Purification, Characterization and Molecular Cloning of a Corresponding PCR Product and cDNA Transcript of a Wound Inducible Tomato Leaf Protein

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Purification, Characterization and Molecular Cloning of a Corresponding PCR Product and cDNA Transcript of a Wound Inducible Tomato Leaf Protein.

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology and Chemistry at Carroll College, Helena, Montana.

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March 25, 1996
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This thesis, rather than just a product of science, is a symbol of my time at Carroll College and what I have learned, how I have grown, and all of those whom I have known. Most important and to whom this page is dedicated are those many persons who have helped me along the way. I wish now to express my sincere thanks to Bud Ryan, Dan Bergey, Andreas Schaller, and the Ryan crew for helping shape my future. John Addis, my advisor and thesis director, I thank for his dedication to teaching, confidence and trust. Ed Noonan I thank for helping as a reader and for all the amazing energy and time he puts into Carroll College and the students. Art Westwell I thank for also helping as a reader and for being one of the best and most interesting teachers I have ever known. Thank you also to all of those wonderful friends who have entered my life and have helped and supported me through my trials. Finally and most importantly, I would like to thank my wonderful and unique family. My mother I thank for her uncountable acts of unselfish support and concern. My father I thank for showing me what it means to have integrity, dedication, and self-confidence. My only brother, Brandon, I thank for all the cool things he does for me and for being himself better than anyone I know. My sister, Terra, I thank for the many laughs, support, confidence and love. And my little sis, Bert, I thank once again for continuing to remind me of what it means to be young. Thank you all and take care.
ABSTRACT

Tomato plants respond to wounding caused by herbivorous insects with the induction of certain defensive genes. This wound response is mediated by systemin, an 18 amino acid polypeptide derived from a larger precursor called prosystemin. Transgenic plants that overexpress the systemin precursor exhibit a constitutive wound response allowing the accumulation of high levels of proteins produced by these defensive genes. Reported here is the purification of one of these defense related proteins from transgenic plant extracts by polyacrylamide gel electrophoresis. The N-terminal amino acid sequence was obtained after blotting to PVDF-membranes. An oligonucleotide corresponding to the amino acid sequence was then designed and used as a primer in the polymerase chain reaction (PCR). Three specific PCR products were obtained. The largest one (900 bp) was used to screen a tomato leaf cDNA library. A cDNA clone of 2.1 kb was retrieved. Preliminary sequence data has identified the clone as a ketol-acid reductoisomerase. The second largest PCR product (500 bp) was cloned via a plasmid vector and preliminary sequence data has identified it as an acyl-CoA binding protein. The role that these proteins play in the systemic wound-response remains to be found.
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INTRODUCTION

The wounding of leaves in several plant families by chewing insects or other mechanical means induces the synthesis of defensive proteinase inhibitor proteins in both wounded leaves, and in distal unwounded leaves throughout the plant (1). The proteinase inhibitors cause malnutrition and starvation by inhibiting the insect's digestive proteases (9). This defense response is mediated by a mobile proteinase inhibitor inducing factor which has been shown to be an 18 amino acid polypeptide hormone called systemin (2). This short polypeptide is derived from an inactive precursor molecule called prosystemin. Processing of prosystemin at the C-terminal region yields the active polypeptide systemin which travels throughout the plant and elicits the defense response in tissues distant from the site of wounding (3,4). Perception of systemin in the target tissue leads to the release of linolenic acid from membranes which is subsequently converted to jasmonic acid via the octadecanoid pathway (5). Jasmonic acid is then thought to activate defense gene transcription (Figure 1).

Transgenic plants which overexpress the prosystemin cDNA show a constitutive wound response (6). This manifests in the accumulation of abnormally high levels of proteinase inhibitor proteins and other defense related proteins. Reported here is the purification and characterization of one of these proteins and the cloning of both a corresponding cDNA and PCR product.
HERBIVORE WOUNDING

\[ \Downarrow \]

PROSYSTEMIN PROCESSING

systemic signal

\[ \Downarrow \]

SYSTEMIN

\[ \Downarrow \]

LIPASE + receptor

\[ \Downarrow \]

LINOLENIC ACID

\[ \Downarrow \]

OCTADECANOID PATHWAY

\[ \Downarrow \]

JASMONIC ACID

\[ \Downarrow \]

GENE INDUCTION

\[ \Downarrow \]

