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MOLECULAR STRUCTURE AND MATURATION OF HEVEIN,
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MOLECULAR STRUCTURE AND MATURATION OF HEVEIN, A CHITIN-BINDING PROTEIN IN RUBBER TREE LATEX

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ABSTRACT

MOLECULAR STRUCTURE AND MATURATION OF HEVEIN, A CHITIN-BINDING PROTEIN IN RUBBER TREE LATEX

BY

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A family of chitin-binding proteins has been isolated from a wide variety of plant species. A common structural motif of these proteins is the chitin-binding domain that consists of 30 to 43 amino acids and contains a high content of glycine and cysteine residues at conserved positions. A chitin-binding domain is often referred to as a "hevein" domain because hevein from the lutoid body (of vacuolar origin) enriched fraction of rubber tree latex possesses only a single chitin-binding domain of 43 amino acids. Although the exact physiological role of hevein *in vivo* is not understood, it has been shown by *in vitro* experiments that this protein inhibits the growth of several chitin-containing fungi.

To understand structure and expression of hevein, a hevein cDNA clone (HEV1) was isolated and characterized. HEV1 encodes a putative signal sequence of 17 amino acids followed by a polypeptide of 187 amino acids: an amino-terminal domain corresponding to the 43 amino acids of mature hevein linked by a hinge

region of 6 amino acids to an extensive carboxyl-terminal domain of 138 amino acids. The difference in polypeptide length between hevein and the HEV1-encoded polypeptide indicates that the formation of mature hevein may result from two proteolytic cleavages of the prohevein *in vivo*. To examine this possibility, domain-specific antibodies were generated. Western blot analysis of the biosynthesis of preprohevein both *in vitro* and in rubber tree latex has revealed that the signal sequence is cotranslationally removed and that the resulting prohevein is cleaved in a subsequent posttranslational processing step.

To further understand the posttranslational processing and targeting of hevein, a cDNA construct was introduced into tomato plants. Northern and Western blot analyses showed that the cDNA-encoded proteins were expressed in transgenic tomato plants. Prohevein was posttranslationally processed in transgenic plants. However, only the C-terminal polypeptide was identified as a cleavage product. Intracellular localization of both proteins suggests that they are most likely localized in vacuoles of tomato plants. It was also found that growth of *Trichoderma hamatum*, a chitin-containing fungus, was retarded in transgenic tomato fruits.

To

My Parents

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CHAPTER 1

INTRODUCTION:

A Family of Chitin-Binding Proteins

Plants synthesize a wide array of proteins that are able to bind reversibly to affinity matrices composed of chitin, a ß-1,4-linked biopolymer of N-acetylglucosamine (GlcNAc). All chitin-binding proteins for which the amino acid sequences are known contain a common structural motif of 30 to 43 amino acids with several cysteines and glycines at conserved positions (Figure 1). This polypeptide motif will henceforth be referred to as the chitin-binding domain. Although the term "chitin-binding proteins" is used to denote the family of proteins containing one or more chitin-binding domains, it must be emphasized that the binding affinity of these proteins is not restricted to chitin but may extend to various complex glycoconjugates containing GlcNAc or N-acetyl-D-neuraminic acid (NeuNAc) as building blocks. Since the natural ligand of chitin-binding proteins is not known with certainty, we can only speculate about their exact physiological role in the plant.

Chitin-binding proteins can be divided into two groups: proteins containing only chitin-binding domain(s) and proteins containing one or two chitin-binding domains and an unrelated domain (Figure 2). The proteins that possess only chitin-binding domains are resistant to heat and proteases. Within this group, the best characterized protein is wheat germ agglutinin (WGA) which is composed of 36-kD homerdimers with four chitin-binding domains per monomer. Although dimerization is apparently required for formation of a functional saccharide binding pocket (Wright, 1984), recent high-resolution X-ray diffraction data indicate that saccharide binding can occur entirely within a single domain (Wright *et al.*, 1991). Other Gramineae lectins (36-kD) isolated from rye (*Secale cereale*; Peumans *et al.*, 1982b), barley (*Hordeum vulgare*; Peumans *et al.*, 1982b), rice (*Oryza sativa*; Tsuda, 1979),

Figure 1. Aligned amino acid sequences of the chitin-binding domains of several chitin-binding proteins.

The one-domain proteins are aligned with domain A of the four-domain protein WGA and the two-domain protein UDA is aligned with domain A and B of WGA. Only those residues that differ from the sequence top line are shown, and sequence identity is indicated by vertical line and conservative substitutions by two dots. Gaps introduced for optimal alignment are represented by dashes. Abbreviations and references: WGAA, WGAD, WGAB, wheat germ agglutinin isolectins (Raikhel and Wilkins, 1987; Smith and Raikhel, 1989); BARL, barley lectin (Lerner and Raikhel, 1989); RICL, rice lectin (Wilkins and Raikhel, 1989); UDA, *Urtica dioica* agglutinin or stinging nettle lectin (Beintema and Peumans, 1992); POTL, tryptic peptide from potato lectin (Broekaert, 1988); HEV, hevein (Walujono *et al.*, 1975); WIN1, WIN2, wound induced proteins from potato (Stanford *et al.*, 1989), BEAC, bean basic chitinase (Broglie *et al.*, 1986), POTC, potato basic chitinase (Gaynor, 1988), TOBC, tobacco basic chitinase (Shinshi *et al.*, 1987), POPC, poplar wound induced chitinase (*win* 8) (Parson *et al.*, 1989), ARAC, *Arabidopsis thaliana* basic chitinase (Samac *et al.*, 1990); RICC, rice basic chitinase (Zhu and Lamb, 1991); BEAC-PR4, bean acidic PR4 chitinase (Margis-Pinheiro *et al.*, 1991); MAIC, maize seed chitinase A (Huynh *et al.*, 1992); Ac-AMP2, *Amaranthus caudatus* antimicrobial peptide (Broekaert *et al.*, 1992).

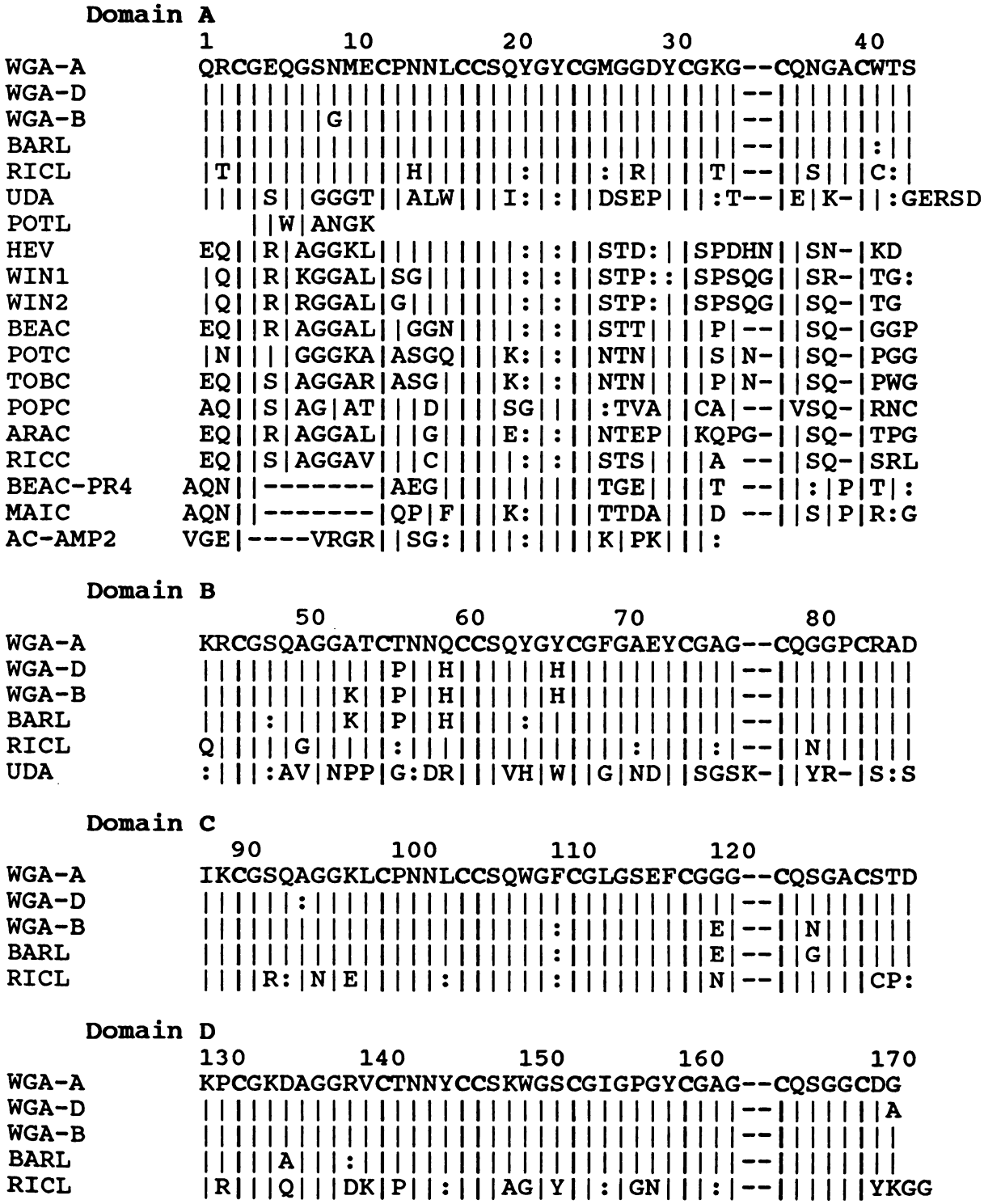

Domain A
1 10 20 30 40
WGA-A QRCGEQGSNMECPNNLCCSQYGYCGMGGDYCGKG--CQNGACWTS
WGA-D
WGA-B
BARL
RICL
UDA
POTL
HEV
WIN1
WIN2
BEAC
POTC
TOBC
POPC
ARAC
RICC
BEAC-PR4
MAIC
AC-AMP2
Domain B
50 60 70 80
WGA-A KRCGSQAGGATCTNNQCCSQYGYCGFGAEYCGAG--CQGGPCRAD
WGA-D
WGA-B
BARL
RICL
UDA
Domain C
90 100 110 120
WGA-A IKCGSQAGGKLCPNNLCCSQWGFCGLGSEFCGGG--CQSGACSTD
WGA-D
WGA-B
BARL
RICL
Domain D
130 140 150 160 170
WGA-A KPCGKDAGGRVCTNNYCCSKWGSCGIGPGYCGAG--CQSGGCDG
WGA-D
WGA-B
BARL
RICL

Figure 2. The primary structure of chitin-binding family and related proteins[a,b].

