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Jasmonate-Induced Proteins in Cotton: Immunological Relationship to the Respective Barley Proteins and Homology of Transcripts to Late Embryogenesis Abundant (Lea) mRNAs

S. Reinbothe,[1,*] A. Machmudova,[2] C. Wasternack,[1] C. Reinbothe,[1] and B. Parthier[1]

[1]Institute for Plant Biochemistry, Weinberg 3, 0-4050 Halle/Saale, Federal Republic of Germany; and [2]Institute for Bioorganic Chemistry, Usbekian Academy of Sciences, Gorki Street 83, SU-700143 Taschkent, USSR

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Abstract. (−)-Jasmonic acid methyl ester (Ja-Me) causes a drastic alteration of gene expression in excised cotton (*Gossypium hirsutum* L.) cotyledons. After in vivo labeling with L-[^{35}S]methionine, soluble proteins of Ja-Me-treated cotyledonary segments were analyzed by two-dimensional polyacrylamide gel electrophoresis. Several different classes of polypeptides were observed, corresponding to molecular sizes of Mr 67, 40, 35, 30, 25, 23, 20, 18 kDa, which were absent in water-treated (control) segments. Western-blot analysis with antibodies raised against 66, 37, and 23 kDa jasmonate-induced proteins (JIPs) of barley (Herrmann et al. 1989) revealed that only the 23 kDa JIPs of cotton are immunologically related to those of barley. The Ja-Me-induced alteration in the protein pattern correlated with the appearance of novel in vitro translatable mRNAs which accumulated transiently or steadily during the incubation period. Three of the Ja-Me-induced mRNAs hybridized with a synthetic oligonucleotide probe that was complementary to a highly conserved sequence motif found in the cotton Lea (late embryogenesis abundant) gD-7 gene (Baker et al. 1988) and in some other Lea genes (Dure et al. 1989). The level of the 2.17, 1.46, and 0.71 kb transcripts detected at a melting temperature (Tm) −15°C criterion did not change significantly in the water-control cotyledons, but they increased three- to fourfold in jasmonate-treated cotyledons.

*Present address: Institute for Plant Sciences, ETH Zentrum, Universitätsstraße 2, CH-8092 Zürich, Switzerland.

(−)-Jasmonic acid and its derivatives (e.g., the methyl ester or certain amino acid conjugates) constitute a novel class of naturally occurring widespread substances regarded as putative plant growth regulators (Parthier 1990, Sembdner and Gross 1986, for recent reviews). Since discovery of (−)-jasmonic acid methyl ester (Ja-Me) in the essential oil of *Jasminum grandiflorum* (Demole et al. 1962) and *Rosmarinum officinalis* (Crabalona 1967), this substance and other related compounds have been isolated from a large number of higher plant species belonging to almost all families (Meyer et al. 1984, Yamane et al. 1981). Like other well-characterized plant growth substances, jasmonates exert a vast variety of biological effects in plants, both inhibiting and promoting physiological processes (Parthier 1990, Sembdner and Gross 1986).

From the molecular point of view two main jasmonate effects have been described for excised leaf segments: (1) the promotion of senescence as determined inter alia by the loss of chlorophylls and ribulose-1,5-bisphosphate carboxylase (Parthier et al. 1987, Satler and Thimann 1981, Ueda and Kato 1980, 1981, 1982, Weidhase et al. 1987b), and (2) the accumulation of novel abundant proteins (Anderson 1988, Herrmann et al. 1989, Müller-Uri et al. 1988, Parthier et al. 1987, Weidhase et al. 1987a). Both aspects reflect a dramatic alteration of gene expression in jasmonate-exposed leaves. The molecular changes seem to be species-specific, since no immunological relationship could be detected between barley leaf jasmonate-induced proteins (JIPs) and respective polypeptides of other monocotyledonous, as well as dicotyledonous plants (Herrmann et al. 1989).

