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The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*)

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Abstract

Jasmonic acid (JA) and its methyl ester (JA-Me) are able to introduce the accumulation of several specific polypeptides in cut leaf segments of barley. Two of the most prominent JA-induced proteins of M_r 15000 and 23000 have been characterized by isolating and sequencing complete cDNA sequences. While the sequence of the M_r 23000 polypeptide shows no similarity to published sequences, the sequence of the M_r 15000 polypeptide corresponds to the higher-molecular-weight precursor of a leaf thionin previously characterized.

Transcripts for the M_r 23000 and M_r 15000 polypeptides accumulate in leaf segments shortly after the beginning of JA treatment. JA and JA-Me induce the appearance of the two proteins not only in leaf segments but also in intact barley seedlings. However, in seedlings the accumulation of JA-induced proteins occurs much more slowly and requires high concentrations of volatile JA-Me. Thus, in barley it seems unlikely that volatile JA-Me is involved in the interaction between different members of this species, as has been proposed recently for tomato seedlings.

Introduction

Jasmonic acid (JA) and its methyl ester (JA-Me) have been isolated from a large number of plant species [24, 43]. The wide distribution of JA and its derivatives suggests that these compounds may have a physiological role in plants. Previous studies have shown that JA-Me or JA, when applied directly to plants, can produce various responses including growth inhibition and promotion of senescence and/or abscission [39, 40]. JA also induces the accumulation of several polypeptides [27]. JA-induced proteins were first described in segments of primary leaves of barley [40]. The possible functions of these proteins in barley are not known yet: JA-induced proteins have been described also in other plant species: vegetative storage proteins of soybean [23, 37], the seed-specific storage proteins napin and cruciferin of rapeseed [42] and a proteinase inhibitor of tomato [15] have been identified among these proteins.

In the present work we have started to characterize JA-induced proteins of barley leaves by

isolating and sequencing cDNAs. The amino acid sequences of JA-induced proteins of M_r 23000 and M_r 15000 have been deduced from the nucleotide sequences of near-full-length cDNAs and have been compared with known sequences of other plant proteins. While the derived amino acid sequence of the M_r 23000 JA-induced protein does not relate to amino acid sequences of other known plant proteins, the M_r 15000 polypeptide could be identified as a prepropolypeptide of barley leaf thionins. Leaf thionins of barley have been identified previously as a group of highly abundant polypeptides with antifungal activity. The toxicity of these proteins to plant pathogenic fungi and the fact that their synthesis can also be triggered by pathogens strongly suggests that leaf thionins are involved in the mechanism of plant defence against microbial infection [9].

Materials and methods

Growth and treatment of plants

Seedlings of barley (*Hordeum vulgare* L. cv. Carina) were grown on vermiculite at 22 °C under continuous light (3000 lx). After 7 days 5 cm segments of primary leaves were harvested and incubated for 1–4 days on water in the absence or presence of JA-Me (4.4–88 μM) under continuous white light (600 lx) at 20 °C.

In some experiments 5-day-old barley seedlings were placed in closed glass chambers and were exposed to various concentrations of volatile JA-Me at various concentrations as described by Farmer and Ryan [15]. Incubation was done in the light (3000 lx) at 20 °C.

Protein analysis of total leaf extracts

Primary leaf segments of control and JA-Me-treated samples were ground under liquid nitrogen. The resulting powder was immediately suspended in preheated buffer containing 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1% SDS, 0.1% bromophenol blue and 40 mM Tris-HCl pH 6.8 and was kept for 10 min at 95 °C. Insoluble cellular debris was separated from the solubilized protein by centrifugation for 15 min at 10000 × *g* [12]. Polypeptides were separated electrophoretically on a 10–15% polyacrylamide gradient gel according to Laemmli [21]. In some experiments proteins were transferred electrophoretically from the polyacrylamide gel onto nitrocellulose according to Towbin *et al.* [38]. Leaf thionins were detected immunologically as described by Forreiter *et al.* [17] using an antiserum against water-soluble leaf thionins of barley [31].

Isolation of nucleic acids

RNA was isolated as described by Chirgwin *et al.* with some modifications [11]. The plant material was ground under liquid nitrogen with a mortar and a pestle and homogenized in 4 ml lysis buffer (4 M guanidinium thiocyanate, 50 mM HEPES-KOH pH 7.4, 2% lauroylsarcosin, 1% 2-mercaptoethanol). Cell debris and denatured protein were pelleted and the nucleic acids in the supernatant ethanol-precipitated. The pellet was homogenized in 4 ml TES buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS) with a Potter homogenizer and the homogenate was extracted with phenol/chloroform and chloroform. Total RNA was precipitated overnight with 2 M LiCl at 4 °C. Poly(A)-containing RNA was isolated according to Aviv and Leder [5].

Genomic DNA was isolated from dark-grown barley seedlings according to Murray and Thompson [26]. Restriction digests, agarose gel electrophoresis and Southern blotting of DNA fragments were performed according to Maniatis *et al.* [22].