The preproproteins of Gramineae lectins (Raikhel and Wilkins, 1987; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989 and Smith and Raikhel, 1989) consist of a signal sequence (solid box), four chitin-binding domains (open boxes) and carboxyl-terminal extension propeptide (CTPP) domain (horizontal lines) containing an N-linked high mannose glycan.

The deduced amino acid sequence of Ac-AMP2 cDNA contains a signal sequence, mature Ac-AMP2 domain and a CTPP domain possessing a putative N-glycosylation site (W. F. Broekaert, unpublished results).

Hevein cDNA encodes a signal sequence, a chitin-binding domain (mature hevein) and a carboxyl-terminal domain (stippled box) (Walujono *et al.*, 1975; Broekaert *et al.*, 1990).

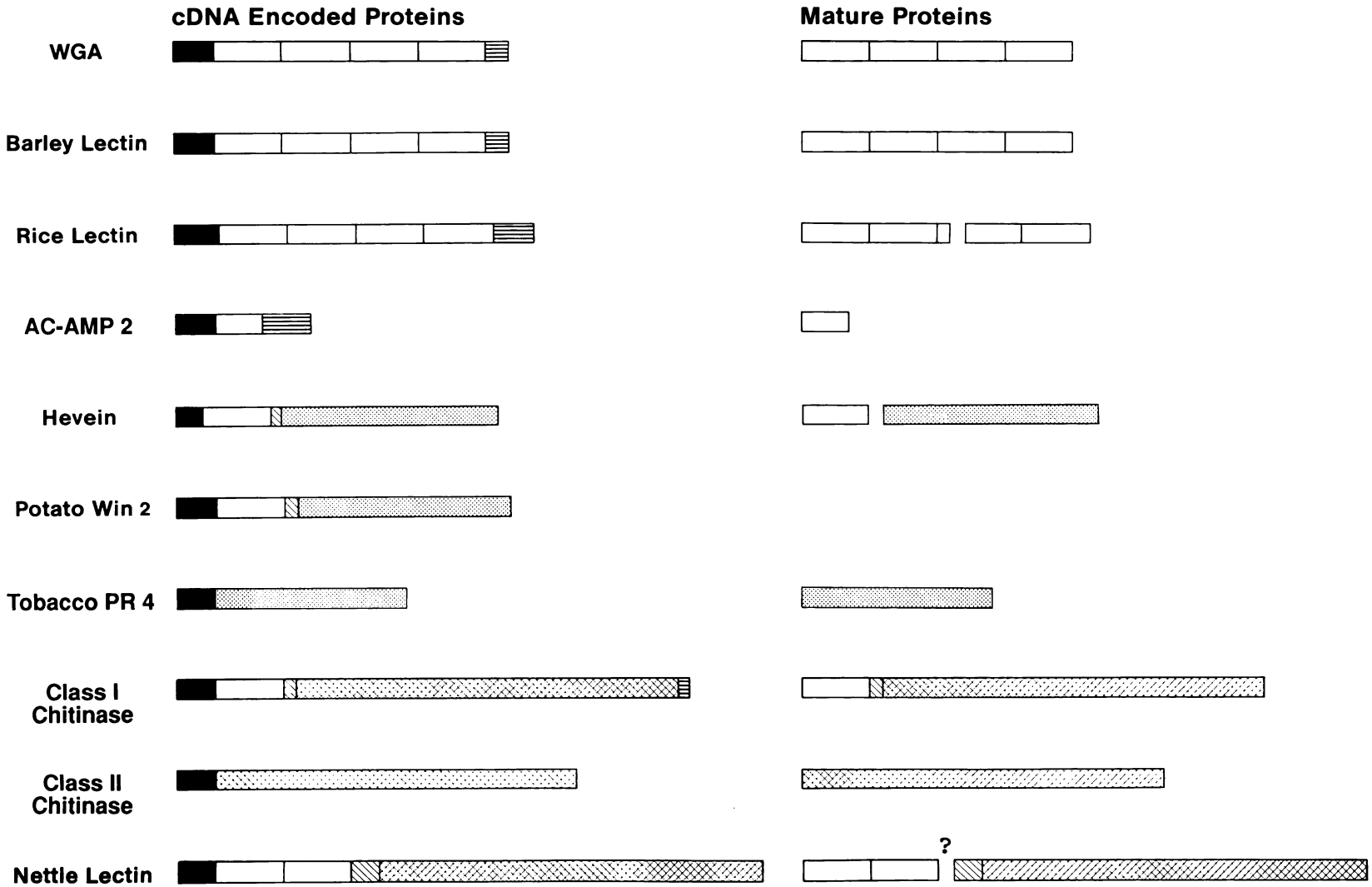
The deduced amino acid sequence of potato *win* genes (Stanford *et al.*, 1989) shows high degree of homology to both domains of prohevein.

[a]The deduced amino acid sequence of tobacco PR-4 cDNA consists of a signal sequence and carboxyl-terminal domain (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991).

The deduced amino acid sequence of class I chitinases (Broglie *et al.*, 1986; Shinshi et al., 1987; Parsons *et al.*, 1989; Gaynor, 1988) shares a similar structure: signal sequence, a chitin-binding domain, a variable hinge region (diagonal box) and chitinase domain (cross-hatched box).

[b]The open reading frame of class II chitinases (Payne *et al.*, 1990; Linthorst *et al.*, 1990) contains a signal sequence and chitinase domain.

Stinging nettle lectin cDNA encodes a signal peptide, two chitin-binding domains (UDA) and a chitinase domain (Lerner and Raikhel, 1992).


cDNA Encoded Proteins
Mature Proteins
WGA
Barley Lectin
Rice Lectin
AC-AMP 2
Hevein
Potato Win 2
Tobacco PR 4
Class I Chitinase
Class II Chitinase
Nettle Lectin
?

couch grass (*Agropyrum repens*; Cammue *et al.*, 1985), and false brome grass (*Brachypodium sylvaticum*; Peumans *et al.*, 1982a) are immunologically and biochemically similar to WGA. In cultivated rice species, the majority of the 18-kD monomer is proteolytically processed to form two subunits of 8-kD and 10-kD which associate via intramolecular disulfide bridges (Stinissen *et al.*, 1985; Figure 2). The smallest chitin-binding proteins are antimicrobial peptides (Ac-AMPs) of 3-kD isolated from the seeds of amaranth (*Amaranthus caudatus*; Broekaert *et al.*, 1992). Since the Ac-AMPs are shorter than the canonic 43-residue chitin-binding domain and contain three disulfide bridges instead of four, they appear to be a truncated variant of the chitin-binding domain. In fact, the Ac-AMP sequences correspond to the "inner conserved core region" of the consensus chitin-binding domain as defined by Wright *et al.* (1991). It is also noteworthy that all residues of the WGA domains that are implicated in saccharide binding are conserved in the Ac-AMPs. The binding affinity of Ac-AMPs to chitin matrices supports the current view of Wright (1992) and coworkers (Wright *et al.*, 1991) that a single "inner core domain" is responsible for ligand binding.

The second group of chitin-binding proteins includes proteins composed of the chitin-binding domain and a number of functionally and structurally unrelated domains. The class I chitinases from bean (Broglie *et al.*, 1986), tobacco (Shinshi *et al.*, 1987), potato (Gaynor, 1988), poplar (Parsons *et al.*, 1989), and rice (Zhu and Lamb, 1991) contain an N-terminal chitin-binding domain of 39-42 amino acids fused by a short hinge region to the catalytic domain (Shinshi *et al.*, 1990; reviewed in Meins *et al.*, 1992), whereas class II chitinases contain only a catalytic domain

(Linthorst *et al.*, 1990). Since the presence of the chitin-binding domain in class I chitinases confers the binding affinity for chitin, only class I chitinases can be classified as chitin-binding proteins. Hevein is a 43-amino acid chitin-binding protein in the latex of rubber tree (Walujono *et al.*, 1975). From the deduced amino acid sequence of the hevein cDNA, as described in this dissertation (Chapter 2), prohevein consists of mature hevein linked by a short hinge region to the C-terminal domain. Prohevein is homologous to proproteins encoded by wound-induced (win) genes of potato (Stanford *et al.*, 1989; Figure 2). In addition, the C-terminal domain of prohevein shares homology with the pathogenesis-related proteins from tobacco (PR-4) and tomato (P2) (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Figure 2). In particular, unlike other chitin-binding proteins, prohevein is partially processed to yield mature hevein and the C-terminal polypeptide (See Chapter 3). Stinging nettle lectin (*Urtica dioica* agglutinin, UDA) consists of two chitin-binding domains. Like hevein, a proUDA-encoding cDNA contains the C-terminal catalytic domain in addition to N-terminal chitin-binding domains (Lerner and Raikhel, 1992). It is not known, however, whether proUDA is stable or is just a transient precursor of UDA in stinging nettle. Although little protein sequence data are available, Solanaceae lectins from potato tubers (Allen *et al.*, 1987), tomato fruits (Nachbar *et al.*, 1978), and thorn apple seeds (Broekaert *et al.*, 1980) are chitin-binding proteins with a highly glycosylated hydroxyproline-rich domain at their C-termini. These C-terminal domains share homology with other hydroxyproline-rich glycoproteins (HRGPs).

Chitin, the most obvious natural ligand, has never been detected in higher plants. Rather, it is an important structural component of the cell wall of fungi and the exoskeleton of many invertebrates, such as insects and nematodes. This fact, and other lines of circumstantial evidence discussed below, point to a role in host defense, although this does not exclude additional functions (Chrispeels and Raikhel, 1991). The locations of chitin-binding proteins in plants indicates that they may interact with plant pathogens. The Gramineae lectins are expressed in peripheral tissues which expand into new areas of soil as the embryo germinates and the root system grows. The patterns of cell-specific localization in peripheral tissues of plant organs have also been reported for class I chitinases in tobacco leaves (Keefe *et al.*, 1990; Mauch *et al.*, 1992), thorn apple lectin in seeds (Broekaert *et al.*, 1988), and stinging nettle lectin in roots and rhizomes (Broekaert *et al.*,1989). The preferential accumulation of these proteins in tissues that are in direct contact with the stress source is consistent with their postulated function in defense. The vacuolar localization of most chitin-binding lectins (except Solanaceae lectins) indicates that these proteins act after the invasion of plant tissue and the release of the cellular contents. Chitin-binding proteins such as chitinases, Gramineae lectins, hevein and *win* proteins are induced by wounding, microbial infection, and the plant 'stress' hormones. Moreover, all chitin-binding proteins examined *in vitro* have demonstrated antifungal, insecticidal or a combination of these activities (Table 1). Finally, an analysis of transgenic plants that overexpress class I chitinases or Gramineae lectins indicates that these proteins can contribute to the protection of

Table 1. Summary of in vitro biocidal activities of chitin-binding proteins.