This obvious specificity of JIP genes or their expression is surprising under the assumption that JIPs represent accumulated products of a common metabolic way by which plant cells respond to either external stresses or internal developmental changes (Parthier 1990). According to the stressor hypothesis of jasmonate action, a close molecular relationship among JIPs of different species would be anticipated in relation with evolutionary-conserved genes similarly to those coding for heat shock and other stress proteins (Nover et al. 1990). A similar view can be referred to JIPs as specific proteins involved in foliar senescence, although we do not know yet whether their physiological functions, if there are any, are related to the induction of senescence. JIP formation in barley leaves clearly precedes senescence symptoms (Müller-Uri et al. 1988).

In the particular case of cotton, studies on correlations between JIP formation and foliar senescence might have practical implications, since the agronomic importance of this plant relies on manipulated abscission of presenescent leaves for facilitated cotton ball harvest.

We demonstrate herein that excised cotton cotyledons respond to Ja-Me treatment by the formation of different classes of JIPs, whose appearance results from the accumulation of novel mRNAs. Three of the induced mRNAs were recognized by a synthetic oligonucleotide probe complementary to a conserved sequence motif of the cotton Lea (late embryogenesis abundant) gD-7 gene described by Baker et al. (1988) and Dure et al. (1989).

Material and Methods

Labeling, Extraction, and Immunodetection of Proteins

Segments (5 mm diameter) of 8-day-old cotyledons of light-grown *Gossypium hirsutum* L. seedlings were floated on an aqueous solution of Ja-Me (225 μM) or water. After various periods of time they were labeled for 2 h with 3.7 MBq L-[^{35}S]methionine (Isocommerz GmbH, Berlin, Germany). Proteins from 10 pieces of segments were extracted by phenol treatment, washed, and prepared for two-dimensional gel electrophoresis (see below) as described by Meyer et al. (1988). Western-blot analyses were performed as described by Herrmann et al. (1989).

Extraction and In Vitro Translation of RNA

Total RNA was prepared by guanidinium hydrochloride extraction (Chirgwin et al. 1979) from 30 deep-frozen cotyledonary segments. After precipitation by ethanol and further purification of LiCl treatment (Reinbothe et al. 1990), high molecular weight RNAs were translated into polypeptides in the presence of 11.2 MBq L-[^{35}S]methionine (37 TBq × mmol^{-1}; Amersham, England) in a wheat germ cell-free translation system (Erickson and Blobel 1983; modified by Reinbothe et al. 1990).

Electrophoretic Separation of RNAs Under Denaturing Conditions

For RNA electrophoresis in the presence of formaldehyde (Meinkoth and Wahl 1984), 7 μg of RNA were loaded onto horizontal 1% (wt/vol) agarose gels. After electrophoresis at 50 mA for 4–5 h, the separated RNAs were blotted onto nitrocellulose filters by standard procedures (Sambrook et al. 1989).

Synthesis and Labeling of a Lea-Specific Oligonucleotide Probe

The oligonucleotide probe 5′ GGC GGC CTT CTG CTT GGC CGC CTC TGC A 3′ was kindly synthesized by Dr. R. Walther, Institute of Biochemistry, University of Greifswald, Germany, using an Applied Biosystems DNA Synthesizer, model 380-A. Except for the *Pst* I site, TGCA, which was added for further work, this oligonucleotide is complementary to the sequence 5′ GAG GCG GCC AAG CAG AAG GCC GCC 3′ that corresponds to the amino acid sequence N-Glu-Ala-Ala-Lys-Gln-Lys-Ala-Ala-C of an abscisic acid-induced protein of barley aleurone layers (Hong et al. 1988).

The probe was labeled by [^{32}P]-γ-ATP by the T4-kinase reaction as described by Sambrook et al. (1989). [^{32}P]-γ-ATP not incorporated at the 5′ terminus of the oligonucleotide was removed by centrifugation through disposable 1-ml columns packed with Sephadex G 50 m in TE [10 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris), pH 7.5; 1 mM EDTA].

Hybridization of Filter-Bound RNA with ^{32}P-Labeled Lea Oligonucleotide Probe

The hybridization conditions (Wahl et al. 1979) were estimated from the calculated melting temperature (Tm = 40°C) of DNA–RNA hybrids at the chosen salt concentrations (Sambrook et al. 1989).