In vitro *translation of mRNA*

Poly(A)-containing RNA was isolated and translated in a cell-free protein-synthesizing system of wheat germ according to Pelham and Jackson [30]. In some cases the *in vitro* translation products were immunoprecipitated as described by

Batschauer *et al.* [6] and separated electrophoretically on a SDS-polyacrylamide gel [21]. Radioactively labelled polypeptides were detected by autoradiography [10].

Construction of cDNA libraries

Two different cDNA libraries were constructed and screened for cDNAs that represent JA-induced transcripts. Poly(A)-containing RNA was isolated from leaf segments JA-treated for 24 h. Double-stranded cDNA was synthesized from 1 μg poly(A)-containing RNA using a cDNA-synthesis kit (Amersham, Braunschweig, FRG) according to the supplier's protocol. The *Eco* RI sites of the cDNA were blocked with 25 μM S-adenosylmethionine and 20 units *Eco* RI methylase during 30 min at 37 °C. The reaction mixture was heated to 70 °C for 10 min, phenol-extracted and passed over a Sephadex G-50 column and the cDNA was ethanol-precipitated. *Eco* RI linkers were ligated in a 10 ml reaction volume at 14 °C. After 16 h, the ligase was heat-denatured and the reaction mixture diluted to 20 μl. Concatemeric linkers were digested with 20 units of *Eco* RI for 3 h. After phenol extraction the unattached linkers were removed from the cDNA on a Sephadex G-50 column. 500 ng of λgt10 arms (Promega, Heidelberg, FRG) were ligated with 50 ng cDNA in a 10 μl reaction volume with 1 unit T4-ligase at 14 °C for 16 h. The ligated DNA was packaged [22] and plated with *Escherichia coli* C 600 hfl according to Huyn *et al.* [20].

Up to 10 000 phage were plated on a 23 cm × 23 cm plate. Replica plaque lifts were prepared on a PALL A Biodyne membrane and treated as suggested by the manufacturer. The differential screenings were performed with oligolabelled DNA probes [16] that were prepared from ss-cDNAs originating from RNAs of control and JA-Me-treated barley leaves. The filters were prehybridized overnight in a hybridization solution (6 × SSC, 5 × Denhardt's solution [13], 0.5% SDS, 100 μg/ml salmon sperm DNA) at 62 °C. Hybridization was performed in the same but freshly added solution with $1-2 \times 10^7$ cpm/ml labelled cDNA for 36 to 60 h. Filters were washed twice with 2 × SSC and 0.5 × SSC with 0.1% SDS at 62 °C and exposed to Kodak XAR films for 1–3 days. Positive plaques were purified through a second and third screening at lower plaque density. Inserts of the purified phage were subcloned into Bluescribe plasmids (Stratagene, Heidelberg, FRG) by standard methods [22].

Full-length cDNA clones were isolated from a second cDNA library. After linker ligation the cDNA was subjected to a size separation step on a Sepharose 4B column (Pharmacia, Freiburg, FRG). Fractions containing cDNA of at least 500 bp were selected for library construction in λgt10. For the non-radioactive labelling of cDNA inserts of the clones pHvJ25 and pHvJ30, the hybridization and the signal detection the digoxigenin labelling and detection kit from Boehringer (Mannheim, FRG) was used. The largest cDNA inserts of positive clones were subcloned in the plasmid Bluescript (Stratagene, Heidelberg, FRG). cDNA inserts were sequenced according to Sanger *et al.* [34].

Hybrid selection

Hybrid selection of mRNA was done as described by Gollmer and Apel [18]. Northern blot and dot blot analysis of RNA samples were done as described by Maniatis *et al.* [22].

Results

JA-induced proteins of barley leaf segments

Freshly cut segments of primary leaves of barley floated on water containing various concentrations of JA-Me ranging from 4.4 μM to 88 μM. After incubation for 72 h the leaf material was homogenized under liquid nitrogen. The total protein extracted in the presence of 1% SDS was separated electrophoretically on a 10–15% polyacrylamide gradient gel. Several major polypeptides were present in samples of JA-treated leaf

segments that could not be detected among the proteins of the control sample (Fig. 1). The apparent molecular weights of JA-induced polypeptides were similar to those reported earlier for JA-induced proteins of a different variety of barley [19, 41]. Even at the lowest concentration of JA-Me (4.4 μM) the levels of most JA-induced proteins were close to their maximum (Fig. 1). The polypeptide of M_r 23000 was by far the most abundant JA-induced protein.

