The octadecanoid pathway is required for pathogen-induced multi-functional acetyl-CoA carboxylase accumulation in common bean (Phaseolus vulgaris L.)

Berenice García-Ponce, Mario Rocha-Sosa *

Instituto de Biotecnología, Universidad Nacional Autónoma de México. Apdo. Postal 510-3, Cuernavaca, Morelos 62250, Mexico

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Abstract

A partial cDNA clone corresponding to the multi-functional acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) was isolated using RNA extracted from methyl jasmonate (MeJA)-induced common bean cell cultures. Most of this clone corresponds to the 3' untranslated region and it showed high identity to alfalfa and soybean ACCase sequences. Southern hybridization revealed one copy of this gene in the common bean genome. In addition to being induced by MeJA in cell cultures and leaves, ACCase mRNA accumulated after yeast elicitor or Pseudomonas syringae pv tabaci treatment. Inhibitors of the octadecanoid pathway severely reduced ACCase mRNA and protein accumulation induced by yeast elicitor or P. syringae pv tabaci, indicating that jasmonates or a precursor mediate ACCase induction after pathogen infection. These results provide a role for the eukaryotic ACCase during the defense response to pathogens in common bean. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) synthesizes malonyl-CoA in an ATP-dependent carboxylation of acetyl-CoA. Two types of ACCase have been reported in plants: the so-called 'prokaryotic' multi-subunit form, built by three dissociable polypeptides present in the chloroplast of most higher plants and involved in fatty acid biosynthesis, and the multi-functional or 'eukaryotic' form, a homodimer of ≈ 250 kDa, which has been localized both in the cytosol of all plants analyzed and in the chloroplast of Gramineae and Brassica napus [1–3]. The function of the cytosolic isoform seems to be the elongation of the fatty acid chain [4] and the synthesis of flavonoids [5] and stilbenes [6], since malonyl-CoA is one of the chalcone synthase and stilbene synthase substrates.

In plants, one response to pathogen attack is the de novo synthesis of a number of small molecular weight compounds with antimicrobial activity, called phytoalexins. These compounds contain different chemical structures: phenylpropanoids, isoprenoids and alkaloids. Phytoalexins synthesized by legumes belong to the phenylpropanoid (flavonoid) group. In common bean whole plants and cell cultures, the accumulation of phytoalexins is induced by pathogens [7] or elicitor treatment [8]. In parallel with the rise of phytoalexin produc-
tion, there is an increased accumulation of the transcripts and the corresponding enzymes involved in the synthesis of flavonoids. In common bean, the expression pattern of key flavonoid biosynthetic enzymes, such as chalcone synthase (ChS) and phenylalanine ammonia lyase (PAL), in response to pathogen infection or elicitor treatment, has already been well documented [7,9–11]. ChS [12] and PAL [13] genes in soybean and naringenin-7-O-methyltransferase in rice [14] are also induced by jasmonic acid (JA) treatment, suggesting a role for JA as a mediator in flavonoid synthesis. There is evidence involving this growth regulator or its precursor 12-oxophytodienoic acid (PDA) in plant defense against pathogens. JA or PDA causes an increase in the synthesis of phytoalexins in plant cell cultures [13,15,16]. In addition, elicitor treatment of a number of different plant cell suspension cultures induces an increase of JA [13,16] or PDA [17] levels. Strong support on the role of JA in the defense mechanism in plants has come from the analysis of Arabidopsis thaliana mutants affected in JA synthesis or perception. These mutants also have an altered response to pathogen attack [18–20].

In spite of ACCase importance in flavonoid biosynthesis, little is known about its response to pathogen, elicitor or JA treatments. It has been reported that alfalfa cell suspension cultures accumulate ACCase mRNA when yeast or Colletotrichum lindemuthianum elicitor is added to the culture media [21]. However, the role of phytohormones proposed to be mediators in the signal transduction pathway between pathogen attack and ACCase gene expression has not been investigated.