PROTEINASE INHIBITORS I AND II

*Figure 1. The systemic wound response. Herbivorous wounding leads to the processing of systemin from prosystemin which travels throughout the body of the plant. When systemin reaches receptors in the plant cell membranes, linolenic acid is released from the membrane and is converted to jasmonic acid through the octadecanoid pathway. Jasmonic acid is then thought to activate genes for the defensive proteinase inhibitors.*
In light of the functional role that plants play in society, the area of plant biochemical research has become increasingly important in the scientific community. Much research has been done to understand the biochemical nature of these organisms. Special attention has been given by certain laboratories to the nature of plant defense. There have been many discoveries in plants' abilities to defend themselves from animal and pathogen attack. One such discovery began with the finding of a wound-induced proteinase inhibitor in plant leaves (13). It was found that tomato leaves, when wounded by herbivorous insects or mechanical means, induce the production of proteinase inhibitor proteins (1). These proteins are found to accumulate to high levels within the leaf tissue of these wounded plants appearing first within about two hours after wounding and accumulating for over a twenty-four hour period (8). These proteinase inhibitor proteins have been found throughout nature and have been shown to form complexes with proteases to inhibit their peptide bond hydrolyzing ability (9). These proteolytic enzymes are highly valuable to many forms of life. Virtually all organisms (animal, microorganism, and plant) require the hydrolysis of proteins in order to replenish free amino acids supplies needed for new protein synthesis. Further, these enzymes are important for other processes such as the proteolysis of the protein coat that surrounds the nucleic acid of the AIDS virus after host cell entry. Since these proteolytic enzymes are so widespread in nature, a means of protecting proteins from these potentially destructive enzymes has also evolved. That is, introduction of foreign proteases into fluids and tissues may lead to unwanted proteolysis, and thus, a means by
which this hydrolytic activity may be halted has developed over time. For example, protection from foreign proteolytic activity of particularly vulnerable tissues such as blood serum, pancreatic acinar cells, and storage tissues of plants have shown high concentrations of protease inhibitors (9). The protease inhibitors are known to act on each of the four classes of proteolytic enzymes and are divided into two categories based on the proteases upon which they act. Both endopeptidases and exopeptidases are included in the term protease, but only endopeptidases are described by the term proteinase. Thus, there are more generally, protease inhibitors, and more specifically, proteinase inhibitors (9). It is proteinase inhibitors that have been the focus of the research in tomato.

In moving back to the discovery of proteinase inhibitor accumulation in wounded tomato leaves, the implications of this event as a defense response can be seen. As an insect begins feeding on the leaves of these plants, wounding occurs. This wounding leads to the production and accumulation of proteinase inhibitors. The insect continues to feed on the leaves as these proteins accumulate, and thus begins to ingest these proteins. Further, if the insect feeds on the plant for a short time, stops, and then resumes feeding within the next twenty-four hours or so, it will consume leaf tissue that is highly concentrated in these proteins--upwards of 100 micrograms per gram of leaf tissue (1). These ingested proteinase inhibitors will proceed to interact with the insect's digestive proteinases in a competitively inhibitory manner, resulting in a slowing of the insect's digestive processes (9). Studies have shown that insects are then unable to efficiently derive nutrition from proteins when their digestive enzymes are being blocked. This manifests in limited growth of the insect and
even death when the insect's only source of nutrition are plants that are producing inhibitory proteins (9).

The detailed research that has been done on tomato has yielded a more specific understanding about the action of proteinase inhibitors. To begin, two proteinase inhibitors have been found—proteinase inhibitor I and proteinase inhibitor II (10). Both are induced by wounding and accumulate to comparable levels, with proteinase inhibitor II accumulating to about one-half of the percentage of leaf weight that proteinase inhibitor II composes (8). The interest in tomato proteinase inhibitor production in response to wounding became even greater when it was found that their accumulation did not just occur in the local area of wounding, but rather throughout the entire body of the plant. It was found that distal non-wounded leaves produced nearly as much proteinase inhibitor I and II as did wounded leaves (1). Thus, there must be some type of signal that is traveling throughout the body of the plant and inducing distal proteinase inhibitor production. Early ideas for this signal being electric, hydraulic, and hormonal led to experiments centered around discovering the wound-response signal, termed the proteinase inhibitor inducing factor (PIIF) (10). Primary candidates for the signal were oligogalacturonides, which are active sugar fragments derived from the plant cell wall (10). These sugar oligomers, or large chains made by bonding up to twenty smaller sugar units together in various arrangements, induce the synthesis of antibiotic phytoalexins which protect the plant from bacterial and viral attack (10). These were ruled out as the signal, however, because the enzymes that are needed to degrade the cell wall and release these oligogalacturonides were not found in tomato leaves
Further, when these fragments were observed for their ability to function as a signal, fragments greater than six units in length were found to be immobile. Thus the search went on for a signal that was mobile and free from carbohydrates. The search ended with the purification of a very small polypeptide as the proteinase inhibitor inducing factor. Chromatographic purification led to the isolation of 1 mg of the signal polypeptide from nearly 60 pounds of induced tomato leaf tissue. The polypeptide was analyzed and sequenced, revealing that this proteinase inhibitor inducing factor was a novel polypeptide of only 18-amino acids in length and was named systemin because of its apparent systemic mobility.

With the search for the PIIF over, experimentation became focused on systemin as a biologically active molecule. That is, what parts of this molecule are important for its action, how does it travel throughout the plant, where does it come from, and ultimately, how does it interact with plant tissues to induce the production of proteinase inhibitors? To show that the 18-amino acid was the full length polypeptide needed for induction of the wound-response, a synthetic molecule of the same primary structure, or amino acid sequence, was produced and fed to non-wounded tomato plants. These feeding experiments were done by severing the plant at the stem about 1 cm below the level of the first leaves and then placing the plant quickly in a small bottle containing various solutions. It was found that solutions containing the synthetic systemin in femtomolar concentrations (100-1000 fmol/plant) resulted in the full production and accumulation of both proteinase inhibitors I and II. Thus, systemin was shown to be an extremely powerful inducer of the wound response and, in some
way, was able to confer a signal throughout the body of the plant. Exhibiting this
mobility further, experiments using $^{14}$C-labeled synthetic polypeptides have
shown that, when placed on wound sites, within 30 min the radioactivity had
moved throughout the leaf, and into the phloem exudate within 1 to 2 hr (10).
Since systemin is both mobile and an inducer of the wound response,
investigation into the relation between the primary sequence of systemin and its
ability to induce the wound-response was undertaken. In particular, a series of
synthetic system molecules were produced with the amino acid alanine being
substituted for each amino acid separately (14). These alanine substituted
synthetic systemin molecules were fed to young tomato plants. The plant leaves
were then assayed for proteinase inhibitor production, yielding information that
suggested each amino acid in the molecule is essential for full activity and that a
substitution in the 17th position (threonine) produced no detectable proteinase
inhibitor production (14).

