Pergamon

PII: S0031-9422(96)00562-6

Phytochemistry, Vol. 44, No. 4, pp. 589–592, 1997
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0031-9422/97 $17.00+0.00

METHYLJASMONATE-INDUCED ACCUMULATION OF COUMAROYL CONJUGATES IN BARLEY LEAF SEGMENTS

JUSTIN LEE, THOMAS VOGT, JÜRGEN SCHMIDT, BENNO PARTHIER and MARIAN LÖBLER*

Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle, Germany

(*Received in revised form* 9 *July* 1996)

Key Word Index—*Hordeum vulgare*; Poaceae; barley; 4-coumaroylagmatine; 4-coumaroylputrescine; methyljasmonate.

Abstract—The effect of methyljasmonate on the induction of phenolic components in barley leaf segments was investigated. RP-HPLC of methanol extracts showed that three compounds accumulate to high concentrations in response to methyljasmonate treatment. Two of them were identified as *N*-(*E*)-4-coumaroylputrescine and *N*-(*E*)-4-coumaroylagmatine by UV-spectroscopy and mass spectrometry. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Jasmonate has the potential to change the pattern of gene expression in many plant species (for a review see ref. [1]). Some of the jasmonate-induced genes play a role in the wound response of tobacco and tomato [2] and others are believed to be part of the defence system against pathogens [3]. In barley, the scenario seems to be quite different. Most jasmonate-induced proteins [4, 5] are not detected after wounding or powdery mildew infection [6], nor are the pathogenesis-related proteins detected after jasmonate treatment [7]. However, we found an increase in the mRNA level of jasmonate-regulated genes [8], two of which are thought to be involved in defence reactions [9]. One is homologous to chalcone synthase that delivers the precursor for the biosynthesis of flavonoid phytoalexins [9]. Another gene is homologous to caffeic acid *O*-methyl transferase needed for lignin biosynthesis [10] and cell wall strengthening [11]. Besides an increase in wall-bound ferulic acid (unpublished observation) the methanol-soluble phase contained high amounts of other UV-absorbing components. Three major jasmonate-inducible components were purified by HPLC and their structures determined by combined HPLC/electrospray mass spectrometry.

RESULTS AND DISCUSSION

We have recently reported a methyljasmonate-induced (JAME-induced) accumulation of a transcript encoding a protein homologous to caffeic acid-*O*-methyl transferase [8]. While a JAME-induced increase of ferulic acid is observed in the cell wall-associated fraction only minor or no changes of ferulic acid concentration were found in the methanolic fraction (unpublished observation). On the other hand, three jasmonate-induced phenolic compounds were found to accumulate to high amounts in the methanol-soluble fraction (Figs 1(a) and 2). The compounds appear in the chromatogram as three prominent peaks A, B and C, and are hardly detected in water treated leaf segments (Fig. 1). The levels of all three compounds rose over a 48 hr time period of jasmonate treatment (Fig. 2) while the levels in water floated leaf segments remained below 200 pmol mg dry wt^{-1} (data not shown). All three substances accumulate between 15- and 85-fold after 48 hr of jasmonate treatment (Fig. 2) when compared to the water controls. Therefore, we were interested to determine their identity. These phenolics from the methanol-soluble fraction were purified on RP-HPLC.

The purified substances were investigated by combined HPLC/electrospray-mass spectrometry as well as HPLC/MS-MS. Compounds **1** and **2** (Fig. 1(a), peaks A and B) show $[M+H]^+$ ions at *m/z* 235 and 277, respectively. The collision induced dissociation (CID) spectra of both $[M+H]^+$ ions exhibit a prominent ion *m/z* 147 (base peak, coumaroyl moiety). While in the CID spectrum of **1** the ions at *m/z* 89 and 72 are derived from the putrescine unit, ions at *m/z* 260, 218, 217, 131, 114 and 72 in the CID spectrum of **2** characterize the presence of an agmatine moiety [12]. Therefore, **1** and **2** are 4-coumaroylputrescine and 4-coumaroylagmatine, respectively, on the basis of their HPLC/UV and HPLC/MS data and a comparison

* Author to whom correspondence should be addressed.