In this paper, we report the isolation of a cDNA fragment of the bean multi-functional ACCase, obtained by differential display using RNA from a cell suspension culture treated with 10 μM MeJA. This transcript also accumulated as a result of yeast elicitor or Pseudomonas syringae pv tabaci (Pst) treatment. Inhibitors of JA synthesis reduced substantially ACCase mRNA accumulation in response to yeast elicitor in common bean cell suspension cultures. In Phaseolus vulgaris embryonic leaves, ACCase mRNA levels increased in response to MeJA or Pst. Pathogen-induced ACCase transcript and protein accumulation in leaves is partially blocked by the application of piroxicam, an inhibitor of JA synthesis.

2. Materials and methods

2.1. Plant material

Plants of common bean, P. vulgaris L. cv Negro Jamapa, were grown in vermiculite in a growth chamber under 16 h light and 8 h dark cycles and 12 000 lux at 25°C until 13 days after imbibition.

Bean cell suspension cultures were obtained as described by León et al. [22]. Cultures (50 ml) were maintained in Shenk and Hildebrandt medium (modified by León et al. [22]) on a rotary shaker (150 rpm) in the dark at 25°C. Each induction was performed 3 days after subculture.

2.2. Assay conditions

Methyl jasmonate (MeJA) purchased from Apex Organics, Leicester, UK (≥90% pure) and salicylic acid (SA), salicylhydroxamic acid (SHAM) and piroxicam (prx) purchased from Sigma, St. Louis, MO, were dissolved in N,N-dimethylformamide stock solutions. For common bean cell culture, MeJA and the JA synthesis inhibitors were used at 10 and 20 μM final concentration, respectively. Diethyldithio-carbamic acid (DIECA) was dissolved in water and used at 500 μM final concentration. Seedlings were both sprayed and watered with 50 μM MeJA or 100 μM prx containing solutions.

Bean cell cultures were exposed to yeast-derived elicitor at 70 μg glucose equivalents per millilitre, final concentration. Pst inoculation was performed at ≈ 1 × 10⁷ or 1 × 10⁸ colony-forming units (CFU) per millilitre, for cell culture and seedlings, respectively. Leaves were infiltrated on the underside using a 1-ml plastic syringe without a needle.

2.3. RNA extraction

For common bean cell culture, the extraction buffer contained: 5 M guanidine-thiocyanate, 0.5% N-lauroylsarcosine, 50 mM sodium citrate pH 7 and 72 mM 2-mercaptoethanol (98% pure) and the procedure was as Ref. [23]. For leaves, the extraction buffer and procedure was the same as in Logemann et al. [24].

2.4. Differential display

Differential display was performed essentially as
described by Liang et al. [25]. Total RNA (1 μg) (DNA-free) from control and 6 h MeJA-treated common bean cell cultures was reverse-transcribed using a mix of three degenerate anchored oligonucleotides (dT)$_{11}$MC (Operon Technologies Inc., Alameda, CA) and later amplified by PCR using an arbitrary decamer (Operon, OPM-07) with the sequence 5'-CCGTGACTCA. cDNA fragments were cloned in the pKS II$^+$ vector. One of the clones, J2-b, was selected for further analysis.

2.5. Sequencing and alignment

DNA was sequenced by the dideoxy chain termination method using a Sequenase II kit (Amerham Pharmacia Biotech, Buckinghamshire, UK). Computer analysis was performed with the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI.

2.6. Southern blot hybridization

Genomic DNA (12 μg) from P. vulgaris was digested with the enzymes indicated in Fig. 2. The DNA was electrophoresed and blot transferred to nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech) as described by Sambrook et al. [26]. Hybridization was performed in PSE buffer: 0.3 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA at 65°C and the blot was washed at high stringency with 0.2 M sodium phosphate pH 7.2, 0.67% SDS, two times at 55°C and another at 65°C for 15 min each. Membranes were exposed overnight to X-ray film (Kodak XAR-5).

2.7. RNA analysis

Northern blot analysis was performed with 30 or 50 μg of total RNA per lane from common bean leaves and cell culture, respectively, gel was transferred to nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech) and fixed both in a gel drier at 80°C for 2 h and with 0.05 M NaOH for 5 min. Hybridization was done in PSE buffer. Blots that were hybridized with the ACCase (J2-b) probe were washed at low stringency with 1 M sodium phosphate pH 7.2, 0.67% SDS. Blots that were hybridized with the ChS [9] and 28S rRNA probes were washed at high stringency as the Southern blot.