JA-induced mRNAs of barley leaf segments

Poly(A)-containing RNA was isolated from leaves of intact barley plants and from leaf segments that had been incubated on water in the absence or in the presence of 44 μM JA-Me for 24 h. The RNA was used as a template for protein synthesis in a cell-free protein-synthesizing system of wheat germ extracts [cf. 25]. Proteins were separated electrophoretically and ^{35}S-labelled *in vitro* products were detected by autoradiography. Several *in vitro* products were present in the sample taken from JA-Me-treated leaf segments that were not detectable among the *in vitro* products of the two control samples (Fig. 2). There were only minute differences in the ^{35}S-labelled polypeptide compositions of the two controls. Thus, the cutting of leaf segments and the subsequent incubation on water had only very little effects on the composition of the poly(A)-containing RNA and apparently were not responsible for the appearance of major translatable mRNAs in JA-treated leaf segments. The appar-

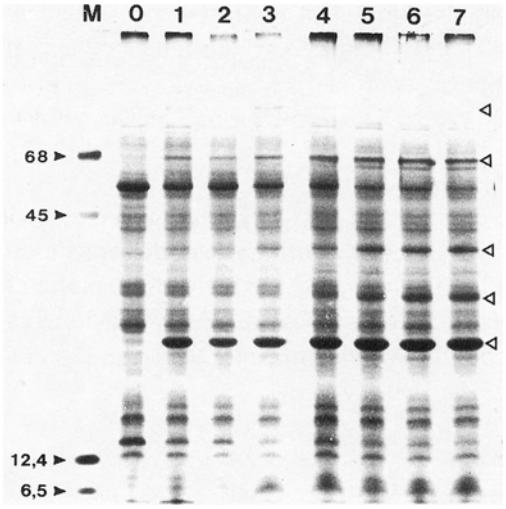

Fig. 1. The effect of JA-Me on the polypeptide composition of barley leaf segments. Barley seedlings were grown for 7 days in the light. Segments of primary leaves were incubated for 3 days in the absence (0) or in the presence of 4.4 μM JA-Me (1), 8.8 μM JA-Me (2), 22 μM Ja-Me (3), 31 μM JA-Me (4), 44 μM JA-Me (5), 66 μM JA-Me (6) and 88 μM JA-Me (7). Total leaf proteins were extracted, separated electrophoretically on a polyacrylamide gradient gel and stained with Coomassie blue. Open triangles indicate the position of JA-induced polypeptides. M = marker polypeptides: bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), cytochrome c (M_r 12400) and trypsin inhibitor (M_r 6500).

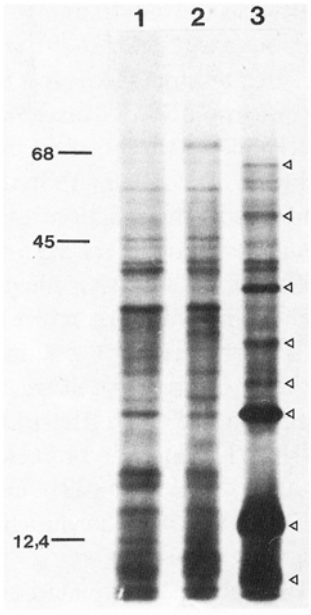

Fig. 2. The effect of JA-Me on the composition of poly(A)-containing RNA of barley leaf segments. Barley seedlings were grown for 7 days. Segments of primary leaves were incubated for 1 day in the absence (2) or in the presence (3) of 44 μM JA-Me. 1: control sample of seedlings prior to the cutting of leaves. Poly(A)-containing RNA was isolated and translated *in vitro* in a cell-free synthesizing wheat germ extract. The resulting ^{35}S-labelled *in vitro* products were separated electrophoretically and visualized by autoradiography. Open triangles indicate the position of JA-induced *in vitro* products. Numbers on the left side indicate the M_r of the marker proteins bovine serum albumin ovalbumin and cytochrome c.

ent molecular weights of most JA-induced *in vitro* products were similar to the sizes of JA-induced leaf proteins as shown in Fig. 1. There was only one notable exception, the polypeptide of M_r 15000 was by far the most prominent *in vitro* product synthesized in the presence of poly(A)-containing RNA from JA-treated leaf segments. However, a JA-induced polypeptide of a similar size could not be detected among the extracted leaf proteins.

Synthesis and cloning of cDNAs

An aliquot of the poly(A)-containing RNA of JA-treated leaf segments was used for the synthesis of double-stranded cDNA. The cDNA was inserted into the vector phage λgt10. JA-specific cDNA clones were identified by differential screening. ^{32}P-labelled cDNAs were prepared from poly(A)-containing RNA fractions of JA-treated and untreated barley leaf segments and used as hybridization probes. Out of 10000 recombinant phage initially more than 30 phage were picked for further analysis and subjected to two subsequent rounds of plaque purification. After the second purification step 19 positive phage were recovered. Equal amounts of cDNA inserts from these selected recombinant phage were dotted onto nitrocellulose sheets. Two cDNA samples (pHvJ8 and pHvJ13) were chosen randomly and used as hybridization probes. Thirteen out of 19 cDNA clones hybridized with the cDNA of clone pHvJ8. A very similar hybridization pattern was obtained when the cDNA of clone pHvJ13 was used as a hybridization probe (Fig. 3). Many of the selected cDNAs seem to represent one major JA-specific transcript or a group of closely related JA-specific transcripts. Two cDNAs, pHJ6 and pHJ25, that did not fall into this first group of closely related JA-specific cDNAs were selected and used as probes for a second hybridization experiment. Both cDNAs hybridized with each other and also with other cDNAs that did not react with members of the first cDNA group (Fig. 3). Only two cDNA clones (pHvJ14, pHvJ18) were unique and apparently not related to either one of the two groups of JA-specific cDNAs mentioned above.