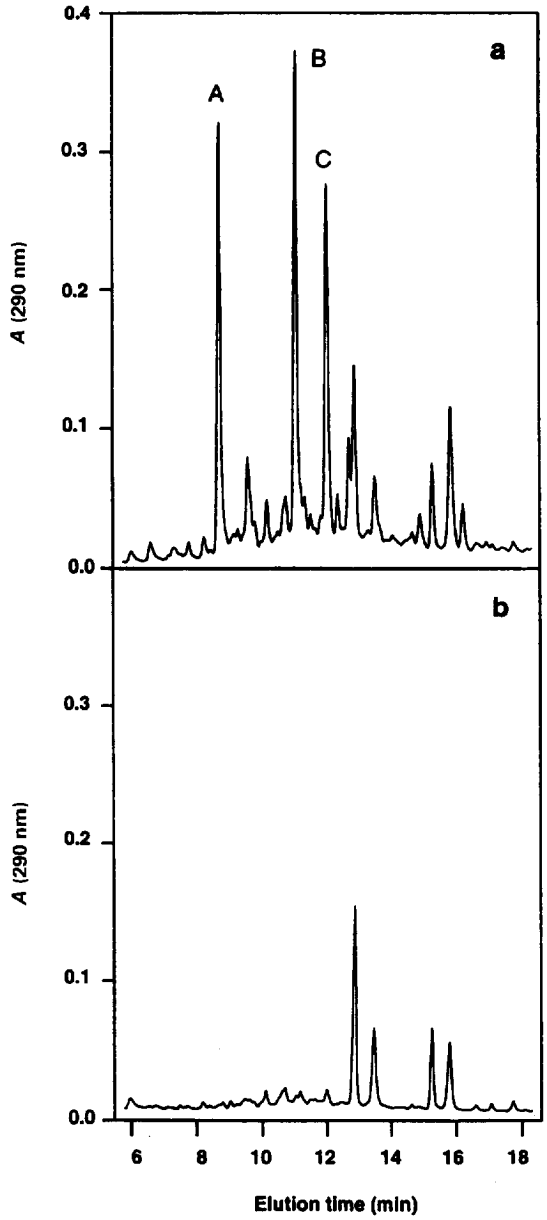


Fig. 1. Analytical RP-HPLC of methanolic extracts of barley leaf segments floated for 48 hr on 45 μM methyljasmonate (a) or on water (b). The peaks A, B and C correspond to the three major jasmonate-induced 4-coumaric acid conjugates.

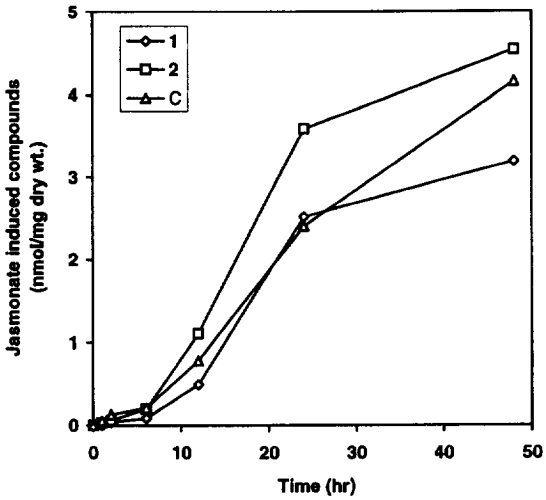
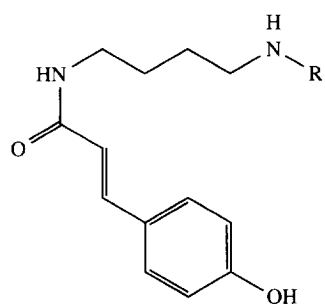


Fig. 2. Time course of methyljasmonate-induced accumulation of compounds **1**, **2** and C. Leaf segments were floated for 48 hr on 45 μM methyljasmonate. In the control experiments (water-floated leaf segments) the concentration of the compounds never exceeded 0.2 nmol mg dry wt^{-1} (not shown). The data presented here are the means of two experiments.



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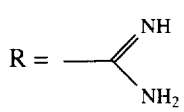
2 R = 

of these data with those of the respective conjugates identified by Peipp *et al.* [12]. Compound C (Fig. 1a, peak C) shows a $[M+H]^+$ ion at *m/z* 331 and a collision induced daughter ion at *m/z* 147 in the electrospray-mass spectra as well as the same UV-spectrum as **1** and **2** and, therefore, also represents a coumaroyl derivative, but its full structure elucidation needs further investigation.