After hybridization, the filters were washed with 5× SSPE (0.9 M NaCl, 50 mM Na_2HPO_4, pH 7.7; 5 mM EDTA) and 3× SSPE for 30 min each. More stringent washings of filters were done successively in 2× SSPE, 1× SSPE, and 0.5× SSPE at 25°C.

Two-Dimensional Gel Electrophoresis of In Vivo and In Vitro Translated Polypeptides

Two-dimensional separations included isoelectric focusing gels (O'Farrell 1975) in the first dimension in combination with sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels in the second dimension (Laemmli 1970, Nover and Scharf 1984, Scharf and Nover 1982).

Results

Accumulation of JIPs in Cotton Cotyledons

The polypeptide composition of cotyledonary segments floated on either Ja-Me or water for 5 days was compared by two-dimensional gel electrophoresis. As indicated by Coomassie staining, Ja-Me-treated cotyledons (Fig. 1B) accumulate a large number of proteins which do not occur in the water-treated (control) cotyledons (Fig. 1A). Of the newly

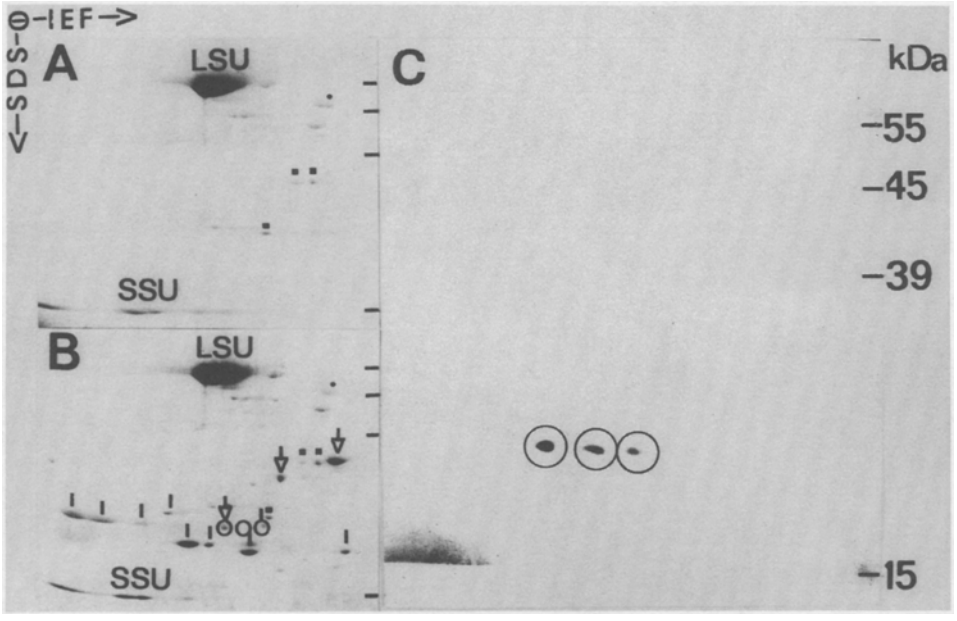

Fig. 1. Two-dimensional polypeptide patterns of cotton cotyledons floated for 5 days on water (**A**) or a 225 μM solution of Ja-Me (**B**). After isoelectric focusing (*IEF*) and denaturing polyacrylamide gradient gel electrophoresis (*SDS*) the proteins were detected by Coomassie staining (**A, B**) or by autoradiography following immunoreaction of polypeptides from Ja-Me-treated cotyledons with the barley anti-23 kDa JIP antibodies and ^{125}I-protein A binding (**C**). Polypeptides detectable only in the Ja-Me-treated cotyledons are marked by *bars* (|) or *arrows* (↓). Proteins cross-reactive to the barley 23 kDa JIPs are encircled in (**B**) and (**C**) [(**C**) is a twofold magnification of the autoradiogram corresponding to the gel shown in (**B**)]. *Dots* (●) and *boxes* (■) indicate constitutive polypeptides, and *arrows* Ja-Me-induced polypeptides, which are likewise detectable in gels of in vivo and in vitro translated proteins (see Fig. 2). Positions of large (*LSU*, 55 kDa) and small subunits (*SSU*, 15 kDa) of RuBPCase, ovalbumin (45 kDa), and aldolase (39 kDa) used as molecular weight standards are included.