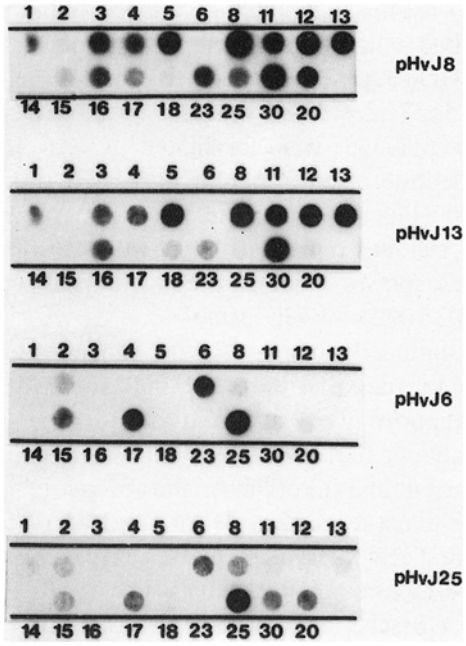

Fig. 3. The relationship of various cDNAs encoding JA-induced polypeptides as revealed by their hybridization to the cDNA inserts of the recombinant clones pHvJ8, pHvJ13, pHvJ6 and pHvJ25. Equal amounts of recombinant plasmid DNA were dotted onto nitrocellulose and hybridized with the ^{32}P-labelled cDNA inserts. Numbers refer to the different cDNA clones.

Characterization of cDNAs encoding JA-induced proteins

The two major groups of JA-specific cDNAs were tested further by hybrid selection of mRNA from total poly(A)-containing RNA of JA-treated leaf segments. Different members of each group, cDNAs of clones pHvJ16 and pHvJ30 (group I), and of clone pHvJ25 (group II) were used for the hybrid selection of mRNA. mRNAs selected by the cDNA inserts of clones pHvJ16 and pHvJ30 directed the synthesis of a polypeptide whose apparent molecular weight was indistinguishable from that of the JA-specific M_r 23000 *in vitro* product synthesized in the presence of poly(A)-containing RNA from JA-treated leaf segments

(Fig. 4). This polypeptide could be immunoprecipitated with an antiserum raised against the M_r 23000 JA-induced polypeptide of barley [19] (Fig. 5). The mRNA selected by the cDNA of clone pHvJ25 was translated *in vitro* into a polypeptide of an apparent molecular weight of 15000 (Fig. 4). Thus, the two main groups of cDNA clones represent the two most prominent mRNA species encoding the JA-induced proteins of M_r 15000 and M_r 23000.

JA-induced changes in the concentration of mRNAs encoding the M_r 23000 and M_r 15000 polypeptides were analysed by northern blots. Freshly cut barley leaf segments were incubated for various lengths of time in the absence (Fig. 6K) or in the presence (Fig. 6J) of 44 μM JA-Me and the total RNA from these leaf samples was separated electrophoretically. The ^{32}P-labelled cDNA inserts of clones pHvJ13 (group I) and pHvJ6 (group II) were used for hybridization.

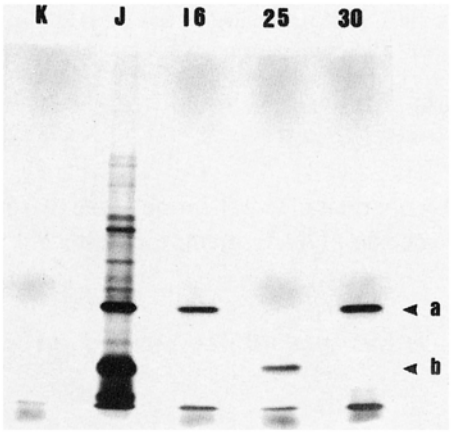

Fig. 4. Cell-free translation of mRNA selected by hybridization to the recombinant plasmid DNAs of clones pHvJ16 (16), pHvJ25 (25) and pHvJ30 (30). Plasmid DNAs were denatured and immobilized on nitrocellulose filters. The filters were then hybridized to the poly(A)-containing RNA from JA-treated leaf segments of barley seedlings grown in the light. The mRNA activity was assayed using cell-free translation. ^{35}S-labelled polypeptides were displayed by SDS-polyacrylamide gel electrophoresis and autoradiography. J: total *in vitro* products synthesized in the presence of poly(A)-containing RNA of JA-treated barley leaf segments. K: control, *in vitro* translation without the addition of exogenous mRNA. Arrowheads indicate the positions of the M_r 23000 (a) and the M_r 15000 (b) JA-induced proteins.