In independently performed experiments, Peipp *et al.* [12] found an increase of several phenolic substances, including the observed 4-coumaroylagmatine and 4-coumaroylputrescine in mycorrhized barley roots, and postulated that they are part of a transient defence response in mycorrhiza formation. This is particularly interesting in view of a proposed signalling role of jasmonate in plant pathogen interactions [3] and the ability of jasmonate to mimic elicitor induction of low-M_r compounds [13]. Coumaroylputrescine has been found in several plant species and it exhibits slight antiviral activity against tobacco mosaic virus [14]. Coumarylagmatine has previously been isolated from barley and its weak antifungal activity was described [15]. However, the homodimer, hordatine A, possesses strong antifungal activities [16] that have been compared to the biological effects of streptomycin [17]. A heterodimer of 4-coumaroylagmatine and feruloylagmatine, hordatine B, also exists [18], with a similar antifungal potency to that of hordatine A [19].

Interestingly, the hordatines can be formed *in vitro*

by oxidative coupling in the presence of peroxides [20]. Since oxidative events are often found in plant pathogen interactions [21] the jasmonate-induced 4-coumaroylagmatine may easily be dimerized to yield the antifungal hordatines. Indeed, hordatines are induced in powdery mildew infected barley seedlings [22]. Endogenous jasmonate concentration does not rise after powdery mildew infection of barley [6] and it does not necessarily induce all jasmonate regulated genes [23, unpublished observation] and hence the physiological function of jasmonate remains obscure. Nevertheless, our results show that exogenous jasmonate can induce the synthesis of precursors of antifungal substances and the massive induction of these compounds might be an alternative explanation of the jasmonate-induced growth inhibition of powdery mildew on barley leaves which was attributed to a direct effect of methyljasmonate on the fungus [7].

EXPERIMENTAL

Five cm leaf segments were cut from the primary leaves of 5-day-old barley (*Hordeum vulgare* L. cv. Salome) plants and floated on water or 45 μM methyljasmonate solution as previously described [24]. Freeze-dried leaf segments were homogenized in a bead-beater and extracted with 80% aq. MeOH (400 μl for up to 25 mg of dry matter, equivalent to 5 leaf segments). After centrifugation, the pellet was analysed for cell wall-bound phenolics [25] and an aq. NaOH soln was added to the supernatant to a final concn of 1 M. Saponification was performed for 1 hr at 80°C under a N_2 atmosphere while stirring continuously. After cooling to room temp, the mixt. was acidified with H_3PO_4 and centrifuged. Of the supernatant 20 μl was subjected to analytical HPLC on a Nucleosil RP-C_{18} column (5 μm particle size, 250 mm length, 4 mm inner diameter, Macherey & Nagel, Düren, FRG). The column was developed within 25 min at a flow rate of 1 ml min^{-1} with solvent A (1% aq. H_3PO_4) and a linear gradient of 10–60% solvent B (80% aq. MeCN). UV absorption was recorded at 290 nm with a photodiode array detector. Quantification was relative to an external standard of 4-coumaric acid.

Prep. HPLC was performed on a Waters RP_{18}RCM 25 column (10 mm particle size, 250 mm length, 25 mm inner diameter, Waters, Eschborn, FRG). The column was developed within 30 min at a flow rate of 8 ml min^{-1} with solvent A (1% aq. HCO_2H) and a linear gradient from 5 to 55% solvent B (80% aq. MeCN). Individual peaks were collected, concd in a speed vac concentrator and immediately analysed by HPLC/MS.

The positive ion electrospray ionization (ESI) mass spectra and the ESI-MS/MS measurements were carried out using a Finnigan TSQ 7000 instrument (electrospray voltage 4.5 kV, sheath gas and auxillary gas: nitrogen) combined with a constMetric 4100 HPLC instrument equipped with a LiChrospher 100 RP_{18}-column (5 μm, 2 × 100 mm). The following HPLC conditions were used: eluent MeCN–H_2O (containing 0.2% HOAc), 2:3, flow rate 0.2 ml min^{-1}; R_t = 8.23 min (**1**), 10.85 min (**2**) and 11.83 (C). The CID-MS during the HPLC run were performed under the following conditions: collision energy, −25 eV; collision gas, argon; collision pressure, 1.7×10^{-3} Torr [26].

Acknowledgements—We want to thank Professor Dieter Strack for critically reading the manuscript. The financial support of the Deutsche Forschungsgemeinschaft to M. L. (Lo 351/2-1) and the Bundesministerium für Bildung und Forschung (BEO/21-0310649) to J. S. is gratefully acknowledged.

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