formed, Ja-Me-induced polypeptides, the most prominent ones show molecular masses of 35, 30, 25, 23, 20, and 18 kDa (JIPs 35, 30, 25, 23, 20, 18). The other polypeptides, among them the large (LSU) and small subunits (SSU) of ribulose-1,5-bisphosphate carboxylase (RuBPCase), are found in similar abundances as under control conditions. Since no detectable loss of RuBPCase is observed (Fig. 1), the appearing JIPs do not seem to be proteolytic degradation products of this major leaf protein.

Immunological Relationship of Cotton and Barley JIPs

Possible molecular relationships between cotton and barley JIPs were checked by Western-blot analysis. After two-dimensional gel electrophoresis, total soluble proteins were blotted onto nitrocellulose filters that were successively treated with antisera raised against the 66, 37, and 23 kDa JIPs (anti-66, anti-37, anti-23) from barley (Herrmann et al. 1989). Autoradiography of immunocomplexes detected by ^{125}I-protein A shows that the anti-66 and anti-37 antibodies of barley did not recognize any cotton polypeptide. However, by using the anti-23 antibodies we traced three polypeptides of 23 kDa, which probably represent isoforms of different isoelectric points (Fig. 1C). Their structural relationship to each other and, unexpectedly, to the barley proteins of very similar molecular mass might be an indication that they are encoded by evolutionary conserved genes.

In Vivo and In Vitro Synthesis of Cotton JIPs

In order to address the question of whether the appearance of novel proteins on Ja-Me treatment results from protein synthesis, leaf proteins were pulse-labeled in vivo with [^{35}S]-methionine for 2 h before the harvest of segments. The autoradiogram in Fig. 2B demonstrates that Ja-Me-treated cotyledons synthesize several polypeptides de novo, in comparison to the water-control cotyledons (Fig. 2A). Most of the newly synthesized proteins could be observed as abundant Coomassie-stained spots (Fig. 1B) suggesting that their high level resulted from an increased rate of synthesis rather than a diminished rate of degradation. Some other JIPs were only found in the autoradiograms (Fig. 2B) but not in the respective Coomassie stains (Fig. 1B).