2.8. RT-PCR

In order to obtain a larger ACCase fragment, 5 μg of total RNA extracted from Pst infiltrated plants was subjected to RT-PCR. RNA was reverse transcribed with the antisense primer 5'-CA-GAGTCTTTAACCAGTACC (which contains the stop codon) and amplified in combination with the sense primer 5'-ATCCTCGTGCTGC-CATTTC. PCR was carried out according to the Boehringer expand high fidelity PCR system manufacturer protocol. Annealing was for 30 s at 55°C, and elongation for 2 min at 72°C in each cycle. One band of the expected 1.2 kb was obtained and cloned. Partial sequencing showed that this clone corresponds to the region encoding the ACCase carboxy terminus.

2.9. Protein analysis

Leaves were homogenized at 4°C in 3X extraction buffer [27] and centrifuged at 13 000 × g for 20 min. Protein content from each supernatant was measured by the method of Bradford et al. [28], using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (Sigma) as standard. One volume of Laemmli 2X loading buffer was added to the extracts and boiled for 4 min. Total protein (7 μg) per lane was subjected to SDS-PAGE containing 7.5% polyacrylamide [29]. Separated proteins were blotted onto nitrocellulose membranes. These were treated as described in Ref. [27], prior to being probed with streptavidin conjugated to peroxidase (Amersham Pharmacia Biotech). Blots were developed with an enhanced chemiluminescence kit (ECL kit; Amersham Pharmacia Biotech).

3. Results

3.1. Isolation of a multi-functional ACCase cDNA fragment

Our interest is in the characterization of common bean molecular responses to pathogen attack and other stresses. Therefore, we decided to search for genes induced by MeJA, one of the compounds postulated to serve as an intermediate between these stimuli and gene expression. Using the differential display technique [25] with RNA
extracted from control or 10 μM MeJA-treated common bean cell suspension cultures, we were able to isolate a number of cDNA fragments present only in conditions where MeJA was applied. These fragments were cloned into the vector Bluescript pKS II+ and sequenced. The nucleotide sequence of one 415 bp clone, termed J2-b, showed 69.6% identity to the *Medicago sativa* multi-functional ACCase cDNA [21] and 87% identity to the *Glycine max* genomic multi-functional ACCase DNA [30] (Fig. 1).

3.2. A single multi-functional ACCase gene in the *P. vulgaris* genome

Southern blot hybridization using the ACCase cDNA (J2-b) fragment as a probe for total *P. vulgaris* DNA digested with either *Bam*HI, *Eco*RI, *Hind*III or *Bgl*II showed a single hybridization band (Fig. 2). Because J2-b clone corresponds mostly to the 3' untranslated region (Fig. 1), a 1.2-kb fragment containing the region encoding for the carboxy terminus of ACCase was obtained by RT-PCR, using oligonucleotides derived from

Fig. 1. Sequence alignment of the *Phaseolus vulgaris* cDNA fragment (Pv) with the multi-functional type ACCase DNA sequences of *Medicago sativa* (Ms) and *Glycine max* (Gm). The deduced amino acid sequence for the *P. vulgaris* sequence is shown in the bottom line. The stop codon is indicated by * and the putative polyadenylation signals are boxed.

Fig. 2. Southern blot of *P. vulgaris* genomic DNA hybridized with the ACCase cDNA fragment. Genomic DNA (12 μg per lane) was digested with the enzymes indicated at the top, resolved on a 0.8% agarose gel, transferred to a nylon membrane and probed with the ACCase ‘differential display’ fragment (J2-b). On the left, the molecular markers are indicated.
the ACCase soybean sequence (5') and the J2-b sequence (3'). This DNA fragment was used as probe in a Southern blot and only one hybridization band was detected (data not shown). Therefore, it is likely to be a single gene encoding for the multi-functional ACCase in the *P. vulgaris* genome, in contrast to what is found in other plant species where at least two genes are present.