With a clearer understanding of the signal molecule itself, studies
pertaining to where this molecule is derived became important. In an attempt to
clone the cDNA for systemin from a wound-induced tomato leaf cDNA library, a
cDNA clone of 839 nucleotide base pairs was found (3). This led to the thought
that systemin may be part of a larger, precursor molecule. It was found after
sequencing the 839 bp clone that systemin occupied the 179th through 196th
amino acids of a larger, 200-amino acid precursor, prosystemin (3). The
discovery of this novel prosystemin led to further interesting experimentation and
questions. The primary structure of prosystemin revealed not only the systemin
molecule at the carboxy-terminal region of the precursor, but also a 6- to 9-
amino acid sequence repeat that shows up in five different regions throughout prosystemin (3). This has led to current investigation in the possibility of further processing of prosystemin after systemin release, yielding other biologically active molecules. After the prosystemin discovery, the possibility of itself being wound-inducible was investigated. Northern blot analysis showed that prosystemin mRNA accumulated in distal leaves of tomato plants whose lower leaves had been wounded, showing that the prosystemin gene is wound-inducible in much the same way that proteinase inhibitors are (3). Unwounded tomato plants were also analyzed, showing that there is a small, constitutive amount of prosystemin mRNA in unwounded plants in contrast to proteinase inhibitor mRNA, suggesting that a continuous supply of systemin may be provided for immediate response to wounding (3).

With the cloning of the prosystemin cDNA, an experiment of particular importance was performed. This experiment deals with the expression of an antisense prosystemin gene in tomato plants. This antisense expression is produced by creating transgenic plants in which the prosystemin antisense cDNA is incorporated into the plant's genome. This sequence is complementary to the sense sequence, and thus complementary to the mRNA that is translated into the prosystemin molecule. Thus, when the antisense construct is transcribed in the cell, it is free to anneal with anything that is complementary to itself, namely the prosystemin mRNA. Since single stranded mRNA (that which is not bound to a complementary strand) is necessary for translation, complementary binding of antisense mRNA should knock out the production of prosystemin. In order to insure that the antisense cDNA would be transcribed in
high levels, the construct was made to include the constitutive cauliflower mosaic virus 35S promoter. Analysis of the transgenic plants showed that expression of the prosystemin antisense cDNA severely decreased the plants ability to produce both proteinase inhibitors I and II in response to wounding (11). This provided both strong evidence that prosystemin is needed for the wound-inducible synthesis of proteinase inhibitors I and II and the opportunity for an intriguing experiment. That is, now that these plants have the wound-response virtually absent, what response will insect pests exhibit when they are exposed to these plants? Since the wound-response is thought to act as a natural plant defense mechanism to deter insect pests, the idea exists that the insects will prefer the transgenic, defense-impaired plants and derive higher levels of nutrition from such plants. In order to determine if this actually occurs, and thus say something more about the wound-response in regards to a means of defense, two specific experiments were performed. The first experiment was conducted by comparing *Manduca sexta* (tobacco hornworm) larvae that were exposed to either transgenic or control, non-transformed tomato plants for 14 days. The average weight of the larvae fed only transformed plants was approximately three times that of those fed only non-transformed plants after 14 days. Secondly, the total amount of leaf tissue that was consumed by the larvae feeding on the control plants was much lower than that consumed by the larvae feeding on the transgenic plants (11). These results indicate that the plants expressing the antisense prosystemin gene have a severely compromised natural defense system and thus provide a much better source of food for the larvae.
It has been shown that the production of proteinase inhibitors in response to leaf wounding is mediated by a mobile polypeptide signal, systemin, that is produced from a larger precursor molecule, prosystemin, that is necessary for the response. This provides some insight as to the beginning of the wound-response, but to this point, the details of what occurs between the entry of systemin into distal leaf tissue and the transcription of the proteinase inhibitor genes has not been discussed. That is, when the plants are wounded, systemin is processed from prosystemin and moves throughout the body of the plant. But what happens when systemin reaches these distal tissues that induces the production of proteinase inhibitors I and II? Much past and present research has been and is focused on this aspect of the systemic wound-response.

