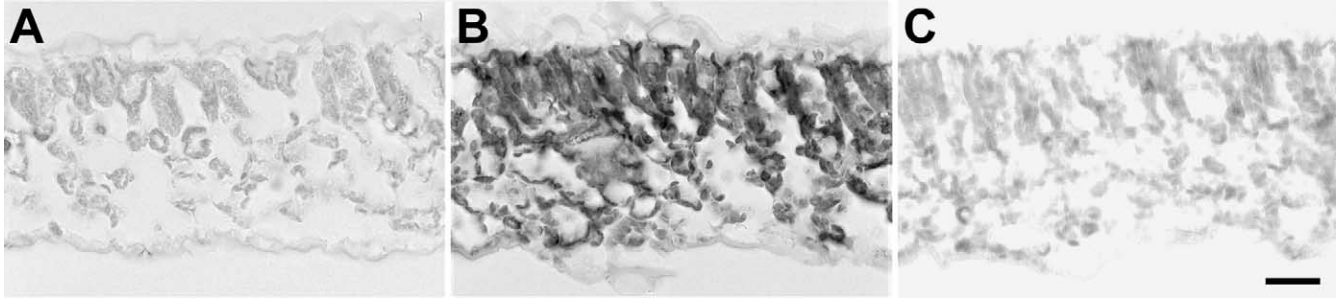

Fig. 2. Accumulation of PIN2 mRNA in excised leaves of 6 week old tomato plants cv. Lukullus after JA treatment. Excised leaves were floated for 24 h on water (A) or on 50 μM JA (B,C) and embedded in paraplast. Cross-sections were subjected to in situ hybridizations with a DIG-labeled antisense RNA probe (A,B) or a DIG-labeled sense RNA probe (C). Note the positive staining with the antisense RNA probe in all mesophyll cells of a JA-treated leaf (B). Bar represents 25 μm for all micrographs.

ment with JA (Fig. 2B), whereas in untreated leaf tissues no label could be detected (Fig. 2A). The control performed by hybridizations of treated leaves with the DIG-labeled PIN2 sense probe did not show any label (Fig. 2C).

In barley leaf segments, untreated or treated with JA, in situ hybridizations were performed by using DIG-labeled JIP23 cDNA. The untreated leaf showed a clear staining of the vascular bundles (Fig. 3A,B, untreated). This corresponds to the constitutive expression of *JIP23* during development of barley seedlings, where JIP23 mRNA is detectable in the scutellum and in the companion cells of the scutellar node and the primary leaf [7]. After JA treatment, JIP23 mRNA accumulated in all mesophyll cells of the leaf (Fig. 3A,B, treated). A clear signal within the epidermis was not detectable in leaf cross-sections, presumable due to the thin cytoplasmic seam of these cells. However, whole-mount in situ hybridizations of isolated epidermal strips showed that JIP23 mRNA is also clearly detectable in epidermal cells of leaves treated with JA for 24 h (Fig. 3C). These JA responses indicate a complete JA signal transduction in both plant species irrespective of whether radioactivity accumulated extracellularly or intracellularly.

3.3. JA application to tomato leaves leads to PIN2 mRNA accumulation in the absence of a JA biosynthesis

Tomato leaves treated with JA accumulate mRNAs encoding enzymes of JA biosynthesis such as AOC [12]. To test whether the extracellularly accumulating jasmonates (Fig. 1A–C) are sufficient for an intracellular response (Fig. 2B), the expression of *PIN2* was examined in transgenic tomato plants expressing *AOC* in an antisense direction. These plants are strongly reduced in their capacity to synthesize JA upon wounding [12]. In leaves of the *AOCantisense* plant PIN2 mRNA could be detected at a similar level as in a wild type plant (Fig. 4). These data suggest that *PIN2* expression does not need endogenous JA biosynthesis if JA is applied exogenously.

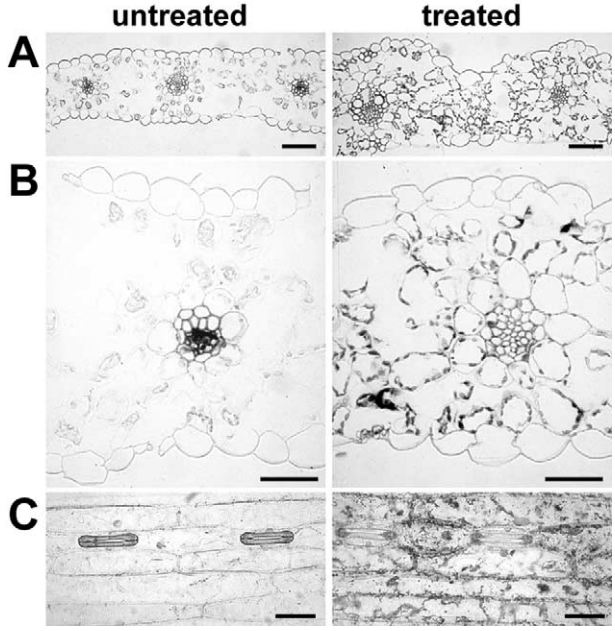

Fig. 3. Accumulation of JIP23 mRNA within barley leaf segments. Segments of primary leaves of 6 day old barley seedlings were processed for in situ hybridization directly or after a treatment with 50 μM JA for 24 h. Labeling was performed with a DIG-labeled cDNA encoding JIP23 using cross-sections (A,B) or isolated epidermal strips (C). The label is visible within all mesophyll and epidermal cells after treatment of leaf segments with JA, whereas in untreated leaves only the phloem exhibits staining. Bars represent 50 μm in A and 25 μm in B,C.