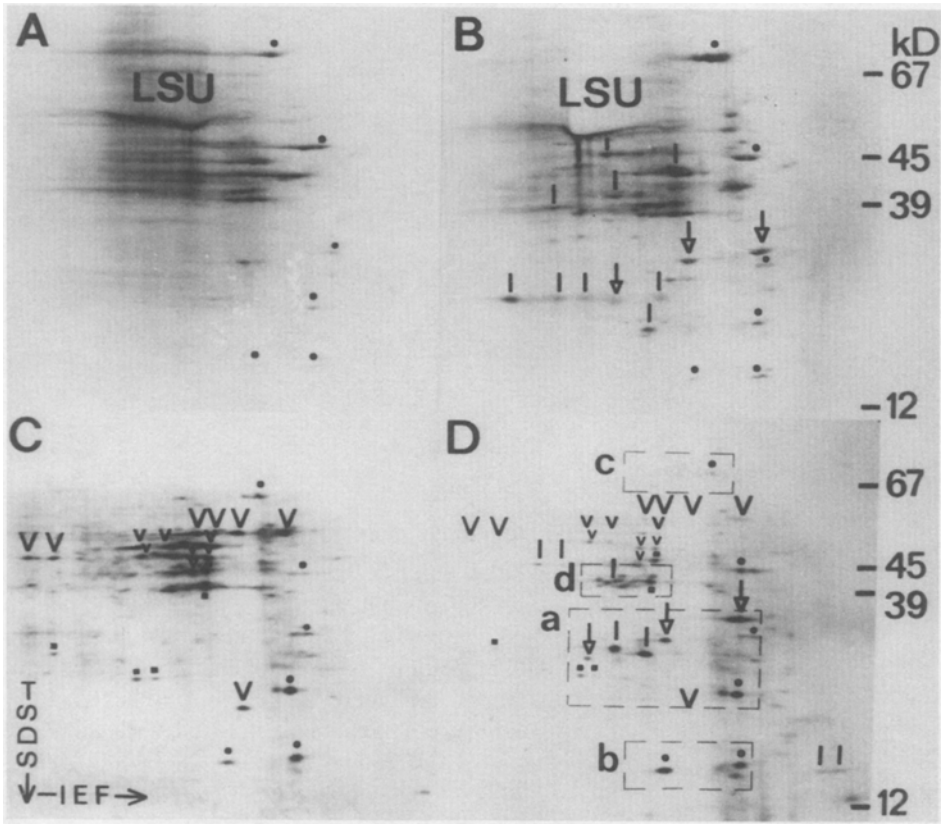

Fig. 2. Two-dimensional polypeptide patterns of in vivo (**A, B**) and in vitro synthesized (**C, D**) proteins. Autoradiograms of gels separating equal cpm of proteins from control (**A**) or Ja-Me-treated (**B**) cotyledons are compared with those of gels separating equal counts of polypeptides formed by cell-free translation of RNA from control (**C**) or Ja-Me-treated cotyledons (**D**). Polypeptides marked by *vertical bars* (|) or by *arrows* (↓) represent Ja-Me-induced in vivo or in vitro translation products. *Open arrowheads* (∨) indicate in vitro translated proteins and, thus, mRNAs disappearing from the RNA population. *Boxes* (■) and *dots* (●) mark constitutive polypeptides. The latter proteins and those marked by arrows are likewise detectable after in vivo and in vitro translation. The framed parts of the gel (*a–d*) are separately shown in Fig. 3. Positions of molecular weight standards (bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; aldolase, 39 kDa; cytochrome c, 12 kDa) are indicated. Same details of separation as in Fig. 1.

Most of the polypeptides synthesized under control conditions (control proteins, e.g., the large subunit of RuBPCase) were labeled at almost unchanged rates in the Ja-Me-treated cotyledons.

To correlate the formation of novel proteins with that of new mRNAs, in vitro translations of RNAs from cotyledons floated for 5 days on water or Ja-Me were included. Analysis of in vitro translated polypeptides indicated that only a very limited number of in vitro translatable mRNAs directed the synthesis of proteins that were electrophoretically indiscernible from those found after in vivo translation (dots and arrows in Fig. 2). Obviously, most cotton proteins translated in vitro had changed isoelectric points and relative molecular masses, which suggests their posttranslational modification in vivo.

By comparing the pattern of polypeptides translated in vitro from RNA of control and Ja-Me-treated cotyledons, three main classes of mRNAs could be distinguished (Fig. 2C and D): (1) constitutive mRNAs (dots and boxes), (2) disappearing mRNAs (open arrowheads), and (3) appearing mRNAs (bars and arrows).

The overwhelming number of mRNAs for control proteins belonged to the second class. Certain control mRNAs were reduced in amount to a low but still detectable level in Ja-Me-treated cotyledons, whereas certain others vanished from the messenger population during the Ja-Me treatment of the cotyledons.

The temporal pattern of appearance of JIP mRNAs was studied by in vitro translation of mRNAs from cotyledons incubated for 6, 12, 18, 24, and 48 h in the presence of Ja-Me (Fig. 3). The mRNAs for JIPs 35, 30, and 23 (vertical bars in Fig. 3a) accumulate steadily and are already detectable about 6 h after the Ja-Me treatment. On the other hand, mRNAs for JIPs 67, 40, and 15 (Fig. 3b–d)