It was found some years ago that the two jasmonates, methyl jasmonate and jasmonic acid, when topically applied to leaf surfaces of unwounded plants, induced the wound response (5). This discovery led to the speculation that jasmonates may possibly be components of the wound-response cytoplasmic signalling pathway. Methyl jasmonate is a volatile fatty-acid compound that differs from jasmonic acid by only a methyl group and can induce the wound-response by being sprayed onto tomato leaves. Jasmonic acid was shown to induce the response by being applied in solution to leaf surfaces. A previously proposed pathway for the synthesis of jasmonic acid from Vick and Zimmerman (1984) shows that jasmonic acid is synthesized through a biosynthetic pathway that originates with the fatty acid linolenic acid. It is thought that linolenic acid is a membrane derivative that is released into the cytoplasm by lipase, an enzyme that is activated by signal interaction with plasma membrane receptors.
Although lipase activity has not been specifically linked to linolenic acid production in response to wounding, linolenic acid and other metabolites in the pathway that convert linolenic acid to jasmonic acid have been shown to have a specific link to proteinase inhibitor production. This metabolic pathway, termed the octadecanoid pathway, proceeds as follows: Linolenic acid, an 18-carbon fatty acid containing three double bonds (18:3), is first converted to 13(S)-hydroperoxylinolenic acid by lipoxygenase. 13(S)-Hydroperoxylinolenic acid is then converted to 12-oxo-phytodienoic acid (PDA) by the enzyme, hydroperoxide dehydrogenase (presumably an enzyme complex consisting of allene oxide synthetase and allene oxide cyclase). PDA is subsequently acted on by reductase and then undergoes a series of beta-oxidations to produce the 5-carbon ring containing fatty-acid derivative, jasmonic acid. The metabolic intermediates of the pathway (linolenic acid, 13(S)-hydroperoxylinolenic acid, and 12-oxo-phytodienoic acid) were tested for their specific ability to induce proteinase inhibitors I and II (5). It was found that these compounds have a strong ability to induce proteinase inhibitors I and II when applied to plant leaves (5). Further, compounds that are very similar to these in structure were shown to have no influence on proteinase inhibitor production, except for linoleic acid, which can be converted by the plant into linolenic acid (5). Thus, a very specific relationship exists between the octadecanoid pathway and the wound response. Further, an interesting parallel exists between the octadecanoid pathway in plants and the eicosanoid pathway in animals (5). Eicosanoid signaling in animals, consisting of many compounds, including prostaglandins and leukotrienes, has been shown to often be involved in animal cells' responses to
local stresses (5).

Now that the signaling pathway that connects the plasma membrane to gene induction has been somewhat defined, the question "what does systemin do that actually starts the octadecanoid pathway?" comes into play. Research proposed by Schaller and Ryan (1994) has shown that systemin specifically interacts with a plasma membrane receptor protein, SBP50. SBP50 is a 50 kD plasma membrane protein that requires the amino-terminal portion of systemin for binding. It is interesting that the N-terminal region of systemin is essential in receptor binding, yet it is the C-terminal region that is absolutely necessary for bioactivity. This leaves open an area of investigation into why the C-terminal portion is so important for biological activity.

It is appropriate now to focus on a line of experimentation that made possible the research that is presented here. As discussed, the phenotypic results of knocking out prosystemin through the antisense experimentation yielded plants with a handicapped defense to the tobacco hornworm. Another experiment with prosystemin that is the flip side of the antisense experiment was performed. This experiment actually uses the same method of genetic engineering, but instead of using the antisense prosystemin cDNA for the genetic construct, it makes use of the sense orientation of the prosystemin cDNA. Tomato plants were similarly transformed with prosystemin cDNA in the sense orientation led by the same constitutive cauliflower mosaic virus 35S promoter. Interestingly enough, the transgenic plants that were produced yielded a wound-response that was constitutively turned on. These plants have been shown to overexpress the prosystemin gene and consequently,
constitutively induce the synthesis of proteinase inhibitors I and II (6). Further, when protein extracts were taken from the leaf tissue of these plants, and analyzed by polyacrylamide gel electrophoresis, interesting observations were made. When extracts from sense plants were compared to extracts from control, non-wounded, untransformed plants, a series of induced proteins was observed. Two of these induced protein bands were identified as the proteinase inhibitors I and II (Bergey, unpublished). But a number of other induced bands were noticed. The characterization of these clearly induced bands remained to be accomplished. One such band is the subject of the research presented here.
MATERIALS AND METHODS

The steps involved in the work described here are shown in Figure 2.

Purification and Sequencing of 10 kD Protein

Protein extracts from non-transformed tomato plants and from plants over-expressing prosystemin were prepared (7) and protein profiles were compared after electrophoresis and silver staining on a 15% urea-SDS polyacrylamide gel. The 30 cm X 10 cm X 1.5 mm upright slab gel was prepared with a separating gel of 29.7 mL urea acrylamide gel solution (see appendix), 33.3 mL gel buffer (see appendix), degassed, filtered, 3.3 mL 1.5% ammonium persulfate/water, 50 μL Temed, and a stacking gel of the same composition but with the urea acrylamide gel solution diluted by 1/2. The separating gel was poured to 4 cm from the top of the gel, overlaid with water saturated n-butanol for 1 hr, replaced with gel buffer, and allowed to polymerize overnight at 4°C. After polymerization, the gel buffer was poured off, rinsed, and filled to 1 cm from the top with activated stacking gel. After 1 hr of polymerization, the comb was removed, and the gel was overlaid with n-butanol for 1 hr, replaced with gel buffer, and again allowed to polymerize overnight at 4°C. After polymerization, the wells were rinsed with running buffer (see appendix) and the gel was pre-electrophoresed with 5 mM glutathione (Sigma) in the anode chamber. After loading protein samples, fresh running buffer containing 0.1 mM thioglycolate was added to the anode chamber. Protein samples were loaded as in Figure 3. Lanes 1-4 were silver stained for analysis and 5-8 were electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore). The electroblotted portion was soaked in electroblot transfer buffer (see appendix), and the PVDF
Figure 2. Experimentation leading to the cloning of an Acyl CoA-binding protein PCR product and a ketol-acid reductoisomerase cDNA fragment. A wound induced protein of 10 kD was excised and sequenced. This information was used to produce an oligonucleotide primer for PCR. Two PCR products were obtained. The 500 bp fragment was cloned via a plasmid vector in bacteria and the 900 bp fragment was used to screen a wound induced tomato leaf cDNA library. Sequence information determined the 500 bp fragment to be an Acyl CoA-binding protein and the 900 bp fragment to be a ketol-acid reductoisomerase.
membrane was wetted with 100% methanol and then soaked in transfer buffer. The electroblot cartridge was assembled in a sandwich beginning with plastic support, sponge, 2 sheets of Whatman #3 filter paper, gel, PVDF, 2 sheets of Whatman #3 filter paper, sponge, and plastic support. This was placed in the transfer tank containing 1 L transfer buffer with the membrane facing the cathode and electroblotted for 90 minutes at 200 milliamps constant current. The blotted membrane was stained with Coomassie blue (see appendix) for 5 minutes and washed 3x with destain solution (see appendix).