### 3.3. MeJA-induced the accumulation of the ACCase mRNA

The isolation of a MeJA-induced ACCase cDNA fragment from bean suspension cultures was preliminary evidence for the regulation of the ACCase expression by this phytohormone. To obtain a time course for MeJA-induced ACCase mRNA accumulation in bean suspension cultures, we added 10 μM MeJA and took samples at different times. RNA was extracted and analyzed. ACCase mRNA starts to accumulate 2–4 h after MeJA addition, reaches a maximum at 6 h and declines at 8 h (not shown). In Fig. 3A the accumulation of ACCase mRNA at 6 h is shown.

We analyzed ChS mRNA levels by Northern blot with the same membranes previously hybridized with the ACCase probe to see if ACCase mRNA accumulation correlates with the induction of mRNA levels of other enzymes involved in the synthesis of phytoalexins. As shown in Fig. 3A, ChS transcripts also increased in common bean suspension culture after 6 h of MeJA treatment.

To see whether we could detect similar patterns in plants treated with MeJA, RNA was extracted from embryonic leaves of 13-day plants treated with 50 μM MeJA. ACCase transcript accumulation in bean leaves is shown in Fig. 4. In this case, in contrast to the cell culture, maximal transcript accumulation occurred 12 h after the addition of the phytohormone and a re-induction occurred after 48 h treatment. ChS mRNA levels increased by 3 h after MeJA application and remained constant until 72 h. (Fig. 4). From the above results...
we demonstrated that in two different systems, common bean cell cultures and intact leaves, exogenous MeJA is able to induce the accumulation of mRNAs of two enzymes involved in flavonoid biosynthesis, suggesting a role of this phytohormone in the regulation of this pathway.

3.4. ACCase mRNA accumulates in response to yeast elicitor or Pst treatments

Pathogen attack or fungal elicitor application induces the production of phytoalexins by stimulating the accumulation of the transcripts and corresponding enzymes responsible of phytoalexin synthesis [7,9,10]. Common bean suspension cultures were treated with 70 \( \mu \)g glucose equivalents per millilitre of the elicitor and the cells were collected after 6 h of treatment, to address the question whether common bean ACCase mRNA accumulates in response to the application of a yeast-derived elicitor, as has been reported for alfalfa [21]. As seen in Fig. 3A, after elicitor treatment ACCase mRNA accumulated to levels similar to those obtained by MeJA application. However, ChS mRNA was more abundant in elicitor-treated than in MeJA-treated cell suspension cultures.

Common bean cell cultures were treated with 4 \( \times \) 10^7 CFU/ml Pst, to explore whether a non-compatible interaction would induce ACCase mRNA accumulation. The kinetics of appearance of this transcript after the addition of the bacteria was analyzed by Northern blot (Fig. 3B). A weak but reproducible induction of ACCase mRNA accumulation occurred after 4 h of treatment and was maintained until 6 h. ChS mRNA peaked at 4 h and decreased afterwards.

To examine the accumulation of ACCase mRNA in common bean leaves in response to Pst, 13-day old embryonic leaves infected with the pathogen (1 \( \times \) 10^8 CFU/ml) were collected at different times and ACCase transcript was analyzed by Northern blot. ACCase mRNA started to accumulate 3 h after bacterial infiltration and reached a peak 12 h after infection. Similar to MeJA treatment, a re-induction occurred at 72 h. CHS mRNA paralleled ACCase mRNA accumulation pattern (Fig. 4). In addition, we wanted to analyze whether the accumulation pattern of the ACCase protein corresponded to the mRNA kinetics. Total protein was extracted from the same plants treated with Pst and the biotinylated proteins were analyzed by Western blot using streptavidin conjugated to peroxidase (only ACCase protein is shown in Fig. 5). The basal ACCase protein level increased after Pst infiltration. The pattern of accumulation is similar to the one obtained for the corresponding transcript, except for the small amount of ACCase mRNA detected in mock-infiltrated plants at 24 h (Fig. 4), that was not evident at the protein level. This result indicated that during Pst and common bean interaction, the ACCase is not regulated at the translational level. This is the first evidence that ACCase mRNA and protein accumulate after pathogen infection in common bean plants.