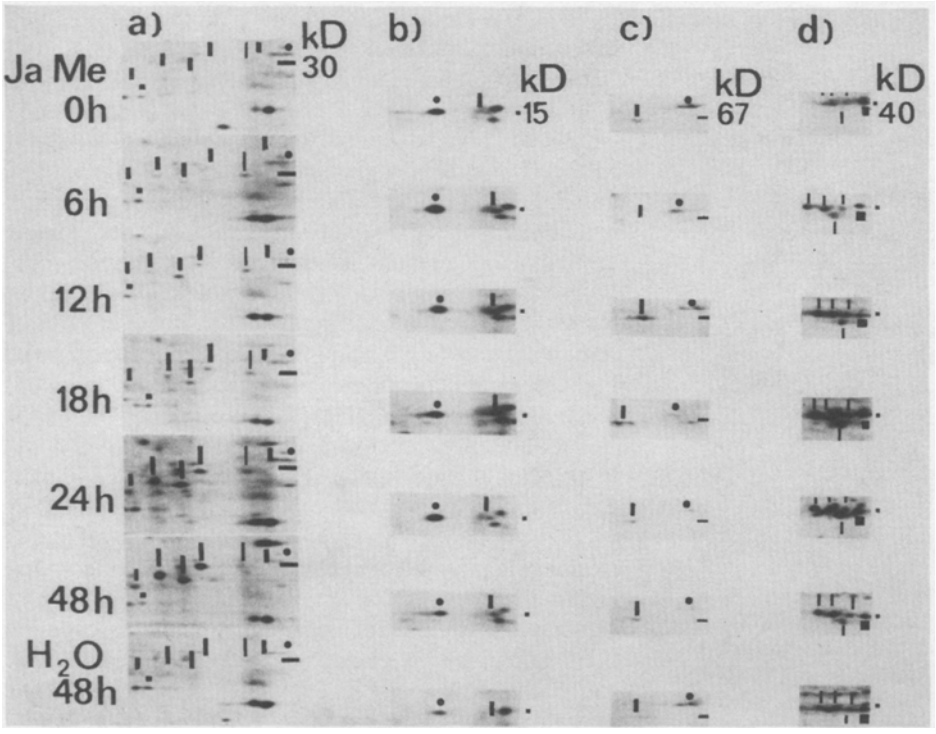

Fig. 3. Kinetic analysis of the mRNA composition of water-or Ja-Me-treated cotyledons. After incubation of cotyledons for the indicated periods of time, RNAs were prepared and translated into polypeptides in the wheat germ system. Equal cpm of L-[^{35}S] methionine-labeled polypeptides were separated in two-dimensional gels and detected by autoradiography. Polypeptides encoded by constitutive or Ja-Me-induced mRNAs are designated in the framed parts as in Fig. 2. Their approximate molecular masses are indicated.

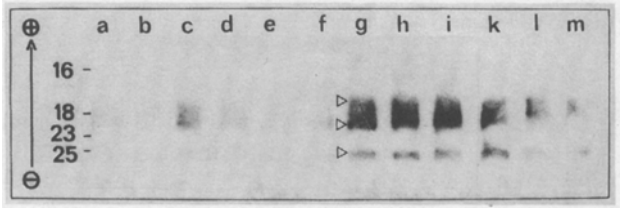

Fig. 4. Temporal pattern of Lea-mRNA expression in water-or Ja-Me-treated cotyledons. Following electrophoretic separation in denaturing agarose gels, the nitrocellulose-bound RNAs from freshly harvested cotyledons (**a**) or cotyledons incubated for 6 h (**g**), 12 h (**b, h**), 18 h (**i**), 24 h (**c, k**), 48 h (**d**), 72 h (**e, l**), and 120 h (**f, m**) in water (**b–f**) or a solution of Ja-Me (**g–m**) were hybridized with the ^{32}P-labeled Lea-oligonucleotide probe (see Materials and Methods). The positions of rRNAs from *Euglena gracilis* used as molecular weight markers are indicated in the autoradiogram. Triangles mark the position of detected Lea-transcripts.

appeared only transiently. None of the mRNAs accumulated to a comparable level in cotyledons during the incubation period (Fig. 3a–d).