The 10 kD band was excised and sequenced by automated Edman degradation using an Applied Biosystems sequenator.

PCR amplification of DNA specific to a 10kD protein specified primer

A 5' primer corresponding to the N-terminal sequence data was synthesized. This primer and a 3' oligo dT primer were used in the polymerase chain reaction (PCR) done by the Hot Start method. The lower mix (see appendix) was prepared and added to a Hot Start tube, heated to 70°C for 5 min, cooled to room temperature, and overlaid with the upper mix (see appendix). PCR was performed at a melting temperature of 96°C for 1 min, annealing temperature of 57°C for 1 min, and an elongation temperature of 70°C for 1 min. This reaction cycle was run 30 times and the DNA products were extracted from the reaction mixture by adding 75 μL Tris-EDTA (TE) and 100 μL phenol/CHCl₃, and subsequently ethanol precipitated (see appendix). The pellet was resuspended in 5 μL TE. The 5 μL DNA sample, mixed with 5 μL sample buffer (dye and TE) and 15 μL TE, was loaded and separated on a 1.0% low melting
point (Imp) agarose gel using Tris-EDTA acetate (TAE) running buffer (see appendix) at constant 100 volts for 2 hr and stained 10 min with 100 mL H2O, 2 mL 50X TAE, and 10 μL ethidium bromide (1000x stock). After electrophoresis, the largest (approximately 900 bp fragment) product was extracted from the agarose by excising the band, suspending in H2O up to 360 μL, and adding 40 μL 3 M sodium acetate. After 5 min, it was heated in 65-70°C H2O bath for 10 min, 2X cold phenol was added, vortexted, and spun 5 min. The H2O phase was removed and 1/20X volume sodium acetate and 2.5X volume ethanol were added. The mixture was allowed to precipitate at -20°C for 2 hr, spun 15 min, washed with 70% cold ethanol, spun 5 min, and resuspended in 15 μL TE.

cDNA library screening

The primary screening of the tomato leaf cDNA library began with probe preparation. The probe was prepared by adding 5 μL of the 900 bp fragment DNA sample to 29 μL H2O, by heat denaturing at 100°C for 3 min, and by chilling on ice. To this denatured DNA the labeling mixture (see appendix) was added and allowed to incubate at 37°C for 15 min after which 2 μL 0.5 M EDTA and 50 μL salt/Tris-EDTA (STE) were added. The labeled probes were retrieved by running the reaction mixture on a Sephadex column. The column was equilibrated by centrifuging the columns at 1600 X g for 3.5 min 5 times: 1) filled column with gel/STE mix, 2) filled column again, 3) 100 μL STE, 4) 100 μL STE, 5) 100 μL STE. The reaction mixture was then loaded and centrifuged through the gel filtration column. The eluted probe fraction was collected and read for
specific and absolute radioactivity.

The plaque lift filters were then pre-hybridized. Nitrocellulose filters were used for plaque lifts from sense plant leaf cDNA library (Bergey, unpublished)—approximately 5 x 10^5 total plaques. Ten filters were pre-hybridized by incubation in a 100 mL pre-hybridization solution (see appendix) at 47°C for 2 hr, after which the filters were hybridized with the probe solution (boiled for 3 min first) at 47°C overnight.

The hybridized filters were washed in a 0.5% SDS, 1% SSC solution three times: once in 350 mL at 42°C for 30 min, once in 350 mL at 80°C for 30 min, once in 350 mL 80°C for 30 min. The washed filters were placed on a sheet of plastic, covered with Saran wrap, placed in an amplification cartridge with Kodak film on top, wrapped in tin foil and exposed for 48 hr at -70°C.