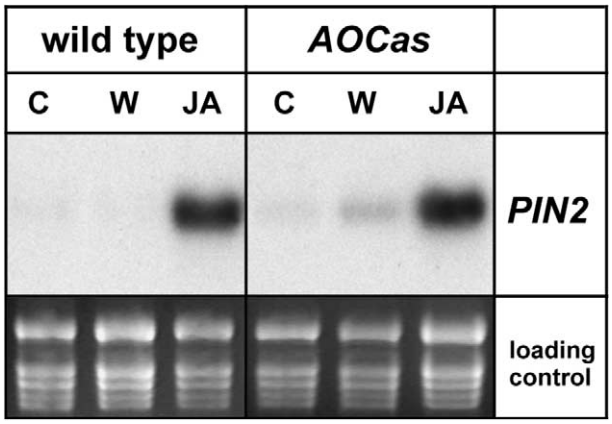

Fig. 4. PIN2 mRNA accumulation in response to JA treatment of tomato cv. Lukullus (wild type) and *35S*::*AOCantisense* (*AOCas*) plants. Leaves of 6 week old plants were freshly excised (C) or were floated on distilled water (W) or on 50 μM of JA (JA) for 24 h. Total RNA was extracted and subjected to Northern blot analysis. Loading of total RNA (20 μg per lane) was controlled by ethidium bromide staining.

4. Discussion

Plants adapt to changes in the environment by altered gene expression mediated by JA in an intra- and intercellular as well as in an interorganismic manner [29]. In particular, upon wounding of tomato leaves, JA was shown to be a signal in local as well as in systemic responses (for review, see [1]). Upon wounding of tomato, JA is synthesized preferentially within vascular tissues [12], whereas the expression of JA-responsive genes is induced in mesophyll cells [4]. Based on these data, a signal transfer from the phloem to the mesophyll cells was proposed [30]. Jasmonates generated in the phloem and released in response to wounding might be readily transported through the apoplast and diffuse to cells surrounding the veins. Here, wound-induced JA may be perceived and cause the expression of *PINs*.

However, nothing is known on the mobility and perception of JA. It is even unknown whether jasmonates are localized extracellularly or intracellularly following application. To get first answers to this question, we localized radioactivity which was applied as ^{14}C-labeled JA to tomato leaves. Using microautoradiography the radioactivity was detectable exclusively within the apoplast, whereas mesophyll cells were free of label (Fig. 1). However, in situ hybridization of PIN2 mRNA, which occurs JA-responsively, indicated a complete perception and transduction in JA signaling. The expression of *PIN2* was detected in all mesophyll cells of a JA-treated tomato leaf (Fig. 2), and was unchanged in leaves of transgenic plants impaired in the biosynthesis of JA by antisense expression of *AOC* (Fig. 4). These data suggest that exogenously applied JA is sufficient to induce the expression of a JA-responsive

gene without additional endogenous biosynthesis. This conclusion is strengthened by the fact that the positive feedback in JA biosynthesis, observed in several plants such as *Arabidopsis* and *Nicotiana tabacum* [31–33], does not lead to measurable quantities of endogenous JA in tomato during the first 24 h of treatment [34].

In the used experimental set-up a metabolic conversion of JA could not be hindered. Up to now, different metabolic routes have been detected, resulting in methylation, amino acid conjugation or adenylation at the carboxylic acid group [35–37], in decarboxylation [38] or in hydroxylation of the pentenyl side chain [39]. It is not known whether the metabolic conversion of JA takes place extracellularly or intracellularly, but most of the metabolic products exhibit biological activity [40]. From the data presented here, it is not possible to shed light on whether or not the radioactively labeled JA entered the cell and where a putative metabolism takes place. Nevertheless, because of the exclusive location of radioactivity within the apoplast in the case of tomato, one can speculate that JA does not enter the cells and that the exogenously applied JA might be perceived at receptors on the plasma membrane. This speculation is supported by the fact that in potato the elevation of the amount of endogenous JA by constitutive overexpression of AOS failed to induce the JA-responsive gene *PIN2*, probably because of jasmonate sequestration [41].

In contrast to the data shown for tomato, the radioactivity could be detected within the mesophyll cells when [^{14}C]JA was applied to leaves of barley. This result highlights significant differences with respect to the role of JA in both species. Contrary to tomato, barley leaves do not exhibit an increase in endogenous levels of JA upon wounding (C. Wasternack, personal communication). Several JA-responsive genes, including JIP23, respond to both exogenous JA and elevation of the amount of endogenous JA, whereas others respond exclusively to exogenous JA [18,19]. The authors supposed that exogenous JA localized in the apoplast might be recognized by plasma membrane receptor(s), and endogenous JA by cytoplasmic receptor(s). Our results support this model: the radioactivity was detected *within* the mesophyll cells when [^{14}C]JA was applied to leaves of barley. Furthermore, it could be confirmed that the expression of *JIP23* was induced in all leaf cells after application of JA (Fig. 3). These data suggest that exogenously applied JA can penetrate the mesophyll cells to be recognized both by plasma membrane receptors and by cytoplasmic receptors in barley leaves.

Binding proteins or receptors for JA have not yet been identified so far, either by biochemical approaches or by screens for mutants [42,43]. This might be due to the existence of several redundant JA receptors, which would hinder their identification by screening for JA insensitivity. Nevertheless, the different sites of accumulation of applied jasmonates shown here for a dicotyledonous and a monocotyledonous plant represent another facet of the complexity of the action of JA.

Acknowledgements: We gratefully acknowledge Ulrike Huth for dependable technical assistance. We also thank Claus Wasternack for helpful discussions and for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Project HA2655/3-1).

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