Accumulation of Lea Transcripts

Lea proteins and mRNAs (Galau et al. 1986) are ubiquitously distributed in seeds of many higher plants (Dure et al. 1989).

Their occurrence and temporal pattern of expression have been intensively studied during cotton seed embryogenesis (Galau et al. 1986, Galau and Hughes 1987). In the last period of embryo development (the desiccation phase) Lea proteins become the most prominent macromolecules of the seed (Galau et al. 1987, Galau and Hughes 1987). During germination they disappear from the cotyledons of the mature embryo.

For this reason we traced Lea transcripts as putative mRNAs for control proteins. This was done by Northern-blot hybridization of cotton RNA with a synthetic oligonucleotide probe corresponding to a sequence motif of class III Lea genes, which is highly conserved in various plant species including barley, rape, carrot, and cotton (Dure et al. 1989).

By using the 28mer oligonucleotide probe indicated above, three transcripts of 2.17, 1.46, and 0.71 kb could be detected at a Tm -15°C criterion in Ja-Me-treated cotyledons (Fig. 4). According to

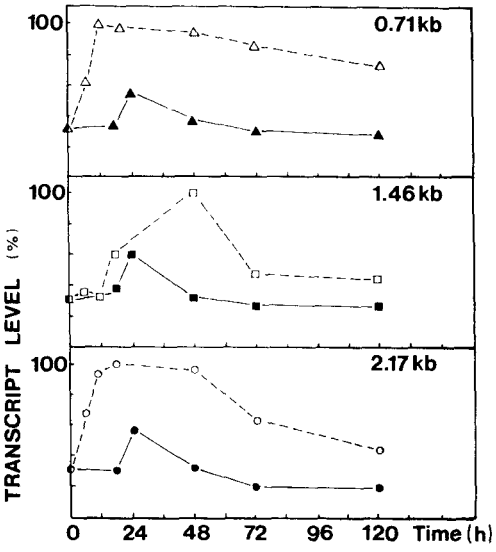

Fig. 5. Relative Lea-transcript contents in Ja-Me- or water-treated cotyledons. After detection of Lea-transcripts by hybridization (see Fig. 4), the relative amount of the detected 2.17 kb (○, ●), 1.46 kb (□, ■), and 0.71 kb (△, ▲) transcript in Ja-Me- (○, □, △) or water (●, ■, ▲)-treated cotyledons was estimated from scanning the optical densities of signals on the x-ray film. The Lea-transcript levels are expressed as percent of the maximum reached by the individual transcript species in the Ja-Me-treated cotyledons at a certain point in time.

the sequence of the probe, they should be homologous to a region of an abscisic acid-inducible mRNA of barley aleurone layers (Hong et al. 1988). The corresponding amino acid sequence N-Glu-Ala-Ala-Lys-Gln-Lys-Ala-Ala-C also occurs with some degree of degeneracy in the cotton Lea D-7 protein (Baker et al. 1988, Dure et al. 1989).

Except for a transient rise after 24 h, all Lea transcript species remained quantitatively almost unchanged in the segments of water-treated cotyledons. In contrast, their amount increased three- to fourfold within 6–12 h (0.71 and 2.17 kb transcript) or 48 h (1.46 kb transcript) in Ja-Me-treated cotyledons. After maximum, the transcript contents declined (Figs. 4 and 5).

Discussion

Obviously, cotton cotyledons respond to Ja-Me treatment by the formation of novel proteins, called JIPs (see Figs. 1 and 2), and respective mRNAs (see Figs. 2–5). In this respect the present paper confirms our findings with barley leaves (Müller-Uri et al. 1988, Weidhase et al. 1987a,b). However, differences for the cotton and barley JIPs were found in at least two aspects.