	Fungicidal Activity	Ref	Insecticidal Activity	Ref
WGA	-	a	+	g
Rice Lectin	ND		+	h
Datura Lectin	ND		+	i
Tomato Lectin	-	b	+	j
UDA	+	c	+	k
Hevein	+	d	ND	
Ac-AMP	+	e	ND	
Class I Chitinases	+	f	ND	

+, activity ; -, no activity ; **ND**, not determined

a: Schlumbaum *et al.*,1986; Broekaert *et al.*, 1989
b: Schlumbaum *et al.*, 1986
c: Broekaert *et al.*, 1989
d: Van Parijs *et al.*, 1991
e: Broekaert *et al.*, 1992
f: Broekaert *et al.*, 1987; Huynh *et al.*, 1992; Leah *et al.*, 1991; Mauch *et al.*,1988; Roberts and Selitrennikoff, 1988; Schlumbaum *et al.*, 1986
g: Czapla and Lang,1991; Huesing *et al.*, 1991a, Murdock *et al.*,1990
h: Huesing *et al.*,1991b
i and **j**: L.L. Murdock and J.E. Huesing, personal communication
k: Huesing *et al.*, 1991b

plants from fungal diseases (Broglie *et al.*, 1991) or insect attack (Maddock *et al.*, 1991).

I am interested in understanding the structure of hevein and the mechanism(s) involved in its maturation at the cellular and molecular levels. As a first step, a hevein cDNA clone (HEV1) was isolated and characterized as described in Chapter 2. In chapter 3, I describe the posttranslational processing events involved in the formation of mature hevein. In Chapter 4, an analysis of transgenic tomato plants, which served as a system to study posttranslational modification, targeting and potential function of prohevein and its cleavage products, is described.

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CHAPTER 2

Wound-Induced Accumulation of mRNA Containing a Hevein Sequence in Laticifers of Rubber Tree (*Hevea brasiliensis*)

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ABSTRACT

Hevein is a chitin-binding protein which is present in laticifers of the rubber tree (*Hevea brasiliensis*). A cDNA clone (HEV1) encoding hevein was isolated by polymerase chain reaction using mixed oligonucleotides corresponding to two regions of hevein as primers and a *Hevea* latex cDNA library as a template. HEV1 is 1018 bp long and includes an open reading frame of 204 amino acids. The deduced amino acid sequence contains a putative signal sequence of 17 amino acid residues followed by a 187 amino acid polypeptide. This polypeptide has two striking features. The amino-terminal region (43 amino acids) is identical to hevein and shows homology to several chitin-binding proteins and to the amino-termini of wound-inducible proteins in potato and poplar. The carboxyl-terminal portion of the polypeptide (144 amino acids) is 74-79% similar to the carboxyl-terminal region of wound-inducible genes of potato. Wounding, as well as application of the plant hormones abscisic acid and ethylene, resulted in accumulation of hevein transcripts in leaves, stems and latex, but not in roots.

INTRODUCTION

Latex of the rubber tree (*Hevea brasiliensis*) is produced by highly specialized cells known as laticifers (de Fay and Jacob, 1989). The contiguous end walls of adjacent laticifer cells are perforated, thus forming an anastomosing system. Upon wounding, the cytoplasmic content of these cells is expelled in the form of latex. Sealing of wound sites occurs by coagulation of the outflowing latex. This process involves bursting of the lutoid bodies (organelles of vacuolar origin) and subsequent interaction of the released cationic proteins with the negatively charged rubber particles (d'Auzac and Jacob, 1989). Wound plugging may be important in preventing entry of pathogens into the phloem.

One of the major proteins in the lutoid bodies of rubber tree latex is hevein (Archer *et al.*, 1969). Hevein is a small, single chain protein of 43 amino acids unusually rich in cysteine and glycine (Walujono *et al.*, 1975). Recently, hevein has been shown to bind chitin and to inhibit the growth of several chitin-containing fungi (Van Parijs *et al.*, 1991). It has therefore been suggested that hevein plays a role in the protection of wound sites from fungal attack (Van Parijs *et al.*, 1991).

Various classes of chitin-binding proteins have been reported to contain polypeptide domains homologous to the hevein sequence. The lectins from the monocotyledonous species wheat, barley and rice are composed of four repetitive hevein-like domains (Wright *et al.*, 1984; Raikhel and Wilkins, 1987; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989), whereas a related lectin from the dicotyledonous *Urtica dioica* is thought to be composed of two such domains

arranged in tandem (Chapot *et al.*, 1986). Basic chitinases from bean (Broglie *et al.*, 1986; Lucus *et al.*, 1985), tobacco (Shinshi *et al.*, 1987) and poplar (Parsons *et al.*, 1989) have a single hevein-like domain located at the amino-terminus which is fused to an unrelated carboxyl portion. Likewise, two wound-induced genes from potato encode proteins with a hevein-like domain located at the amino-terminus and a carboxyl-terminal extension that differs from the chitinase carboxyl-terminus (Stanford *et al.*, 1989).

Here we describe the isolation and sequence analysis of a cDNA clone encoding hevein. However, the cDNA clone (HEV1) encodes a protein with an extensive carboxyl-terminal portion not present in the mature hevein. We also present data on the accumulation of hevein mRNA in response to wounding and hormonal treatment.

MATERIALS AND METHODS

Plant Material

Latex for construction of the cDNA library was obtained from rubber trees twenty years of age (*H. brasiliensis* RRIM600). These trees were regularly tapped for latex and were treated with 0.1% ethephon (2-chloroethylphosphonic acid) two weeks prior to tapping, a procedure which enhances latex production (Coupe and Chrestin, 1989). For studying the regulation of hevein gene expression by Northern blot analysis, seedlings four months of age of *H. brasiliensis* RRIM600, grown in a growth chamber, were used.

RNA Isolation and Construction of Latex-specific cDNA Library

Total RNA from the latex was extracted as described by Kush *et al.* (1990) and poly(A)$^+$ RNA was purified by oligo-dT cellulose affinity chromatography using the method of Silflow *et al.* (1979). Double-stranded cDNAs were prepared from 10 μg of poly(A)$^+$ RNA according to Gubler and Hoffman (1983) with the cDNA Synthesis System (Amersham, Arlington Heights, IL). The cDNA was ligated into lambda gt10 (Stratagene, San Diego, CA) with EcoRI linkers (New England Biolabs, Beverly, MA) and packaged *in vitro* using Gigapack Gold (Stratagene). The host strain used for screening plaques was *E. coli* C600hfl. (The cDNA library was constructed by Dr. Anil Kush.)

Polymerase Chain Reaction (PCR)

The oligonucleotide mixtures were synthesized by the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University. DNA amplification was carried out on a Perkin-Elmer thermal cycler, in a 100 μl vol containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 1 μM of each primer (Fig. 1), 200 μM each dNTP, 2.5 units of *Thermus aquaticus* polymerase (Cetus), and 1 μg of lambda gt10 cDNA library. Twenty five cycles of amplification were performed (96° C for 1 min, 47° C for 2 min, 72° C for 3 min with a final polymerase extension step at 72° C for 7 min) and 10% of the product was analyzed on a 1% agarose (SeaKem; FMC) gel. The product was excised from the gel and reamplified. The reamplified band was isolated as above, digested with *Eco*RI and *Bam*HI, and ligated into pUC119 (Vieira and Messing, 1987).

Isolation of the HEV1 Clone and DNA Sequencing

The amplified PCR fragment was labeled with [^{32}P]dATP by the random primer technique (Feinberg and Vogelstein, 1985). Approximately 200,000 plaque forming units of the lambda gt10 cDNA library were screened with the [^{32}P]-labeled PCR product as the probe by *in situ* plaque hybridization at high stringency conditions (Raikhel *et al.*, 1988). Plaques producing positive signals were selected and rescreened using the same probes under the same conditions. Inserts from purified plaques were subcloned into the *Eco*RI site of pUC119 and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using [α-^{35}S]-dATP and 7-deaza-dGTP in place of dGTP (Mizusawa *et al.*, 1986). The complete sequence of the clones was obtained by sequencing overlapping deletions generated by T4 DNA polymerase (Dale and Arrow, 1987). Sequence analysis was performed by Editbase software (courtesy of N. Nielsen, Purdue University, West Lafayette, IN).

Northern blot analysis

Total RNA from the various parts of four-month old seedlings was prepared by the method of Kirk and Kirk (1985) using aurincarboxylic acid. Microtapping was done to collect the latex, by making an incision on the stem and collecting the drops of exuded latex. Total RNA was separated in 2% agarose gels containing 6% formaldehyde and blotted onto Hybond-N membrane (Amersham). The conditions of blotting, prehybridization and hybridization were as recommended by the manufacturer. Blots were hybridized with the cDNA HEV1 labeled by the random

primer method of Feinberg and Vogelstein (1985).

Wounding of Hevea Stems, Ethephon and ABA Applications

For wounding, a spiral cut about 0.5 cm deep was made on the stem of seedlings and RNA was extracted from stem, leaf and latex 24 hours later. Ethephon 0.1% (v/v) was sprayed on young seedlings and RNA was isolated from stem, leaf and latex after 24 hours. ABA (50 uM) dissolved in sterile water containing 0.01% ethanol was sprayed on the plant and RNA was extracted from stems, leaf and latex after 24 hours as described above. Control plants were sprayed with water/ethanol solution alone. Autoradiograms were scanned with a Gilford (Oberlin, Oh., USA) densitometer.

RESULTS

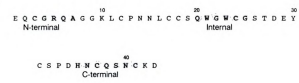
Isolation and Sequence of a Full-Length cDNA Clone

The primary structure of the hevein protein has been previously reported (Fig. 1A; Walujono *et al.*, 1975). We synthesized three sets of oligonucleotide primers corresponding to the amino-terminal, carboxyl-terminal and internal regions of the hevein protein (Fig. 1A; Walujono *et al.*, 1975). Each primer was a degenerate mixture of oligonucleotides encoding the shaded amino acids (Fig. 1). The amino-terminal and carboxyl-terminal primers were used in the PCR. The template for the PCR was phage DNA prepared from a *Hevea brasiliensis* lambda gt10 cDNA

Fig. 1. Complete amino acid sequence of mature hevein and nucleotide sequences of the primers used in PCR.

A) Amino acids of hevein are depicted as single letter codes. Peptide sequences corresponding to the PCR primers are indicated by the shaded boxes. B) The nucleotide sequences of the PCR primers are represented by the following codes: M, either A or C; N, A, G, C, or T; R, either A or G; S, either C or G; W, either A or T; Y, either T or C. The orientation relative to hevein mRNA is sense for the amino-terminal primer, and antisense for the internal and carboxyl-terminal primers. Restriction enzyme sites are underlined (*Hin*dIII) or double underlined (*Bam*HI).