After the film was developed, 10 positive plaques were picked for the secondary screening. The plaques picked were placed each in 1 mL SM (see appendix) 3 drops CHCl₃ added, labeled 91-910, and placed at 4°C. These plaque suspensions were diluted 1/500 in 1.0 mL SM and the bacteria to be infected (XL1-Blue MRF') were grown in a maltose/MgSO₄ 2x YT broth (see appendix) to an optical density of 0.500. The diluted plaque suspensions (20 μL) were used to inoculate 200 μL of the bacteria and were incubated at 37°C for 15 min. After incubation, 200 μL of the plaque/bacteria inoculant was added to 2.5 mL of 48°C tempered top agar (see appendix), mixed, poured to NZY agar base plates (same as top agar, except without agarose), and incubated at 37°C for 12 hr. Nitrocellulose filter plaque lifts were made of the 10 plates, dried for 1 hr, and baked in an autoclave at 110°C for 2 min.
The filters were pre-hybridized according to the procedure in the primary screening, using 10X SSPE in place of 5X SSC. Probes for the 900 bp fragment were prepared using the template produced in the previous PCR reaction and the procedure for probe preparation used in the primary screening. After overnight hybridization the filters were exposed to film in a similar manner. One plaque from each plate was picked, suspended in 1 mL SM, 3 drops CHCl₃, labeled 91-910, and stored at 4°C.

In vivo excision

Excision of the insert containing phagemid from lambda DNA was performed by the Strategene ExAssist/SOLR system. The XL1-Blue MRF' and SOLR strains of bacteria were inoculated to 2x YT broth, supplementing the XL1-Blue MRF' cells with 300 μL maltose and 300 μL MgSO₄, 24 hr previous to the excision and was incubated at 30°C. The phage suspensions were vortexed and diluted by 1/500, 100 μL of which were inoculated to 200 μL of MRF' cells that were spun for 2-3 min when they reached an O.D.₆₀₀ of 0.500, resuspended in 10 mM MgSO₄, and grown to an O.D.₆₀₀ of 1.0. To the bacteria/phage solution, 1 μL of helper phage was also added. The suspension was incubated at 42°C for 10 min, 5 mL of 2x YT broth was added, and was again incubated at 37°C for 2.5 hr. The suspension was then heated to 70°C for 20 min, spun for 15 min, and supernatant removed.

Of the supernatant, 10 μL was used to inoculate 200 μL of SOLR cells (O.D.₆₀₀ 1.0) in 2x YT broth and incubated at 37°C for 45 min. To AMP agar 100 μL of each culture were plated. From each plate, 2 colonies were used for mini-
preps (see appendix) and labeled 91A, 91B-910A, 910B.

After ethanol precipitation the pellets were resuspended in 200 μL TE to which 50 mg/mL Pancreatic RNase was added and the suspension was incubated at 37°C for 30 min. After incubation the DNA was phenol/CHCl₃ extracted, ethanol precipitated, dried and resuspended in 40 μL TE. After insert analysis on agarose gel, the 94A clone was chosen for subcloning and sequencing.

Subcloning and sequencing of 94A phagemid insert

The 94A DNA contained in the pBluscript SK- phagemid (Strategene) was digested with Xho1 and EcoR1 (New England Biochemical). To the 40 μL of DNA, 5 μL 10x Xho1 buffer (see appendix) and 5 μL Xho1 were added, vortexed, and incubated at 37°C for 1 hr. After incubation, 50 μL EcoR1 buffer (see appendix) 5 μL EcoR1, and 45 μL H₂O were added and the mixture was incubated at 37°C for 1 hr. After digestion, the DNA was electrophoresed in a 1.0% Imp agarose gel from which the cDNA 94A insert was extracted by the phenol/CHCl₃ extraction protocol and resuspended in 100 μL TE. Of this cloned insert, 8 μL was then digested with 1 μL HaeIII and 1 μL New England Biochemical (N.E.B.) buffer#2 for subcloning. After digestion and electrophoresis, four distinct fragments were extracted by the extraction protocol and ligated into the pSK- vector (Strategene) which was prepared by digesting with EcoRV to give a blunt ended vector. The procedure for the ligation began with resuspending the extracted DNA in 5 μL TE, adding 1 μL 10X ligation buffer
(N.E.B.), 1 μL T-4 ligase, 1 μL 10 mM ATP, 2 μL vector (50 μg), and incubating at 14°C overnight.

After ligation, the four inserts (labeled H1-4) were transformed to electrocompetent cells by electroporation, 100 μL of which was plated to AMP plates and incubated at 37°C overnight. After incubation, 2 colonies from each Hae1-Hae4 were used to inoculate mini-preps (resuspended in 40 μL TE) and labeled Hae11, Hae12-Hae41, Hae42. The eight clones were analyzed on a 1.0% agarose gel and Hae21 was chosen for sequencing.

The Hae21 DNA was prepared for sequencing by a sequencing reaction of 3.2 μL T3-3' primer, 1 μL template (Hae21), 6.3 μL H2O and a reaction mixture containing Taq, dNTP's, and ddNTP's. The DNA was sequenced by an Applied Biosystems sequenator model 373A version 1.2.1.

Amplification and cloning of a 500 bp PCR product

The 500 bp PCR fragment that was produced above was reamplified by the Hot Start method in a total reaction volume of 150 μL. Cycles were 95°C-1.5 min, 57°C-1.0 min, 72°C-1.0 min, 33 times. The PCR product was extracted from a 1.0% agarose gel from the above method and ligated with a pSK-(Strategene) vector prepared by the following method: 10 μL of the vector was digested with 5 μL EcoR1 in 5 μL 10X EcoRI buffer (N.E.B.), 1 μL BSA, and 30 μL H2O, incubated at 37°C for 1 hr, ethanol precipitated and resuspended in 45 μL H2O. The vector was then digested with alkaline phosphatase (see appendix) to remove phosphate groups at 37°C for 30 min. After incubation, 1 μL
additional CIAP was added and incubated for 30 min at 37°C. The activity was stopped with 40 μL H2O, 10 μL 10X STE, 2.5 μL 20% SDS and heating to 70°C for 15 min, after which the vector was extracted with 2X phenol, 1X phenol/CHCl₃, ethanol precipitated and resuspended in 200 μL H₂O giving a concentration of 25 M.