3.5. The octadecanoid pathway is required for ACCase induction in response to Pst

The experiments described above showed that in common bean, ACCase transcript accumulated in response to MeJA, elicitor or Pst treatments.
However, it was not clear if JA or a precursor mediates this response. To explore this possibility, common bean suspension cultures were treated with several inhibitors of the octadecanoid pathway 2 h before the addition of yeast elicitor. Six hours after the addition of the elicitor, cells were collected and the accumulation of ACCase mRNA was analyzed. All inhibitors tested strongly reduced ACCase transcript levels induced by the elicitor (Fig. 6). One of the inhibitors, piroxicam (prx), was chosen for the plant experiments in response to Pst, because it was not toxic to bacteria (data not shown) and was very effective in reducing ACCase mRNA accumulation in cell cultures in response to elicitor (see Fig. 6). Prx was both sprayed and added to the irrigation solution 24 h before Pst infiltration and ACCase transcript and protein accumulation was analyzed in leaves infected for 6 and 72 h, respectively. Prx effectively reduced ACCase mRNA and protein levels at both times tested (Fig. 7). These results indicate that oxylipins are required for a complete induction of ACCase mRNA and protein accumulation. Application of oxylipin synthesis inhibitors to cell cultures or embryonic leaves also reduced ChS mRNA accumulation induced by elicitor or Pst.

Fig. 6. Reduction of the elicitor-induced ACCase and ChS mRNA accumulation by oxylipin synthesis inhibitors in bean cell culture. Total RNA (50 μg) extracted from each of the following conditions were hybridized with the ACCase, ChS and 28S-rRNA probes in a Northern blot. At the top, inhibitor and/or elicitor application is depicted by (+). Lane 1, untreated cell culture; lane 2, elicitor induction; lanes 3–4, DIECA application; lanes 5–6, prx; lanes 7–8, SA; and lanes 9–10, SHAM. All samples were taken after 8 h of treatment with inhibitors and 6 h after inoculation.

Fig. 7. Piroxicam reduction of Pseudomonas-induced ACCase mRNA and protein levels in common bean embryonic leaves. (A) ACCase and ChS detected mRNAs by Northern blot in 30 μg of total RNA extracted from leaves pre-treated with either water or 100 μM prx for 24 h before mock or Pst infiltration for the times indicated below. Same blots were hybridized with 28S-rRNA probe as an RNA loading control. (B) Protein extracts from the same plants used in A were electrophoresed in a SDS-PAGE. At top, proteins were blotted onto nitrocellulose membranes and the ACCase biotin-detected protein by a streptavidin conjugated to peroxidase is shown. Underneath, the corresponding stained gel. Lanes are in the following order: Lanes 1 and 4, Plants pre-treated with prx containing solution/magnesium infiltrated; lanes 2 and 5, plants pre-treated with prx/Pst induced; lanes 3 and 6, Pst infiltration. Mock or Pst infiltration periods are indicated below.

4. Discussion

We report in this paper the isolation of a common bean ACCase partial cDNA clone that responds to MeJA in cell cultures as well as in intact plants. This is the first evidence indicating that the expression of this gene is regulated by this hormone. Two facts suggest that the enzyme encoded by this cDNA belongs to a eukaryotic, or multifunctional type of ACCase: first, the similarity of this cDNA to the M. sativa and G. max genes
encoding the respective multi-functional ACCase (Fig. 1); and second, the size of the mRNA (≈8 kb, not shown) detected by probing Northern blots with this clone, since the mRNAs corresponding to the prokaryotic ACCase subunits are smaller.