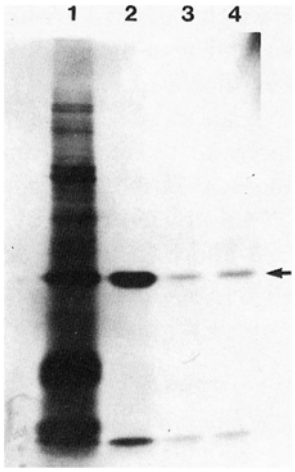

Fig. 5. Immunoprecipitation of *in vitro* translation products of mRNAs selected by hybridization to the recombinant plasmid DNAs pHvJ16 (3) and pHvJ30 (4). Poly(A)-containing RNA was isolated from barley leaf segments treated with JA-Me as described in Fig. 1. ^{35}S-labelled polypeptides were immunoprecipitated with an antiserum against the M_r 23000 JA-induced polypeptide. Controls: total *in vitro* translation products of poly(A)-containing RNA of JA-treated barley leaf segments (1) and immunoprecipitation of (1) with an antiserum against the M_r 23000 JA-induced polypeptide of barley (2). The arrow indicates the position of the M_r 23000 JA-induced protein.

Shortly after the beginning of the JA treatment transcripts encoding the M_r 23000 JA-induced polypeptide began to accumulate rapidly in the leaf segments and reached a maximum after 24 h of incubation. The size of the transcript was approximately 1050 bp. Only very small amounts of this transcript were detectable in leaf segments after they had been incubated for 48 h on water (Fig. 6A). The second transcript encoding the M_r 15000 polypeptide had a size of approximately 750 bp. It was barely detectable in control leaf segments that had been kept floating on water. However, in JA-treated leaf segments this transcript began rapidly to accumulate and reached its maximum concentration within the first 24 h after the beginning of incubation (Fig. 6B). The kinetics of the JA-induced accumulation of both transcripts looked almost identical.

A comparison of the sizes of cDNA inserts of group I and group II cDNA clones with the length of the corresponding transcripts revealed that

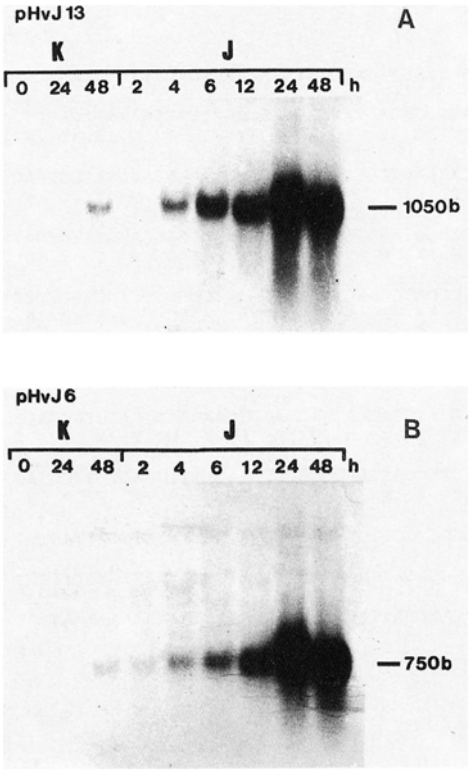

Fig. 6. The effect of JA-Me on the accumulation of transcripts encoding the JA-induced polypeptides of M_r 23000 (A) and M_r 15000 (B) in barley leaf segments. Total RNA was isolated from barley leaf segments incubated for various lengths of time (0–48 h) in the absence (K) or in the presence of 44 μM JA-Me (J). Equal amounts of RNA were denatured and separated electrophoretically on an agarose gel. The fractionated RNA was transferred onto nitrocellulose and hybridized with the ^{32}P-labelled cDNA inserts of clones pHvJ13 (A) and pHvJ6 (B). The numerals indicate the approximate sizes of the transcripts.

none of the cDNAs were complete. Thus, a second cDNA library in λgt10 was constructed and first screened with the digoxigenin-labelled insert of clone pHvJ30 (group I). A large number of positive cDNA clones were identified from which 10 clones were chosen randomly for the determination of the insert size. One of these clones contained a cDNA insert of ca. 1000 bp which is close to the expected size of a full-length cDNA. This cDNA insert was subcloned into the Bluescript plasmid and sequenced. Only one of the three possible open reading frames encoded a polypeptide of approximately 23000 Da (Fig. 7A) while the two others were interrupted by several stop codons. The derived amino acid sequence of the M_r 23000 polypeptide was compared with known protein sequences stored in the Swiss-Prot data bank. No similarity to other protein sequences could be detected.

In a second screening the cDNA library in λgt10 was probed with a digoxigenin-labelled cDNA of clone pHvJ25 (group II). One of the selected cDNAs was of almost full length and contained an open reading frame for a M_r 15000 polypeptide. This polypeptide could be identified as a precursor of a leaf thionin which contained a signal peptide, a thionin domain and an acid polypeptide domain [7]. The nucleotide sequence of the cDNA was identical with the leaf thionin-specific cDNA pHvDG3 (Fig. 7B).

A M_r 15000 polypeptide could not be detected among the JA-induced proteins of barley leaf segments. However, the size of a M_r 6000 JA-induced polypeptide is similar to the apparent molecular weight of the mature leaf thionin. The possible identity of this low-molecular-weight JA-induced polypeptide with a leaf thionin was tested in the following experiment. Total protein was extracted from JA-treated barley seedlings, separated electrophoretically on a 15–20% linear polyacrylamide gradient gel and transferred onto nitrocellulose membranes. The M_r 6000 JA-induced polypeptide crossreacted with the antiserum against water-soluble thionins of barley leaves (Fig. 8, B3). In protein samples of control seedlings thionins could not be detected (Fig. 8, B2).