A)



B)

PCR Primer	Primer Sequence	Complexity of the Primer
N-terminal:	5' AAGCTTGARCARTGYGGNMGNCARGC 3'	512
Internal:	5' CCRCACCANCCCAYTG 3'	16
C-terminal:	5' GGATCCCARTTNSWYTGRCARTTRTG 3'	512

library. The amplified DNA fragment was identified by agarose gel electrophoresis as a band of the expected size of 140 base pairs. To confirm that the amplified DNA was derived from a portion of the hevein cDNA, the 140 base pair fragment was gel-purified and reamplified with the amino-terminal and internal primers. The predicted size of the reamplified PCR product was 80 base pairs. To rescue the 140 base pair fragment, DNA from the entire PCR reaction was digested with appropriate restriction enzymes (recognition sites for *Hin*dIII and *Bam*HI were included at the 5' end of the amino-terminal and carboxyl-terminal primers, respectively, Fig. 1B) and cloned into the plasmid PUC119 between the *Bam*HI and *Hin*dIII sites. Recombinant clones were propagated in *E.coli* and several isolates containing inserts of the appropriate molecular size were chosen. Sequencing of the inserts demonstrated that each contained an open reading frame corresponding to the hevein sequence.

For isolating the full-length hevein cDNA clone, a lambda gt10 cDNA library was synthesized from poly(A)$^+$ RNA isolated from the latex of *Hevea brasiliensis*. Approximately 200,000 primary recombinant phages were screened with the 140-base pair PCR product. Eight positive clones were identified that contained inserts ranging from 1 to 1.2 kbp. Dideoxy sequencing of the longest cDNA (HEV1) showed that this clone was 1018 nucleotides long and contained an open reading frame of 204 amino acids including 17 amino acids at the amino terminus that are not present in the mature protein (Fig. 2). These residues comprised a predicted signal sequence structure (von Heijne, 1985) and the cleavage site between the -1 and +1 amino acid matches the amino terminus found by protein sequence analysis

Fig. 2. Nucleotide and deduced amino acid sequence of hevein cDNA clone (HEV1). The deduced amino acid sequence is numbered on the right. Numbers on the left refer to the nucleotide sequence. The open box indicates the mature hevein domain. The two stop codons at the end of the coding region are marked with asterisks. The potential polyadenylation signal is underlined.

```
                M  N  I  F  I  V  V  L  L  C  L  T  G  V  A  I  A  E  Q      2
1   GGAAGAGTTATGAATATATTTATAGTTGTTTTATTATGTTTAACAGGTGTTGCAATTGCTGAGCAA

    C  G  R  Q  A  G  G  K  L  C  P  N  N  L  C  C  S  Q  W  G  W  C      24
67  TGTGGTCGGCAAGCAGGTGGCAAGCTCTGCCCCAATAACCTATGTTGTAGCCAGTGGGGGTGGTGT

    G  S  T  D  E  Y  C  S  P  D  H  N  C  Q  S  N  C  K  D  S  G  E      46
133 GGCTCCACTGATGAATATTGTTCACCTGATCATAACTGCCAAAGCAATTGCAAAGACAGCGGCGAA

    G  V  G  G  G  S  A  S  N  V  L  A  T  Y  H  L  Y  N  S  Q  D  H      68
199 GGTGTTGGTGGTGGAAGTGCTTCCAACGTTCTTGCGACGTACCATTTGTATAATTCACAGGATCAT

    G  W  D  L  N  A  A  S  A  Y  C  S  T  W  D  A  N  K  P  Y  S  W      90
265 GGATGGGACTTGAATGCCGCAAGTGCATATTGCTCTACATGGGATGCTAACAAGCCATATTCATGG

    R  S  K  Y  G  W  T  A  F  C  G  P  V  G  A  H  G  Q  S  S  C  G     112
331 CGGAGCAAGTATGGCTGGACTGCATTCTGCGGTCCCGTCGGAGCACACGGCCAATCCTCCTGTGGA

    K  C  L  S  V  T  N  T  G  T  G  A  K  T  T  V  R  I  V  D  Q  C     134
397 AAGTGCTTGAGTGTGACAAATACAGGGACTGGAGCTAAAACGACAGTGAGGATTGTGGATCAGTGT

    S  N  G  G  L  D  L  D  V  N  V  F  R  Q  L  D  T  D  G  K  G  Y     156
463 AGTAATGGAGGACTAGATTTGGACGTGAATGTTTCCGTCAACTGGACACAGATGGGAAAGGATAT

    E  R  G  H  I  T  V  N  Y  Q  F  V  D  C  G  D  S  F  N  P  L  F     178
529 GAACGAGGTCATATTACAGTGAACTACCAATTTGTTGATTGTGGAGATTCCTTCAATCCTCTATTC

    S  V  M  K  S  S  V  I  N  *  *                                       187
595 TCCGTTATGAAATCATCAGTAATTAATTAATAACATTGGATTGGATGTATGTTTAAGTCCAATCGT

661 AGTAACTAAGCTTCTCAAGCAATAAGCAACAACAAGGCCAATTAATACTTCGTTGGCCACTATAAG

727 AACTTGTGAAATGTTATGAGTTGTTGAAAGAGTTTGTTGTTGGAAATAATGGCATTTGAGCCAGCT

793 CTGTAAGGTATTGGTGAAGATTATTGGGAAGATCGGCTATCTCTTTAGTGAGATATCCATTGGTTT

859 TCCCTTCCTCCTTCCTAAGTTGGGTGTATTTGAGTTACGATTGTGTGTATTTGAGTTACGATTGTG

925 AGTTCAAGGTTGAGTGGCTTGTTATGAGTGAAAAAAATATTTAATGTTTATATTTTTTTTTTATAT

991 AATAAAAGTTTTGTTTGCAAAAAAAAAA
```

(Walujono *et al.*, 1975). The deduced amino acid sequence of the region following the putative signal peptide (Fig. 2) is identical to the known 43 amino acid sequence of hevein (open box in Fig. 2). In addition to the putative signal sequence and the known hevein protein, the cDNA clone encoded a protein with an additional 144 amino acids (Fig. 2) extending beyond the hevein sequence.

The 3'-untranslated region contained two consecutive in-frame termination codons (TAA, TAA, Fig. 2, stars) and a 316 nucleotide untranslated region. A potential polyadenylation signal (AATAAA, underlined in Fig. 2) began at position 991 and was followed 11 nucleotides downstream by a short poly(A) tail.

Northern blot analysis

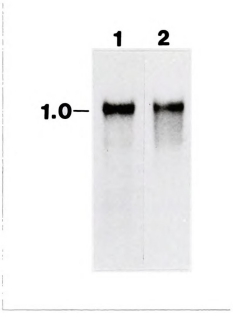
To confirm the identity of the HEV1 cDNA clone, total RNA extracted from *Hevea brasiliensis* latex was fractionated by agarose-formaldehyde gel electrophoresis and transferred to a nitrocellulose filter. A 1.0-kb mRNA was detected (Fig. 3, lane 1) by hybridization with the nucleotide fragment that was specific for the amino-terminus sequence of hevein. The same blot was reprobed with the C-terminal region of the HEV1 cDNA clone which hybridized to the same 1.0-kb mRNA species (lane 2).

HEV1 is Similar to Wound-Inducible Genes in Potato

A comparison of the primary structure of the HEV1 encoded protein with the wound-induced genes *WIN*1 and *WIN*2 of potato (Stanford *et al.*,1989) demonstrated a high degree of homology in both the amino-terminal and carboxyl-terminal portions (Fig.

Fig. 3. Northern blot analysis of total RNA from rubber tree latex.

Lane 1: the blot was probed with a ^{32}P-labelled DNA fragment corresponding to the amino-terminal region of HEV1 (nucleotides 61-182, see Fig. 2). Lane 2: the same blot was stripped and reprobed with a ^{32}P-labelled DNA fragment including the carboxyl-terminal portion of Hev1 (nucleotides 239-610, see Fig. 2).


1
2
1.0

4). The overall sequence identity between the HEV1 and the *WIN* deduced amino acid sequences was as high as 65% (HEV1/*WIN*1) and 68% (HEV1/*WIN*2). No sequence conservation could be observed in the putative signal-peptide region.

Organ-specific Expression of Hevein mRNA

The expression of hevein mRNA was examined by Northern blot analysis. RNA was isolated from intact leaves, stems, roots and latex from four young seedlings of *Hevea brasiliensis*. Hevein mRNA accumulates in leaves, stems and latex (Fig. 5 A-C). However, no hevein mRNA was detected in roots (data not shown).

Accumulation of Hevein mRNA upon Wounding, ABA and Ethylene Treatments

To investigate the extent that hevein mRNA is induced by wounding and hormone treatment, young rubber tree seedlings were locally wounded by applying a spiral cut along their stem. After 24 h the RNA was extracted from leaves, stem and latex of both intact and wounded plants. Northern blot analysis (Fig. 5A) showed that in intact plants, hevein was expressed in all three tissues, reaching the highest expression levels in the latex. However, in leaves, stems and latex of wounded plants the steady-state amounts of hevein mRNA transcripts increased 2 to 5-fold relative to the levels in tissues isolated from control plants (Fig. 5A). A similar increase in hevein mRNA in leaves, stems and latex was observed 24 h after spraying rubber tree seedlings with either 0.1% ethephon (Fig. 5B) to produce ethylene, or 50 uM ABA (Fig. 5C). As a control for the specificity of the observed responses, each blot was reprobed with a probe for beta ATPase (Bounty and

Fig.4. Comparison of the amino acid sequences deduced from the cDNAs of HEV1 and WIN2.

Identical amino acids are marked by vertical lines and conservative substitutions by two dots. Gaps introduced for alignment of homologous domains are indicated by asterisks. The first and the last amino acid of the amino-terminal domain (mature hevein) are designated with arrows.