First, isoelectric points and molecular masses discriminate most cotton JIPs from the respective barley proteins: JIPs 67, 40, 35, 25, 23, 20, 18, and 15 in cotton (see Figs. 1 and 2) versus JIPs 110, 66, 37, 30, 23, 12, and 9 in barley (Herrmann et al. 1989, Müller-Uri et al. 1988). Certain JIPs (i.e., JIPs 67/66 and 23) could be detected in both plant species. But only the 23 kDa JIPs were immunologically related and, for this reason, recognized by antibodies directed against the barley polypeptides. On the other hand, antibodies raised against the most abundant barley JIPs of 66, 37, and 23 kDa did not recognize the respective proteins from oat, wheat, rye, tomato, and cucumber (Herrmann et al. 1989).

Second, most cotton JIPs show extensive post-translational modification in vivo if deduced from the comparison of the migration positions of in vivo- and in vitro-translated JIPs in two-dimensional gels (Fig. 2B and D). Interestingly, one member of the cross-reactive 23 kDa JIPs did belong to the few exceptions of in vitro-translated polypeptides which had a counterpart among the in vivo-translated proteins (Fig. 2B). In barley, the 23 kDa and all other in vivo- and in vitro-formed JIPs occupied very similar positions in two-dimensional gels indicating that little posttranslational modifications changed them in vivo (Müller-Uri et al. 1988). In addition, the accumulation of various JIP mRNAs (Fig. 3) emphasizes a remarkable alteration of gene expression after Ja-Me treatment.

Another aspect refers to a putative relation between JIP formation, foliar senescence, and/or stress response (see Introduction). Since we never detected a significant loss of RuBPCase and chlorophylls (Fig. 1, and Herrmann et al. 1989), a correlation between JIP formation and foliar senescence is doubtful in 5-day-old Ja-Me-treated cotyledons. An alternative hypothesis concerns with JIPs as stress–response molecules (Parthier 1990). Among a large number of stress conditions where specific proteins are synthesized (Nover et al. 1990, Sachs and Ho 1986) desiccation seems to be a particular phenomenon occurring in the ontogeny of most plants (Bewley and Krochko 1982, Gaff 1980). When the embryo becomes dormant during the late phase of embryogenesis, desiccation tolerance is acquired in the seed. Abscisic acid is thought to be involved in this process (for reviews see King 1982, Quatrano 1986, Ross 1984, Walbot 1978) possibly by inducing the synthesis of Lea proteins (Dure et al. 1989, Galau et al. 1986, Skriver and Mundy 1990).

Most abscisic acid-inducible proteins from various plants (for review, see Skriver and Mundy 1990) share common amino acid domains (Dure et al. 1989) which suggests that they might have a similar

function in the acquisition of desiccation tolerance (Baker et al. 1988). Besides being a normal aspect of seed development, the expression of Lea genes might be on-call in nonembryogenic tissues at other times of the life cycle (Baker et al. 1988, Skriver and Mundy 1990, Taylorson and Hendricks 1977).

Our results on the induction of Lea transcripts in Ja-Me-treated cotton cotyledons is in accordance with this idea. We find a selective reinduction of Lea mRNAs in mature plant leaves, where these mRNAs are normally not expressed. Consequently, Ja-Me treatment can be regarded as some kind of stress that induces processes similar to the desiccation response. JIPs might represent "water stress proteins" (Dure et al. 1989) very similar or even identical with the polypeptides induced by abscisic acid. However, the question remains open whether Ja-Me is directly involved in the stress response or in signal transduction.

Our results concerning the three transcripts observed at a Tm -15°C criterion (Fig. 4) are surprising and difficult to interprete, if related to the data of Galau et al. (1986). They never detected any cross-hybridization of the Lea gD-7 probe to other Lea genes and respective transcripts under the same stringent hybridization conditions we used. Additionally, precursor/mature relationships of transcripts coded by the same locus of the cotton Lea gD-7 gene were never observed (Galau et al. 1986). However, since we have used a short synthetic oligonucleotide for hybridization, it might be that the probe recognized highly conserved sequence motifs occurring in the structurally related, but not identical, transcripts. In agreement with the idea of different genes encoding the three detected Lea transcript species, considerably different kinetics of transcript accumulation were observed (Figs. 4 and 5). Further studies have to unravel the nature of the different Lea transcripts.

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