Leaf thionins of barley are encoded by a complex multigene family which consists of more than 50 members per haploid genome [8]. It was of interest to determine the number and the complexity of genes that encode the second major JA-induced protein of M_r 23000: genomic DNA was isolated from barley leaves, cut with various restriction enzymes and the resulting DNA fragments were separated electrophoretically on an agarose gel. Southern blots were hybridized with the ^{32}P-labelled cDNA insert of clone pHvJ30. Depending on the restriction enzymes used one or two genomic fragments were detected that hy-

A

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  1                                                                AGCTAGTACACCTATATATCACAGTGTGTGTGCAAAGCA

 42  ATGGCCTCTGGAGTGTTTGGTACCCCCATTTCAGCGCAGACGGTGATAGCCACTGGAGAGTATAAGGAACCCATTACCCAAAAAGATGTT
      M  A  S  G  V  F  G  T  P  I  S  A  Q  T  V  I  A  T  G  E  Y  K  E  P  I  T  Q  K  D  V    30

132  GCAGACTATGCCATGAAGATGATCAACGCCGGTGGTAAGGATGTTAACGCGCAAAAGTTCGTCGACAACCTCAAGGAGAGGTACGGTAAC
      A  D  Y  A  M  K  M  I  N  A  G  G  K  D  V  N  A  Q  K  F  V  D  N  L  K  E  R  Y  G  N    60

222  GGAATAGCTGTAAAATGCCTCCTCTACAATGCCACTGGTGCCACTTTGAACTTTGCTAAGTACAACGATTGGCACGGCCATATCTATGAT
      G  I  A  V  K  C  L  L  Y  N  A  T  G  A  T  L  N  F  A  K  Y  N  D  W  H  G  H  I  Y  D    90

312  ACACCCTACCCATCAGATATTCAGAATGGGCAATGGGGTGCATTCCTCCACGTCCACCCAAGTGGAGCTGCCGCTGGTTCAGCTGGTGCC
      T  P  Y  P  S  D  I  Q  N  G  Q  W  G  A  F  L  H  V  H  P  S  G  A  A  A  G  S  A  G  A   120

402  GTTGTGTATCGTAGCAAGATCCCCTCCAGCAGCAGCTCCTGCGATTGGTTGTTCTCTTGGACCGTCCCCTACATTGGTGGCAACGGGGTG
      V  V  Y  R  S  K  I  P  S  S  S  S  S  C  D  W  L  F  S  W  T  V  P  Y  I  G  G  N  G  V   150

492  TACACTGAAATCCGCGAGGAAGGGCACTACCCAAGTGTGGGAAGCTGGGATTATATCTACAATGTGAAGCTGAAAAATTCAAGTGTCACC
      Y  T  E  I  R  E  E  G  H  Y  P  S  V  G  S  W  D  Y  I  Y  N  V  K  L  K  N  S  S  V  T   180

582  TCTATTGATAGCAACTATGGATACGTTTCCAAGGCTGACATCGGTGAAGGCACTACCATGAACGCACGTGGAGTTTTCGAGTTTCCCTAC
      S  I  D  S  N  Y  G  Y  V  S  K  A  D  I  G  E  G  T  T  M  N  A  R  G  V  F  E  F  P  Y   210

672  TAGATCTCGTTGCAAGCAGCGAGTTGTTATTGTGGTCTTCAAGTCTTGTGTGGCGTCCATGTACGCTTGTGTAGTTTTTTTCTTTACTGT

762  CTTTATGGTGCATTTTTATAGATCTGATGATCCACGAATAAGCGAAGGAAGATGGGGACCAAAGAGATGTACCGTGTAATTGTATTTTAT

852  TTTACATGTCGTTGTGTATCAAGTTGTCCTTCTTTTGTATCGGTTACCTTTTTACTTAATAATTAAAGATATAAAAAAAAAAAAAAAA
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B

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  1                                                                GAGAAATTAAGGCCAACCAGCCAACC

 27  ATGGCAACCAACAAGAGTATTAAGAGCGTGGTTATTTGTGTTCTCATATTGGGTTTAGTTCTGGAGCAGGTCCAAGTAGAAGGCAAGAGT
      M  A  T  N  K  S  I  K  S  V  V  I  C  V  L  I  L  G  L  V  L  E  Q  V  Q  V  E  G  K  S    30

117  TGCTGCAAGAACACGACGGGCAGAAACTGCTACAACGCCTGCCGTTTCGCTGGTGGTTCCCGACCAGTCTGCGCAACTGCTTGTGGTTGT
      C  C  K  N  T  T  G  R  N  C  Y  N  A  C  R  F  A  G  G  S  R  P  V  C  A  T  A  C  G  C    60