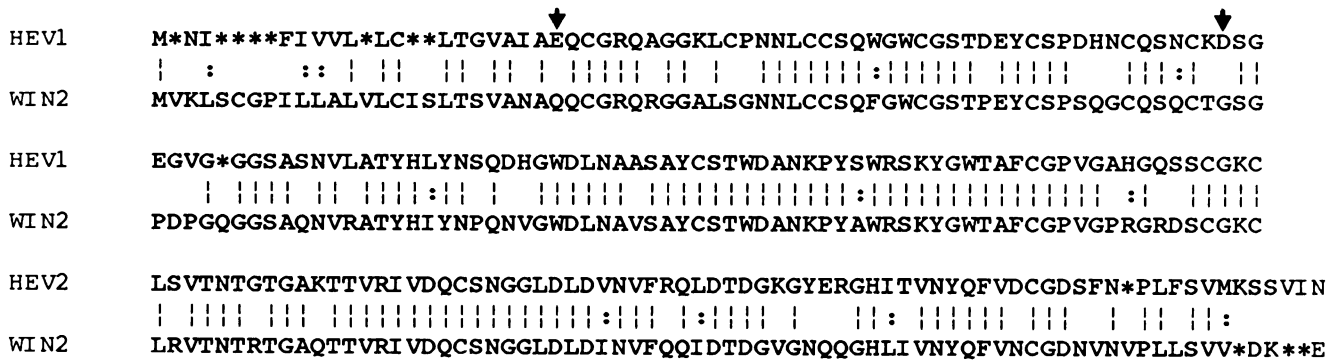
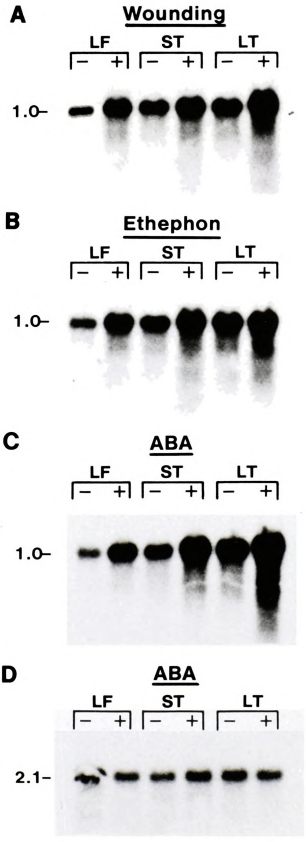

HEV1
M*NI****FIVVL*LC**LTGVAIAEQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSG
WIN2
MVKLSCGPILLALVLCISLTSVANAQQCGRQRGGALSGNNLCCSQFGWCGSTPEYCSPSQGCQSQCTGSG
HEV1
EGVG*GGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRSKYGWTAFCGPVGAHGQSSCGKC
WIN2
PDPGQGGSAQNVRATYHIYNPQNVGWDLNAVSAYCSTWDANKPYAWRSKYGWTAFCGPVGPRGRDSCGKC
HEV2
LSVTNTGTGAKTTVRIVDQCSNGGLDLDVNVFRQLDTDGKGYERGHITVNYQFVDCGDSFN*PLFSVMKSSVIN
WIN2
LRVTNTRTGAQTTVRIVDQCSNGGLDLDINVFQQIDTDGVGNQQGHLIVNYQFVNCGDNVNVPLLSVV*DK**E

Fig.5. Effect of stress treatments on the accumulation of hevein transcripts.

Total RNA (15 ug per lane) was isolated from leaves (LF), stems (ST) and latex (LT) of either untreated (-) or treated (+) rubber tree plants. (A) Treatment: wounding. Probe: coding region of HEV1. (B) Treatment: ethephon (0.1%). Probe: coding region of HEV1. (C) Treatment: 50 uM ABA. Probe: coding region of HEV1. (D) Treatment: 50 uM ABA. Probe: beta-ATPase cDNA. (These experiments were performed by Dr. Anil Kush.)


A
Wounding
LF
ST
LT
− + − + − +
1.0–
B
Ethephon
LF
ST
LT
− + − + − +
1.0–
C
ABA
LF
ST
LT
− + − + − +
1.0–
D
ABA
LF
ST
LT
− + − + − +
2.1–

Chua, 1985) which is known to be constitutively expressed in plants (Kush *et al.*, 1990). None of the treatments affected the level of beta ATPase mRNA in any tissue (Fig 5D). No hevein mRNA could be detected in roots from intact, wounded or hormone treated plants (data not shown).

DISCUSSION

In this chapter, we present the amino acid sequence deduced from a hevein cDNA clone designated HEV1. The first 43 deduced amino acids are identical to the known hevein sequence as determined by direct amino acid sequencing (Walujono *et al.*, 1975). However, the protein deduced from the HEV1 cDNA clone has a striking feature. The DNA sequence of HEV1 encodes a protein that extends 144 amino acids beyond the carboxyl terminus of the hevein protein. Northern blot analysis using the amino-terminal and carboxyl-terminal portions of the HEV1 cDNA clone as probes indicated that they hybridize to the same mRNA species. The results of the Northern analysis and the fact that amino acids deduced from the amino-terminal portion of HEV1 cDNA clone are identical to the known hevein sequence strongly indicate that the HEV1 cDNA clone encodes the hevein protein.

The difference in polypeptide length between purified hevein and the hevein deduced from the cDNA clone may be the consequence of a post-translational modification. Thus, cleavage of the 187 amino acid pro-protein may lead to the

formation of a mature 43 amino acid hevein and a 144 amino acid carboxyl-terminal polypeptide. Alternatively, cleavage of the carboxyl-terminal portion may occur during the purification of hevein and the mature protein may actually contain the full coding region of the HEV1 clone.

The amino-terminus of the protein encoded by HEV1 cDNA shows extensive homology to the N-acetyl glucosamine-oligomer-specific lectins from wheat (Raikhel and Wilkins, 1987), barley (Lerner and Raikhel, 1989), rice (Wilkins and Raikhel, 1989), and *Urtica dioica* (Chapot *et al.*, 1986), to the amino-termini of basic chitinases (Broglie *et al.*, 1986; Lucas *et al.*, 1985; Shinshi *et al.*, 1987) and polypeptides encoded by wound-induced genes (*WIN*1 and *WIN*2) of potato (Stanford *et al.*, 1989). The carboxyl-terminal portion of the HEV1 encoded protein shows homology to the carboxyl-termini of proteins deduced from *WIN*1 and *WIN*2 genes, but not to those of chitinases nor to chitin-binding lectins. The carboxyl-terminus of HEV1 shows 74% similarity to the deduced amino acid sequences from the *WIN*1 gene and 79% to the *WIN*2 gene. The amino terminus (minus the putative signal sequence) has 72% and 74% similarity to *WIN*1 and *WIN*2, respectively. These levels of homology indicate that the carboxyl-terminal portion of these proteins serves a function as important as that of the amino terminus. The study of the role of both HEV1 domains and the relevance of the presumed post-translational cleavage is in progress in transgenic tobacco plants.

Wheat germ agglutinin has been demonstrated to act as an anti-nutrient factor for insect larvae (Murdock *et al.*, 1989). In addition, mature hevein (Van Parijs *et al.*, 1990) as well as the stinging nettle lectin (Broekaert *et al.*, 1989) and

chitinases (Schlumbaum *et al.*, 1986; Broekaert *et al.*, 1988) are known inhibitors of fungal growth *in vitro*. Hence, it appears that in a broad sense this class of related chitin-binding proteins may serve to protect plants from attack by a wide range of potential pathogens. For hevein, however, it remains to be demonstrated that the presumed post-translational cleavage between amino acids +43 and +44 occurs *in vivo*, and that the released amino-terminal hevein portion exerts antifungal activity *in vivo*. It is interesting to note that thionins, a group of hydrophobic defense-related proteins with antifungal properties are also synthesized as large precursors of which the amino-terminal portions correspond to mature thionins (Bohlmann *et al.*, 1988).

Our results indicate that wounding, or exogenous application of the stress-related hormones ABA and ethylene, lead to increased steady-state levels of hevein mRNA in leaves, stems and latex from tapped stems, although not in roots. We do not know at present whether the accumulation of hevein transcripts in leaves and stems is confined to the laticifers or whether other tissues are involved as well. The observed response may be systemic since transcripts accumulate in unwounded leaves upon wounding of the stems. Both wounding and exogenous application of ethylene have been shown to cause accumulation of mRNAs of defense-related proteins such as chitinases in beans (Broglie *et al.*, 1986; Hedtick *et al.*, 1988) and hydroxyproline-rich glycoproteins in carrots (Ecker and Davis, 1987). Recently, ABA has been implicated in the wound-induced response of protease inhibitor II in potato and tomato (Pena-Cortes *et al.*, 1989). In our experiments, both ethylene and ABA mimicked the wound-induced accumulation of hevein mRNAs in different rubber tree tissues. These data support the hypothesis that several separate signal

transduction pathways can lead to the activation of wound-induced and/or defense related genes in plants (Ecker and Davis, 1987; Henstrand and Handa, 1990).

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CHAPTER 3

Co- and Post-Translational Processing of the Hevein Preproprotein of Latex of the Rubber Tree (*Hevea brasiliensis*)

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ABSTRACT

Hevein is a chitin-binding protein of 43 amino acids found in the lutoid body-enriched fraction of rubber tree latex. A hevein cDNA clone (HEV1) [Broekaert, W., Lee, H.-I., Kush, A., Nam, C.-H., and Raikhel, N. (1990) *Proc. Natl. Acad. Sci. USA 87*, 7633-7637] encodes a putative signal sequence of 17 amino acids followed by a polypeptide of 187 amino acids. Interestingly, this polypeptide has two distinct domains: an amino-terminal domain of 43 amino acids corresponding to mature hevein and a carboxyl-terminal domain of 144 amino acids. To investigate the mechanisms involved in processing of the protein encoded by HEV1, three domain-specific antisera were raised against fusion proteins harboring the amino-terminal domain (N domain), carboxyl-terminal domain (C domain) and both domains (NC domain). Translocation experiments using an *in vitro* translation system show that the first 17 amino acid sequence encoded by the cDNA functions as a signal peptide. Immunoblot analysis of proteins extracted from lutoid bodies demonstrates that a 5-kDa protein comigrated with purified mature hevein and crossreacted with N domain and NC domain-specific antibodies. A 14-kDa protein was recognized by C domain and NC domain-specific antibodies. A 20-kDa protein was cross-reactive with all three antibodies. Microsequencing data further suggest that the 5-kDa (amino-terminal domain) and 14-kDa (carboxyl-terminal domain) proteins are posttranslational cleavage products of the 20-kDa polypeptide (both domains) which corresponds to the proprotein encoded by HEV1. In addition, it was found that the amino-terminal domain could provide chitin-binding properties to a fusion protein bearing it either amino-terminally or carboxyl-terminally.

INTRODUCTION

Latex of the rubber tree (*Hevea brasiliensis*) is composed of the cytoplasmic fluid of specialized tube-like cells, called laticifers. These laticifer cells are known to form an anastomosing system in *H. brasiliensis*. Upon wounding, damaged sites are sealed by coagulation of the outflowing latex. This coagulation process involves bursting of the lutoid bodies (vacuolar origin), followed by interaction of the released cationic proteins with the negatively charged rubber particles (d'Auzac and Jacob, 1989).