In bean cell suspension cultures, ACCase mRNA starts to accumulate 2–4 h after MeJA addition, reaches a maximum at 6 h and declines at 8 h (not shown). In embryonic leaves the induction of the accumulation of this transcript is slightly different. There is a peak at 12 h, after which ACCase mRNA level is reduced, a re-induction occurs at 48 h (Fig. 4). The increase in ACCase transcript accumulation after elicitor treatment of common bean cell suspension cultures is comparable to the increase induced by MeJA after the same time treatment (Fig. 3A). Accumulation of ACCase mRNA is induced also by Pst addition to the cell culture (Fig. 3B). Furthermore, ACCase transcript and protein increase their levels after Pst infiltration in embryonic leaves (Figs. 4 and 5). It is interesting that in a long-term treatment where the stimulus is present all the time, a re-induction at the transcriptional level is detected. Since JA can induce its own synthesis [31], this could explain the second peak of ACCase and ChS mRNA accumulation. These results support the idea that ACCase regulation is part of the plant defense response to pathogen attack. Since this enzyme provides the substrate for ChS, ACCase gene expression could be a key regulatory step in flavonoid biosynthesis.

One could expect that genes encoding enzymes involved in the same pathway respond to similar effectors. Evidence in favor of a coordinate regulation between ACCase and other enzymes of the phenylpropanoid pathway has been reported earlier [5,32]. Therefore, we also analyzed the accumulation of the common bean ChS mRNAs. We found that levels of ChS and ACCase mRNAs increase in response to the same treatments.

ACCase and ChS mRNA accumulation in P. vulgaris in response to MeJA or to yeast elicitor and Pst could take place through separate pathways. However, there is a possibility that JA and/or a precursor are mediators in the response to pathogen attack. To test the latter, oxylipin synthesis was inhibited prior to elicitor or Pst treatments. At least three types of inhibitors have been used to block the octadecanoid pathway in plants: (i) drugs interfering with the cyclooxygenase activity of the prostaglandin endoperoxide H synthase such as SA, aspirin, piroxicam and ibuprofen, because of the similarity of the octadecanoid pathway to the pathway leading to prostaglandin synthesis in animals. In plants the targets for these compounds are either lipoxygenase (LOX) [33] or allene oxide synthase (AOS) [34], the first and the second enzymes respectively in the biosynthetic pathway to JA from linolenic acid. Additional sites of inhibition by SA, downstream to AOS activity [35] or after JA synthesis [36] have been proposed as well. (ii) LOX inhibitors such as n-propyl gallate or SHAM [37]. (iii) DIECA, a strong reducing agent, which provokes the reduction of 13(S) hydroperoxylinolenic acid, the LOX product, preventing its subsequent conversion to JA [38]. The application of these compounds prior to wounding or elicitor treatment effectively blocks plant responses to these stimuli [16,36,38–41], suggesting a role for JA or a precursor as mediator in the plant response to these stresses. Although the activity of these inhibitors is not specific, for instance, SHAM is also an inhibitor of alternative oxidase [42]; and SA alter H⁺/K⁺ transport at the plasma membrane [43], the fact that inhibitors of the three types were able to partially block ACCase and ChS mRNA accumulation in response to elicitor treatment (Fig. 6) strongly supports that the cause of this effect is the inhibition of the octadecanoid pathway.

Prx also effectively reduced ACCase mRNA and protein levels in leaves at 6 and 72 h as shown in Fig. 7. All these results strongly suggest that an oxylipin mediates ACCase and ChS gene induction in common bean in response to pathogen attack. In common bean during an incompatible interaction, phytoalexins and ChS or PAL transcripts accumulate only in the infected tissue where the hypersensitive response occurs [44]. Also JA only accumulates in the hypersensitive response lesions and not in adjacent tissue in tobacco leaves [45]. Therefore, JA may participate only as a local signal during the defense response to pathogens. However, we cannot discard the possibility that an intermediate as PDA could be involved in this response.

None of the oxylipin synthesis inhibitors in our conditions could totally block either Pst or yeast elicitor induced mRNA accumulation. One possibility is that the inhibitors used do not completely
block oxylipin synthesis in common bean plants and cell cultures; the other possibility is that additional signals participate. As has been suggested by Penninckx et al. [46], ethylene could act in parallel with JA in the pathogen response of *Arabidopsis* plants.

In conclusion, we have demonstrated that ACCase mRNA and ChS mRNA accumulate in parallel both under stress conditions (elicitor or pathogen induction) and MeJA application, which supports the idea that both enzymes from the phenylpropanoid pathway are regulated in a coordinated manner. Furthermore, we give evidence that oxylipins are involved in signal transduction during the defense response to pathogens in common bean.

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