Ligation by the above ligation procedure using 2 μL vector and 1 μL 500 bp DNA was performed. Electrocompetent cells were prepared and transformed by electroporation. Mini-preps were made of transformed cells and 1 μL of the cloned DNA was used as a template for the sequencing reaction. Sequencing reaction products were sequenced by an Applied Biosystems sequenator model 373A version 1.2.1.
RESULTS AND DISCUSSION

Gel electrophoresis of sense plant and control plant protein extracts yielded a protein profile in which certain clearly induced bands can be seen (Figure 3). The enriched band of interest migrated to a level indicating a molecular weight of about 10 kD. After the gel was electroblotted to a PVDF-membrane and stained, the 10 kD protein was easily recognized. A partial N-terminal sequence obtained from the excised band (Figure 4) when compared to protein sequences corresponded to the N-terminal sequence of a Brassica acyl-CoA-binding protein (ACBP). A comparison of the tomato sequence with the Brassica ACBP sequence, as well as other species, can be seen in Figure 5.

The N-terminal comparison, as well as the estimation of the tomato 10 kD protein's molecular weight have given strong evidence that this protein may in fact be a tomato acyl-CoA-binding protein. The fact that this protein is induced in the wound-response (although Northern blot analysis for mRNA induction has not yet been performed) has led to speculation as to the involvement of this protein in the wound-response.

From the N-terminal amino acid sequence data an oligonucleotide primer was designed for use in PCR. The region indicated in Figure 4 was chosen as a guide for primer construction because of the low degeneracy of the possible nucleotide sequence coding for amino acids 2-7. The polymerase chain reaction yielded three specific products (Figure 6) of about 900, 500, and 400 bp in length. The 500 bp pair fragment was judged to be the most hopeful due to the size of the amino acid in question. The length of the 10 kD protein was
Figure 3. Polyacrylamide gel electrophoresis showing wound inducible proteins. Lane 1, 80 μg sense extract; Lane 2, 85 μg control extract; Lane 3, 40 μg ammonium sulfate cut, DEAE flow through; Lane 4, 40 μg ammonium sulfate cut. DEAE bound.
Protein  $^{+}\text{H}_3\text{N}--\text{Ala}--\text{Leu}--\text{Lys}--\text{Glu}--\text{Glu}--\text{Phe}--\text{Glu}--\text{Ala}--\text{COO}'$

Primer  5'$-----------------------$
        AAA---GAA---GAA---TTT---GAA---GCA------3' 
        G G G G C G

*Figure 4.* Partial amino terminal sequence of 10 kD protein. A corresponding oligonucleotide primer was designed to be used in the polymerase chain reaction (degeneracy is shown).
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<td>38</td>
</tr>
<tr>
<td>Human</td>
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<td>38</td>
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<tr>
<td>Yeast</td>
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<td>Drosophila</td>
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**Figure 5.** Comparison of N-terminal amino acid sequence of a 10 kD wound-inducible protein in tomato to the amino acid sequence of an acyl-CoA-binding protein in cow, human, yeast, *Drosophila melanogaster*, and *Brassica* (15).
Figure 6. Products of the polymerase chain reaction. Lane 1, 5’ primer alone; Lane 2, 5’ and 3’ primers; Lane 3, 3’ primer alone; and Lane 4, size standards.
speculated to be about 90 amino acids in length, thus a gene of about 270 bp would be needed. This plus a usual polyA tail of about 200 bp would yield a total transcript length of about 470 bp. The 900 bp fragment was also considered because of the possibility of post-translational processing.

The primary screening of the wound-induced cDNA library with the 900 bp PCR product derived probe yielde positive plaques (Figure 7), 10 of which were used for the secondary screening. From the secondary screening (Figure 8) 10 positive plaques were chosen for cloning. Cloning resulted in a cDNA full length clone of about 2.1 kb that was used for subcloning and sequencing. Sequence data that was obtained after subcloning showed that the clone had a particular resemblance to an *Arabidopsis thaliana* ketol-acid reductoisomerase (18). Studies on ketol-acid reductoisomerasers have shown that these enzymes are involved in branched chain amino acid biosynthesis in plants. This enzyme appears to be the second enzyme in the isoleucine/valine parallel biosynthetic pathway (18). This enzyme carries out a two-step reaction in which either 2-acetolactate or 2-aceto-2-hydroxybutyrate is converted into either 2,3-dihydroxy-3-methyl-butyrate or 2,3-dihydroxy-3-methylvalerate respectively. This enzyme has been studied for its structure-function relationships because of its use as a target enzyme in herbicide design (18). Two questions about this enzyme remain to be answered in terms of the wound-response: why was this transcript specifically amplified in the polymerase chain reaction when the oligonucleotide primer was designed for a different protein, and is this protein wound-inducible? Detailed analysis of the cDNA sequence of this protein and comparison to the oligonucleotid primer used may answer first question and Northern blot analysis
Figure 7. Primary screen of wound induced tomato leaf cDNA library. Positive plaques are indicated by the arrows.
Figure 8. Secondary screen of wound induced tomato leaf cDNA library. Positive plaques are indicated by arrows.
of wound-induce mRNA may yield an answer to the second.