207  AAAATCATAAGCGGCCCAACATGTCCTAGGGACTATCCTAAGCTGAATCTTCTCCCTGAATCCGGTGAACCAAATGCGACTGAGTACTGC
      K  I  I  S  G  P  T  C  P  R  D  Y  P  K  L  N  L  L  P  E  S  G  E  P  N  A  T  E  Y  C    90

297  ACAATTGGATGCAGGACTTCCGTCTGTGACAACATGGACAATGTTTCCCGTGGCCAAGAGATGAAATTCGACATGGGACTCTGCAGCAAC
      T  I  G  C  R  T  S  V  C  D  N  M  D  N  V  S  R  G  Q  E  M  K  F  D  M  G  L  C  S  N   120

387  GCATGTGCCCGTTTCTGTAATGATGGTGAAGTCATTCAGTCTCTTGAAGCCTAATCATGCCTTCTTGGGCATGATTTCTATGGCCAAGGT
      A  C  A  R  F  C  N  D  G  E  V  I  Q  S  L  E  A                                          137

477  CGTTTTGTCATTTTCTGTTCAACAATCTTGATACCATCACCACATCGAAGCAAATGTATCATCTTAGTTATGTATTTCTGTTTTTTCCCT

567  CGTTCCAATAAGAGTCGCCACGATGAATCTATTTATACCCTTGGCTTAATATAAAAGTTCCATGGATATATTATGTGATACACCACAAAA

657  AAAAAAAA
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Fig. 7. The nucleotide sequences and the derived amino acid sequences of the pHvJ3015 cDNA encoding the M_r 23000 JA-induced protein (A) and the pHvJ256 cDNA encoding the M_r 15000 JA-induced polypeptide (B). Nucleotides and amino acid residues are numbered on the left and on the right, respectively. In B the black triangles mark the beginning and the end of the thionin domain of the M_r 15000 JA-induced polypeptide.

bridized specifically with the cDNA probe (Fig. 9). This result is consistent with the idea that in contrast to the leaf thionin the M_r 23000 JA-induced polypeptide is not encoded by a large and complex multigene family.

The effect of volatile JA-Me on intact barley seedlings

So far, the appearance of JA-induced proteins had been analysed in cut leaf segments. Similar

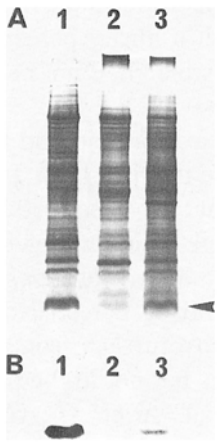

Fig. 8. The effect of airborne JA-Me on the accumulation of leaf thionins in barley seedlings grown in the light. Barley seedlings were incubated for 3 days in the absence (2) or in the presence (3) of 4.5 μM volatile JA-Me in a closed glass chamber. Total leaf proteins were solubilized, separated electrophoretically on a SDS 10–15% polyacrylamide gradient gel and stained with Coomassie blue (A) or transferred electrophoretically onto nitrocellulose and immunostained with an antiserum against water-soluble leaf thionins of barley (B). 1: control sample of proteins extracted from leaves of etiolated barley seedlings. The arrowhead indicates the position of leaf thionins.

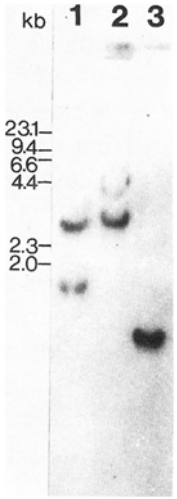

Fig. 9. Southern blot analysis of total DNA of *Hordeum vulgare* cv carina. The DNA was digested with the restriction enzymes *Hin*d III (1), *Bam* HI (2) and *Eco* RI and *Xba* (3). The resulting DNA fragments were separated electrophoretically on a 0.8% agarose gel, transferred onto Hybond N membranes and hybridized with the radioactively labelled cDNA insert of the clone pHvJ30.

effects of JA could also be observed when intact barley seedlings were exposed to a JA-containing atmosphere. Seedlings were kept in a closed glass container to which varying amounts of volatile JA-Me were added. The JA-induced proteins of M_r 23 000 and M_r 6000 could be detected first at a concentration of 4.5 μM jasmonate (Fig. 8). The maximum levels of these proteins were reached at a concentration of 15 μM. In intact barley seedlings the accumulation of JA-induced proteins occurred at a much slower rate than in cut leaf segments. The maximum concentration of JA-induced proteins was reached in intact barley seedlings only after 3 days of incubation while in leaf segments this level was reached already within the first 24 h of incubation.

Discussion

Recently, JA and its possible role in plants has received considerable attention and various modes of JA action have been discussed [27, 28, 36]. JA is widely distributed among higher plants [24]. JA and JA-Me have been implicated in the control of growth and senescence [35, 39, 40]. Other physiological effects were elicited at much lower concentrations of JA [3]. Several proteins which accumulate under various stress conditions such as wounding and water stress are also induced by JA or JA-Me. Few of these proteins have been identified: the vegetative storage proteins in soybeans [1, 2, 23, 37], the seed-specific storage proteins napin and cruciferin of rapeseed [42] and the proteinase inhibitor in tomato [15]. The accumulation of the vegetative storage protein has been shown to be controlled by changes in the source-sink relationship and the nitrogen concentration, by drought stress and by wounding [36]. Seed-specific storage proteins of rapeseed are affected by abscisic acid [42] similar to the effects induced by JA and JA-Me. The concentration of the proteinase inhibitor is primarily affected by wounding [15].