Hevein is one of the major proteins from the lutoid body-enriched fraction, which can be separated by ultracentrifugation (Archer *et al.*, 1960). It is a small, single chain protein of 43 amino acids (Walujono *et al.*, 1975) and is classified as a chitin-binding protein (Van Parijs *et al.*, 1991). Although the exact physiological role of hevein *in vivo* is not understood, it has been shown by *in vitro* experiments that this protein inhibits the growth of several chitin containing fungi (Van Parijs *et al.*, 1991). Recently, we have isolated and characterized a cDNA clone encoding hevein, designated HEV1 (Broekaert *et al.*, 1990). From the deduced amino acid sequence, it appears that HEV1 encodes a polypeptide of 187 amino acids including a putative signal sequence of 17 amino acids. This polypeptide possesses two distinct domains: an amino-terminal domain corresponding to the 43 amino acids of mature hevein and an extensive carboxyl-terminal domain of 144 amino acids. The difference in polypeptide length between hevein and the HEV1-encoded polypeptide suggests that the formation of mature hevein may result from

two proteolytic cleavages of the hevein precursor *in vivo*. The first cleavage would involve removal of the signal peptide, which is necessary for cotranslational translocation into the lumen of the rough endoplasmic reticulum (RER). A subsequent posttranslational processing could give rise to the formation of the amino-terminal portion (hevein) and carboxyl-terminal portion. Alternatively, cleavage of the carboxyl-terminal extension may occur as an artifact during the purification of hevein.

We are interested in understanding the mechanisms and the role of the post-translational modifications that occur during hevein biosynthesis. One approach to address these questions is to make use of domain-specific antibodies. In this report, we describe the production of domain-specific antibodies and present immunological evidence to show that the hevein signal sequence can be cleaved *in vitro* and posttranslational cleavage of the 187 amino acid polypeptide *in vivo* yields the hevein and carboxyl-terminal peptides. Moreover, we demonstrate that the amino-terminal domain can confer chitin-binding properties on polypeptides to which it is fused.

MATERIALS AND METHODS

Source Materials

Freeze-dried bottom fraction of latex (lutoid body-enriched fraction) was a gift of Dr. Anil Kush from National Institute of Singapore, Kent Ridge, Singapore. Alternatively,

lutoid body-enriched factions were also prepared by a single step of ultracentrifugation (50,000*g*, 1h, 4° C) of latex (Moir, 1959; Martin, 1991) which was obtained from 2-3 year old *H. brasiliensis*, grown in a greenhouse at Michigan State University. HPLC-purified hevein was kindly provided by Dr. J. J. Beintema, Rijksuniversiteit, Groningen, The Netherlands. Chitin was prepared by reacetylation of chitosan (Sigma, St. Louis, MO) as described by Molano *et al.* (1977).

Site-directed Mutagenesis, Plasmid Construction, and Preparation of Fusion Proteins and Polyclonal Antibodies

To facilitate subcloning of the amino-terminal (N), carboxyl-terminal (C) and both (NC) domains of HEV1, restriction enzyme sites and stop codons were introduced into desired DNA sequences of the wild type HEV1 clone by site-directed mutagenesis according to Kunkel *et al.* (1987). Two synthetic oligonucleotides, NR26 and NR27, were designed for mutagenesis. The NR26 (43mer) is 5'C_{44}AG GTGTTGCAATTGCT$_{60}$GAATTCCCCGGGG$_{61}$AGCAATGTGGTCG$_{74}$3' (underline: *Eco*RI and *Sma*I sites) and delimits the 5' end of the N domain. The NR27 (61mer) is 5'G_{170}CCAAAGCAATTGCAAAGAC$_{189}$TGATGAAGATCTAAGCTTGAATTCA_{190}GCG GCGAAGGTGTTGG$_{206}$3' (underline: stop codons, *Bgl*II, *Hind*III and *EcoR*I sites) and was used to mutagenize the junction between the N and C domains. Since the wild type HEV1 sequence has a *Hind*III site located downstream of the natural stop codon, the 3' end of the C domain was not altered. HEV1-containing pUC119 (pHEV1) was used to produce a single stranded uracil-containing DNA template in *Escherichia coli* strain CJ236 (*dut*, *ung*). Two mutant constructs were derived from

this template: HEVm1 contained both mutations introduced by NR26 and NR27, whereas HEVm2 only had the NR26 defined mutation. To confirm the identity of the mutagenized sequences, both constructs were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using single stranded DNA obtained from phagemids in the *E. coli* strain MV1193. The DNA fragments encoding different domains were isolated as *Eco*RI-*Hin*dIII fragments either from HEVm1 (for the N domain, 170 bp, and the C domain, 480 bp) or from HEVm2 (for the NC domain, 610 bp), and subsequently subcloned in frame into corresponding restriction sites of the *E. coli* expression vector pIH821 (New England Biolabs, Beverly, MA). This expression vector carries the *MalE* gene lacking its signal sequence (Guan *et al.*, 1987). Fusion proteins were expressed in *E. coli* strain MV1193 and were purified on a crosslinked amylose affinity column (New England Biolabs, Beverly, MA) according to manufacturer's protocol. To produce polyclonal antibodies against specific domains, purified fusion proteins (100 μg per immunization) were injected into New Zealand white rabbits.

In Vitro Transcription, *In Vitro* Translation and Translocation

For preparation of the hevein transcript, the *Eco*RI-*Hin*dIII DNA fragment from HEV1 cDNA was subcloned into pBS SK$^-$ (Stratagene, La Jolla, CA). RNA transcripts were generated from the pBS SK$^-$ construct using T3 polymerase according to the manufacturer's protocol. *In vitro* translation was performed with a wheat germ extract cell-free system (Promega, Madison, WI) according to the manufacturer's instructions. [^{35}S] cysteine (1,200 mCi/mmol; NEN, Boston, MA) at the final

concentration of 0.5 mCi/ml was added into the reaction mixture because of high cysteine contents (fifteen residues) but low methionine contents (two residues at the position -17 and 181) in the HEV1-encoded protein (Broekaert *et al.*, 1990). For cotranslational translocation experiments, the translation reaction mixture was supplemented with either canine pancreatic microsomes (Amersham, Arlington Heights, IL) or maize microsomes that were kindly provided by Dr. Jan Miernyk (USDA, Peoria, IL). For protease protection assays, Proteinase K (0.5 mg/ml) and 1% (v/v) Triton X-100 were added into the translation reaction mixture and were incubated on ice for 1 hour. After 1 hour incubation, 5 mM phenylmethylsulfonyl fluoride (PMSF) was added to inhibit further proteinase K activity.

SDS-PAGE, Fluorography and Immunoblotting

In vitro translation products were immunoprecipitated with NC domain-specific antibodies (Anderson and Blobel, 1983) and were fractionated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), followed by fluorography (Mansfield *et al.*, 1988). SDS-PAGE was performed on 12 to 17.5% (w/v) polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Control experiments were conducted with pre-immune serum or maltose binding protein (MBP) antiserum. For immunoblotting analysis, proteins from the lutoid body-enriched fraction were separated by SDS-PAGE and were transferred on Immobilon P (Millipore, Bedford, MA) using a semidry electroblotting apparatus (Millipore, Bedford, MA). The blots were probed with N, C or NC domain-specific antibodies and cross-reactive bands were visualized with alkaline

phosphatase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) as described by Black *et al.*(1984).

N-terminal Microsequencing

Proteins from the lutoid body-enriched fraction were separated by SDS-PAGE and were electroblotted on Immobilon P membranes according to Towbin *et al.* (1979) with the following modifications. The transblotting buffer was composed of 10mM CAPS and 10% methanol (pH11). Transfer proceeded for 60 min, i.e., 15 min at each of the following settings: 50 mA, 100 mA, 150 mA and 200 mA. The desired protein bands were excised after visualization with Ponceau S. Alternatively, proteins from the lutoid body-enriched fraction were separated by two-dimensional PAGE as described elsewhere (O'Farrell, 1975), transferred on to Immobilon P membranes and then stained with Coomassie blue. After destaining, the desired spot was excised. Protein samples were analyzed on a 477A protein sequencer with an on-line model 120 PTH AA analyzer (Applied Biosystems, Foster City, CA) by the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University.

Chitin Binding Assay

Samples were dissolved in either 50 mM sodium acetate buffer (pH 4, for fusion proteins) or 1X phosphate buffered saline (PBS, for lutoid body proteins) and loaded on a chitin micro-column (2.5 x 6 mm) equilibrated with either sodium acetate buffer or 1X PBS, respectively. The samples were recycled through the

column three times and the flow-through fractions were collected. After extensive washing with 1X PBS, the chitin binding proteins were eluted with 6 M Guanidine-HCl+ 0.1 M NaOH. Flow-through and elution fractions were dialyzed against double distilled water and lyophilized. The proteins were separated by SDS-PAGE and analyzed either directly by Coomassie blue staining or indirectly by immunoblotting as described above.

RESULTS

Expression of Domain-specific Polypeptides and Production of Domain-specific Antibodies

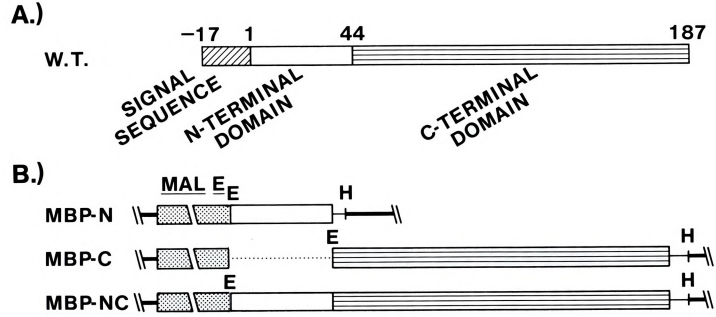
To produce polyclonal antibodies specifically directed against the N domain, C domain and NC domain of the protein encoded by HEV1, the corresponding domain-specific polypeptides were expressed in *E. coli*. For this purpose, the wild type HEV1 cDNA was engineered by site-directed mutagenesis to create restriction sites and stop codons to delimit the different domains. The DNA fragments encoding each domain were subcloned into the *E. coli* expression vector pIH 821 so that their coding sequences were fused to the 3' ends of the *MalE* gene coding for the maltose-binding protein (MBP) (Fig. 1). *E. coli* carrying each construct produced fusion proteins consisting of either N, C or NC domain fused at the carboxyl-terminal of MBP. The fusion proteins allowed for purification by amylose affinity chromatography and were used for immunization of rabbits. As shown by

Figure 1. The construction of expression vectors encoding domain-specific fusion proteins.

Restriction sites and stop codons were introduced into HEV1 by site-directed mutagenesis to delimit N, C and NC domains. The mutagenized constructs were then subcloned into the expression vector pIH821.