The 500 bp PCR product was directly cloned via a plasmid vector and sequenced. The preliminary sequence data was used for gene bank analysis which produced interesting results. The sequence matched nearly identically to that of an acyl-CoA-binding protein (ACBP). This result confirmed that the 10 kD protein is in fact an ACBP. Further exploration into the role that this protein may have in the wound-response is exciting because of what is already known about the functions of these proteins.

The study of ACBPs in other species, including plant, animal, and microorganism, has led to an understanding about the mechanistic nature of these proteins. It has been found that ACBP is a highly conserved cytosolic protein that is between 86-92 amino acids in length (16). ACBP is known to participate in acyl-CoA metabolism by binding with very great affinity acyl-CoA-linked long chain fatty acids. It has been shown to bind with the greatest affinity to chains 12-22 carbons in length. Further, ACBP exists in levels that are about 2-4 times acyl-CoA concentration (17), suggesting that most existing acyl-CoA linked fatty acids are bound up by these proteins. This may be important for a number of reasons. First, it has been shown that when acyl-CoA esters are bound to ACBP, they are highly resistant to ester cleavage by thioesterases (16). This gives ACBP a functional role in the pooling of the esters. Secondly, ACBP has been shown to play an important role in mediating transport of long chain acyl-CoA esters from one membrane and donating them to an acyl-CoA-utilizing enzyme in another membrane for processes such as beta-oxidation (16).

This information allows for speculation into ACBP's role in the wound
response. First, it is particularly interesting that ACBP binds with greatest affinity fatty acid chains 12-22 carbons in length—a number that would include linolenic acid, jasmonic acid, and the other intermediates in the octadecanoid pathway. This suggests that possibly one of these intermediates may be coupled to acyl-CoA and pooled—waiting for release upon the reception of systemin in the tissue. Because ACBP is known to donate long chain esters to beta-oxidation processes is also very interesting since a series of beta-oxidations occurs in the octadecanoid pathway. The role of this protein in the wound-response may well shed new light on what happens after systemin is received by the membrane receptor. Long standing questions such as 'why lipase activity (release of linolenic acid from membranes) is not induced by wounding?' may now be just a bit closer to being answered.
REFERENCES


APPENDIX

Acrylamide gel solution - 29.17% acrylamide, 1.4% bis-acrylamide, 8 M urea

Alkaline phosphatase - 43 μL vector, 5 μL 10X Calf Intestine Alkaline
Phosphatase(CIAP) buffer, and 1 μL CIAP

Coomassie blue - 0.1% Coomassie blue R-250, 50% methanol

Destain - 50% methanol, 10% acetic acid

EcoRI buffer - 50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100 pH 7.5

Electroblot transfer buffer - 700 mL 0.1 mM CAPS (pH 11.0 with 10 N NaOH), 700 mL methanol, 5.6 L water

Ethanol precipitation - 15 min @ -70°C, spin 15 min, wash and spin 5 min with 70% cold ethanol

Gel buffer - 4.84% Tris, 10% glycine, 0.2% SDS, 8 M urea

Labeling mixture - 10 μL 5X reagent mix(dNTP's, buffer, random hexamers), 5 μL 50 μCi α-32P-dCTP, 1 μL T7-DNA polymerase

Lower mix - 10 μL H₂O, 2.5 μL 10X buffer, 1.5 μL 50 mM MgCl₂, 1 μL 10 mM dNTP's, 5 μL 10 μM 5' primer, 5 μL 10 μM 3' primer

Mini-prep - 200 μL 2x YT inoculated, incubated 37°C overnight, 1.5 mL overnight culture spun for 2 min, pellet resuspended in 100 μL mini-prep buffer (25mM tris-HCl pH 7.5, 50 mM glucose, 10 mM EDTA), 150 μL 0.2 N NaOH and 1.0% SDS added, allowed to sit 5 min on ice, 150 μL 3 M potassium acetate added, 5 min on ice, spin 5 min, collect supernatant
and ethanol precipitate

**Pre-hybridization solution** - 50% formamide, 5X SSC, 5 mM KPP, 2 mL

Denhart's solution, 200 μg/mL salmon sperm DNA, 0.5% SDS

**Running buffer** - 0.6% Tris, 2.88% glycine, 0.1% SDS, water

**SM** - 10.5%NaCl, 3.6%MgCl₂, 50.0 mL 1 M Tris-HCl (pH 7.5), 5.0 mL of 2% gelatin

**Top agar** - 5 g NaCl, 2 g MgSO₄.7H₂O, 5 g yeast extract, 10 g NZAmine (casein hydrolysate), 0.7% agarose, pH to 7.5 with NaOH, bring up to 1 L and add 15 g agar

**Tris-EDTA acetate (TAE) running buffer** - 40mM Tris, 2mM EDTA, 10% acetate

**Upper mix** - 12 μL H₂O, 2.5 μL 10X buffer, 0.5 μL Taq DNA polymerase, 10 μL template (amplified Sense plant leaf cDNA library by Stratagene Zap-Clone)

**Xho1 buffer** - 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol pH 7.9, 100 μg/mL BSA

**2X YT broth** - 10 g NaCl, 10 g yeast extract, 10 g bactotryptone up to 1 L