The identification of leaf thionins as one of the main JA-induced proteins in barley adds further

support to the notion that JA is involved in the regulation of stress-induced reactions of higher plants. Leaf thionins of barley have been discovered recently as a novel class of lower-molecular-weight polypeptides that may play an important role in the defence against pathogens [4, 9]. In this respect the effect of JA on barley leaves is similar to its effect on tomato leaves. In both cases JA activates the expression of genes whose products apparently are part of defence mechanisms [4, 33]. Tomato seedlings accumulate rapidly large amounts of proteinase inhibitors when exposed to volatile JA-Me. In barley leaves thionins appear when intact seedlings are treated with JA-Me. Thus, the effects of JA on the synthesis of JA-induced proteins are not confined to the artificial experimental system of a cut leaf segment. Tomato seedlings respond to much lower concentrations of volatile JA-Me than intact barley seedlings. Based on the results with tomato seedlings it has been proposed that JA may be involved in signalling within and between plant communities [15]. Because of the lower sensitivity of intact barley seedlings to exogenous volatile JA and the low level of endogenous JA in barley leaves [29] it seems unlikely that JA is involved in an interplant communication between members of this species.

When mRNAs from JA-treated barley leaf segments were translated in a cell-free protein-synthesizing wheat germ extract the M_r 15000 polypeptide was by far the most abundant *in vitro* translation product. A similar-sized polypeptide was not detectable among the translation products of RNA from control leaf segments. This prominent *in vitro* product has not been described in previous studies of JA-induced proteins of barley leaves [19, 25, 41]. In these studies the *in vitro* translation products of mRNA from JA-treated barley leaf segments have been separated by two-dimensional gel electrophoresis. Since most thionin precursor molecules are basic proteins [7] it seems likely that with this separation technique the precursors of leaf thionins have not been resolved. Also the low-molecular-weight mature thionin was not described among the JA-induced proteins [19, 25, 41]. Polypeptides with apparent molecular weights lower than 10000 are resolved only in gels with a higher percentage of polyacrylamide-SDS, which had not been used in these earlier studies.

The M_r 6000 leaf protein and the M_r 15000 *in vitro* product are the first JA-induced proteins of barley leaves that have been identified. Our results offer an explanation why in contrast to other prominent JA-induced proteins the M_r 15000 polypeptide could be detected only after the *in vitro* translation of mRNA from JA-treated barley leaf segments but not in the protein extract of this leaf material. After its synthesis the prepropolypeptide of leaf thionins is processed to the mature thionin which accumulates to high levels in the central vacuole and in cell walls of barley leaves [31]. Also the JA-induced polypeptide of M_r 6000 has been found to be located within the vacuole (unpublished results).

In the present work also a second JA-induced protein has been characterized. The M_r 23000 polypeptide is by far the most prominent JA-induced protein of barley leaf segments. A full-length cDNA for this polypeptide has been isolated and sequenced. The identification of the cDNA was based on the following criteria. The cDNA contained an open reading frame for a polypeptide of a similar apparent molecular weight as the M_r 23000 JA-induced protein. When the cDNA was used for hybrid selection an mRNA was isolated that encodes a protein of M_r 23000. This product could be immunoprecipitated specifically with an antiserum raised against the JA-induced M_r 23000 polypeptide of barley [19]. The mRNA was hardly detectable in leaf segments of control plants; however, its concentration rapidly increased when leaf segments were treated with JA or JA-Me. The amino acid sequence of the 23 000 polypeptide was deduced from the nucleotide sequence of the cDNA. When compared to known protein sequences no resemblance to other protein sequences could be detected. In recent work by Reinbothe *et al.* [32] a JA-induced protein of M_r 23000 has been described in barley that bears some similarity to the 'late embryogenesis abundant' (LEA) proteins of other plants [14]. The sequence of this barley

protein is not known yet. Since the amino acid sequence of the JA-induced M_r 23 000 polypeptide described in the present work shows no homology to the sequence of 'LEA' proteins it is possible that there may be more than one M_r 23 000 JA-induced polypeptide species in barley.

Besides the two main JA-induced proteins that have been characterized in the present study, there are other prominent JA-induced proteins in barley [41]. Even though the function of these proteins is not known yet, it seems unlikely that all these proteins are involved in the same stress-related reaction as the pathogen-induced leaf thionins of barley. This prediction is in accordance with a wide variety of JA effects that have been described in various higher-plant species. If JA is part of a signal transduction pathway [15, 28] it appears to act at a superior level from which different subordinate branches in the signal transduction pathway may arise which ultimately may regulate different types of stress- and defence-related reactions. It will be necessary to determine the function of more JA-induced proteins before the assumed complex pattern of JA-controlled reactions in higher plants may be understood.

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