A) Organization of wild type (wt) HEV1 coding region.

B) Constructs of pIH821 coding for MBP-N, MBP-C and MBP-NC fusion proteins. *MalE* encodes the maltose binding protein and is represented by stippled boxes. *Eco*RI and *Hind*III sites are abbreviated to E and H, respectively. The thin line represents the untranslated region of HEV1. The thick line indicates the DNA sequence of the plasmid pIH821.


A.)
−17
1
44
187
W.T.
SIGNAL SEQUENCE
N-TERMINAL DOMAIN
C-TERMINAL DOMAIN
B.)
MAL
E
E
H
MBP-N
E
H
MBP-C
E
H
MBP-NC

SDS-PAGE analysis (Fig. 2), the fusion proteins had the expected molecular weights of 45-kDa for MBP-N domain (lane 2), 55-kDa for MBP-C domain (lane 3) and 60-kDa for MBP-NC domain (lane 4). The endogenous 40-kDa maltose-binding proteins from *E. coli* were copurified in all cases along with the MBP-fusion proteins (Fig. 2, lanes 2-4). To assess the specificity of the domain-specific antibodies, HPLC-purified mature hevein (which corresponds to the N domain of HEV1) was used in immunoblotting experiments. The purified hevein cross-reacted with both the N domain and NC domain-specific antibodies, but not with the C domain-specific antibodies (data not shown).

Processing of the HEV1 Signal Peptide upon *In Vitro* Translation

An *in vitro* translation system was used to investigate whether the putative 17-residue peptide present in the HEV1 encoded protein can effectively function as an ER translocation signal. RNA corresponding to the HEV1 clone was transcribed *in vitro* using the T3-T7 polymerase promoter system and subsequently translated *in vitro* in a wheat germ extract cell-free system. As shown in Figure 3 (lane 1), two translation products of approximately 20.5- and 22-kDa were obtained. The 22-kDa product has the expected size of the primary HEV1 translation product (204 amino acids). The smaller protein probably is a degradation product of the 22-kDa protein. Both polypeptides were specifically immunoprecipitated by antibodies raised against the NC domain (Fig. 3, lane 2). Moreover, none of the translation products could be immunoprecipitated using either preimmune serum or MBP specific antibodies (data not shown). Upon addition of either canine pancreatic microsomes (Figure

Figure 2. Expression of domain specific fusion proteins.

Fusion proteins were over-expressed in *E. coli* strain MV1193, purified by affinity chromatography on an amylose column and separated on an SDS gel. MBP-N (lane 2), MBP-C (lane 3) and MBP-NC (lane 4) proteins were visualized by Coommassie blue staining. Maltose binding protein (lane 1) was used as control. Endogenous maltose binding proteins are also seen in lanes 2-4. (Fusion proteins were prepared by Dr. Willem Broekaret.)

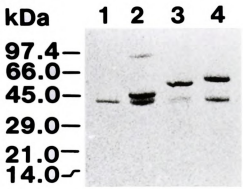
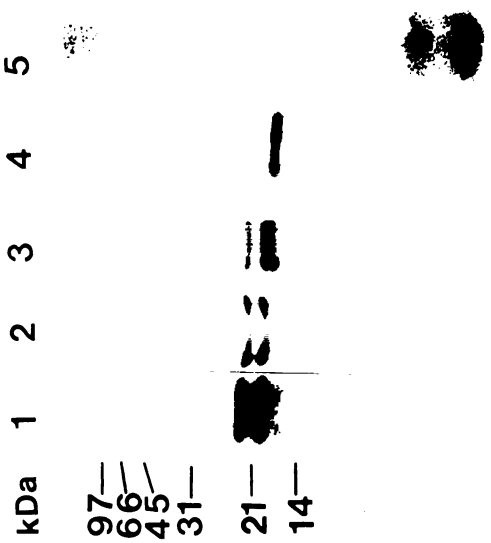

kDa
1
2
3
4
97.4
66.0
45.0
29.0
21.0
14.0

Figure 3. Processing of *in vitro* translation products.

HEV1 transcript was *in vitro* translated with wheat germ extract containing [^{35}S] cysteine in the absence (lanes 1 and 2) or presence of canine pancreatic microsomes (lanes 3-5). After *in vitro* translation, equal aliquotes of reaction mixture were treated with proteinase K (lane 4), or proteinase K in the presence of Triton X-100 (lane 5). The reaction mixtures (lanes 2-4) were immunoprecipitated with NC domain specific antibody. Translation products were analyzed by SDS-PAGE and fluorography.


kDa
1
2
3
4
5
97
66
45
31
21
14

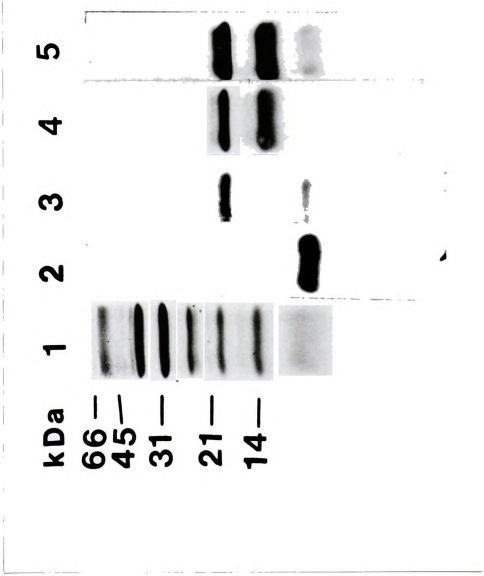
3, lanes 3-5) or maize microsomes (results not shown) to the *in vitro* translation system, most of the 22-kDa primary translation product was processed to a 20-kDa polypeptide (Fig. 3, lane 3). This processing event is consistent with the cleavage of the 17 amino acid signal sequence by a signal peptidase present in the microsomal membranes. The degradation product was also observed in Figure 3 (lane 3), which is slightly higher than the processing product of 20-kDa. Proteinase K digestion of the microsome translation mixture, followed by immunoprecipitation, confirmed that the 20-kDa polypeptide was located in the microsomes (Fig. 3, lane 4). Lysis of the microsomes by Triton X-100 in the presence of proteinase K resulted in complete proteinase-mediated degradation of the translocation products (Fig. 3, lane 5).

Identification of Post-translationally Processed Products *In Vivo*

To identify whether the HEV1 encoded polypeptide is specifically cleaved *in vivo* to yield an amino-terminal (hevein) and a carboxyl-terminal polypeptide, proteins from a lutoid body-enriched fraction of latex were analyzed by immunoblotting using the domain-specific antibodies. From the lutoid body-enriched fraction, at least seven distinct bands were identified on SDS-PAGE stained with Coomassie blue (Fig. 4, lane 1). The HPLC-purified hevein migrated as a 5-kDa protein and was immunoreactive with the N domain (Fig. 4, lane 2) and NC domain-specific antibodies (data not shown). As shown in Figure 4 (lane 3), the N domain-specific antibody recognized two bands which migrated as 5- and 20-kDa proteins. C domain-specific antibody crossreacted with a 14- and a 20-kDa protein, but not with

Figure 4. Immunoblot analysis of proteins in the lutoid body-enriched fraction using domain-specific antibodies.

Approximately 10 ug of total proteins from lutoid body-enriched fraction was separated by SDS-PAGE. After electrophoresis, lutoid body proteins were either stained with Coommassie blue (lane 1) or immunoblotted with antibodies specific for the N (lane 3), C (lane 4) or NC (lane 5) domain. As a control, HPLC-purified hevein was immunoblotted with N domain-specific antibody (lane 2).


kDa
66
45
31
21
14
1
2
3
4
5

the 5-kDa protein (Fig. 4, lane 4). In addition, the NC domain-specific antibody detected all three proteins of 5-, 14- and 20-kDa (Fig. 4, lane 5). These data suggest that the 5-, 14- and 20-kDa bands correspond to mature hevein (amino-terminal domain), to the carboxyl-terminal domain, and to the proprotein containing both domains, respectively. To confirm the identity of the 14- and 20-kDa polypeptides, the primary amino acid sequence of the amino-terminus of each polypeptide was analyzed by microsequencing. The nine N-terminal amino acids of the 20-kDa protein are E-Q-X-G-R-Q-A-G-G (X: unidentified amino acid residue). These sequences are exactly matched to amino acids at position 1 through 9, deduced from HEV1 (Broekaert, 1990), except for the cysteine residue at position 3 which can not be determined by Edmann degradation. The N-terminal amino acid sequence of the 14-kDa polypeptide is G-G-S-A-S-N-M(V)-L-A-T-Y which corresponds to amino acids 50 (G) to 60 (Y) of the HEV1-encoded protein with the exception of the methionine residue at position 56. This methionine residue is most likely due to isoforms which were observed by two-dimensional gel electrophoresis. The N-terminal sequence analysis of the 14-kDa polypeptide indicates that post-translational cleavage *in vivo* occurs between amino acids 43 and 50 of the HEV1-encoded protein and that six amino acids, 44 to 49 (S-G-E-G-V-G), are lost during maturation.

Chitin-binding Properties of Hevein and Hevein-containing Polypeptides

Since hevein (N domain) is known to have chitin-binding properties (Van Parijs *et al.*,1990), we investigated whether the proprotein (NC domain) also had the ability

to bind chitin. The lutoid body-enriched fraction was subjected to affinity chromatography on chitin. The fraction was eluted with 6 M Guanidine-HCl containing 0.1 M NaOH and was subsequently analyzed by immunoblotting using NC domain-specific antibodies. In the chitin-unbound fraction, the 14-kDa protein (C domain) was recovered but both the 5-kDa protein (N domain) and the 20-kDa protein (NC domain) were almost completely depleted (Fig. 5, lane 2). After elution with 6 M Guanidine-HCl containing 0.1 M NaOH, both the 5- and the 20-kDa protein but not the 14-kDa protein were eluted from the chitin column as shown in Figure 5 (lane 3). Thus, it appears that the C domain polypeptide of 14-kDa, which has no affinity toward chitin, acquires chitin-binding properties when it is linked carboxyl-terminally to the hevein domain. It is interesting to note that the proportion of the 5-kDa to 20-kDa protein in chitin-bound fraction (Fig. 5, lane 3) is much higher than that in the lutoid body-enriched fraction (Fig. 4, lane 5). These results may reflect that hevein (5-kDa) has higher affinity to chitin than the 20-kDa protein. In order to investigate whether the hevein domain can also confer chitin-binding properties when it is linked at the carboxyl-terminus of an unrelated polypeptide, we analyzed the chitin-binding activity of the MBP-N domain fusion protein. Figure 6 (lane 2) shows that the amylose-purified MBP-N domain preparation contains the fusion protein as well as the MBP protein itself. Upon passage over the chitin column, the unbound preparation is relatively enriched in MBP and depleted in the MBP-N domain fusion protein (Fig. 6, lane 3). The MBP-N domain fusion protein could be desorbed from the column by 6 M guanidine-HCl containing 0.1 M NaOH (Fig. 6, lane 4).

Figure 5. Chitin-binding properties of proteins in the lutoid body-enriched fraction.

Lutoid body proteins were passed through a chitin column. The flow-through fraction and fraction eluted with 6M guanidine-HCl+0.1N NaOH were analyzed by SDS-PAGE. The lutoid body proteins (lane 1) and the flow-through fraction (lane 2) were stained with Coomassie blue. The elution fraction was immunoblotted with NC domain-specific antibodies (lane 3).

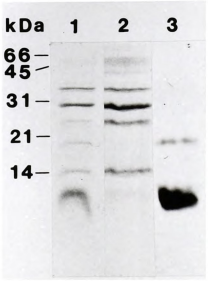
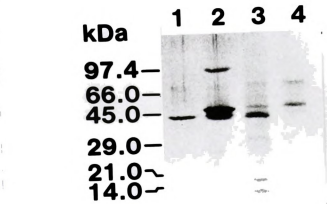

kDa
1
2
3
66
45
31
21
14

Figure 6. Affinity of the MBP-N fusion protein to chitin.

Amylose-purified fusion protein (lane 2) was subjected to chitin affinity chromatography. Flow-through fraction (lane 3) and elution fraction (lane 4) were separated on an SDS gel and stained with Coomassie blue. Pure maltose-binding protein is shown in lane 1. (This experiment was performed by Dr. Willem Broekaert.)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DISCUSSION

In this chapter, we have examined the processing events that are involved in the formation of mature hevein. *In vitro* translocation experiments, in the presence of either canine pancreatic microsomes or maize microsomes, indicate that the preproprotein form of hevein is cotranslationally processed and translocated into the RER. This implies that the HEV1-encoded protein is one of the proteins that traverse the secretory pathway. After cleavage of the signal peptide, this protein (187 amino acids) undergoes further post-translational processing *in vivo*. Immunoblot analysis of proteins from the lutoid body fraction demonstrates that three distinct bands from this fraction crossreact with the domain-specific antibodies: a 5-kDa protein is detected by both the N domain and NC domain-specific antibodies, a 14-kDa protein crossreacts with C domain and NC domain-specific antibodies, and a 20-kDa protein is immunoreactive with all three types of antibodies. These results strongly suggest that the 5-, 14- and 20-kDa proteins are equivalent to the N domain, C domain and NC domain, respectively, of the protein deduced from HEV1. Also, the apparent molecular mass of each of the three immunoreactive proteins is in agreement with that predicted from the deduced amino acid sequence of N, C, or NC domains of HEV1. The identity of all three proteins was further confirmed by several lines of evidence. The 5-kDa protein comigrates upon SDS-PAGE with HPLC-purified hevein and has affinity to chitin as described for mature hevein (Van Parijs *et al.*, 1991). The N-terminal amino acids of the 14-kDa protein correspond to the HEV1 amino acid sequence at position 50

to 60, which belongs to the C domain. The 20-kDa protein possesses chitin-binding ability and its eight N-terminal amino acids are identical to those of the N domain. Collectively, the data presented here demonstrate the identity of the 5-kDa protein as the N domain, the 14-kDa protein as the C domain and the 20-kDa protein as the proprotein (NC domain).

The occurrence of N, C and NC domain proteins in the lutoid body fraction suggests that the 5- and 14-kDa proteins are proteolytic cleavage products of the 20-kDa protein. This cleavage may be either (incomplete) posttranslational processing or reflect an artifactual proteolysis during the isolation of the lutoid body fraction. The latter hypothesis, however, is not favored because preparation of the lutoid body only involves a single centrifugation step starting from freshly tapped latex. Moreover, lutoid bodies immediately dissolved and boiled in an SDS containing buffer produced the same SDS-PAGE pattern as lutoid bodies that had been dissolved in 1X phosphate buffered saline or 50mM MES buffer and incubated at 28°C for 24 hr (data not shown). Surprisingly, *in vivo* cleavage occurs between amino acids at position 43 and 50, but not between amino acids at position 43 and 44 which are border sequences of hevein and the C domain. This result suggests that six amino acids between 44 to 49 of HEV1 may be cleaved off either at the same time during formation of hevein and the C domain polypeptide or by exopeptidases after cleavage either between amino acids 49 and 50 or between amino acids 43 and 44 of HEV1.

A conserved 43 amino acid domain (chitin-binding domain) has been found in a variety of chitin-binding proteins including hevein, cereal lectins and several

basic chitinases (Chrispeels and Raikhel, 1991). Interestingly, the post-translational processing which occurs during hevein (chitin-binding domain) formation is unique in comparison to that of other chitin-binding proteins. The lectins from wheat (Raikhel and Wilkins, 1987), barley (Lerner and Raikhel, 1989), rice (Wilkins and Raikhel, 1989), which contain repetitive hevein-like domains, undergo posttranslational cleavage of the short glycosylated C-terminal portion. The nature of a short C-terminal portion (< 20 amino acids) seems to be distinguished from that of the long unglycosylated C domain (144 amino acids) encoded by HEV1. Basic chitinases (Broglie *et al.*, 1986; Laflamme and Roxby, 1989; Lucas *et al.*, 1985; Parsons *et al.*, 1989; Shinshi *et al.*, 1987), another class of chitin-binding proteins, encode a single hevein-like domain at the N-terminus fused to an unrelated C-terminal domain. These proteins accumulate in vacuoles, but do not undergo post-translational processing to N- and C-terminal polypeptides. Proteins highly homologous to HEV1 are encoded by the *win* genes in potato (Stanford *et al.*, 1989). However, it has not been examined whether posttranslational processing occurs in the potato *win* gene-encoded proteins. Interestingly, a processing event similar but not identical to that found for HEV1 has been described for thionins, a class of small 5-kDa cysteine-rich proteins with antifungal properties (Bohlmann *et al.*, 1988). The thionin genes code for 15-kDa preproproteins that are co- and post-translationally processed to produce N-terminal portions corresponding to mature thionins and carboxyl-terminal polypeptides of 62 or 63 amino acids with unknown function (Bohlmann and Apel, 1987; Gausing, 1987). Similarly, the function and significance of the C-terminal domain of HEV1 is unknown. The 14-kDa C domain

protein does not bind chitin and may fulfill a function different from that of the N domain protein.

It has been shown that hevein (the N domain protein) has chitin-binding properties. We have also found that the affinity to chitin is retained in the proprotein encoded by HEV1 as well as an MBP-fusion protein carrying the hevein domain at the C-terminus. These observations suggest that the hevein domain can provide chitin-binding properties when fused to either the N- or C-terminus of an unrelated polypeptide. Evolutionarily, this supports the idea that chitin-binding proteins, such as basic chitinases and proteins encoded by the potato *win* genes and HEV1, have evolved from fusion of a chitin-binding domain (hevein-like domain) with structurally and functionally unrelated polypeptides (Chrispeels and Raikhel, 1991). The resulting fusion proteins may adopt new properties such as the ability to bind chitin containing structures of fungi, insects or nematodes.

ACKNOWLEDGEMENTS

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CHAPTER 4

Posttranslational Processing of Hevein cDNA-Encoded Proteins in Transgenic Tomato Plants

ABSTRACT

In latex of rubber tree (*Hevea brasiliensis*), prohevein, homologous to potato *win* gene-encoded proteins, is incompletely processed to yield mature hevein composed of one chitin-binding domain and the C-terminal polypeptide homologous to pathogenesis-related proteins such as tobacco PR-4 and tomato P2 proteins. To investigate the processing mechanism and the sorting of prohevein, transgenic tomato plants were used as an experimental system. Immunoblot analysis showed that prohevein was partially cleaved to form the C-terminal polypeptide in these plants. However, mature hevein, the N-terminal cleavage form, was not found. Both prohevein and the C-terminal polypeptide were localized intracellularly in transgenic tomato plants. Prohevein retained affinity for chitin in these plants. Growth of *Trichoderma hamatum* was delayed in transgenic tomatoes constitutively expressing HEV1-encoded proteins.

INTRODUCTION

Plants synthesize proteins *de novo* in response to wounding or invasion by pathogens. In particular, a group of these proteins can be classified as chitin-binding proteins by their affinity for chitin, a polymer of N-acetyl-D-glucosamine that is present in fungi, insects and nematodes, but is lacking in plants. A family of chitin-binding proteins has been found in monocot and dicot plants. The chitin-binding proteins studied possess the chitin-binding domains of 30-43 amino acids that are enriched in glycines and cysteines at conserved positions. The deduced amino acid sequences of all isolated cDNA clones contain a putative signal sequence, and most proteins studied in this family have been shown to be localized in the vacuole, indicating that chitin-binding proteins are secretory proteins.

Hevein is a major protein in the lutoid body-enriched fraction from rubber tree latex (Archer *et al.*, 1960). It is a small, monomeric polypeptide of 43 amino acids (Walujono *et al.*, 1975) that possesses chitin-binding affinity. Thus, a chitin-binding domain is sometimes referred to as a hevein domain. This protein has been shown to inhibit the growth of several chitin-containing fungi in *in vitro* experiments (Van Parijs *et al.*, 1991), suggesting the involvement of this protein in plant defense. A cDNA clone encoding hevein (HEV1) was isolated and characterized (Broekaert *et al.*, 1990). From the deduced amino acid sequence, HEV1 encodes a putative signal sequence of 17 amino acids followed by a polypeptide of 187 amino acids. This polypeptide possesses two distinct domains: an amino-terminal domain corresponding to the 43 amino acids of mature hevein and an extensive carboxyl

terminal domain of 144 amino acids. The difference in polypeptide length between hevein and the HEV1-encoded polypeptide suggests that the formation of mature hevein may result from two proteolytic cleavages of the hevein precursor *in vivo*. *In vitro* translocation experiments showed that the first cleavage involves cotranslational removal of the signal peptide. Immunoblot analysis of proteins in the lutoid body-enriched fraction further demonstrated that subsequent posttranslational processing yields two cleavage products: mature hevein (5-kD) and the C-terminal polypeptide (14-kD) (Lee *et al.*, 1991).

We are interested in elucidating the mechanism of posttranslational processing that occurs during hevein maturation, and understanding the targeting and possible function of the HEV1-encoded proteins. To approach these questions, a hevein cDNA construct was introduced into tomato plants, a biological system that is more convenient for experimentation than rubber tree. In this study, three distinct objectives were pursued: i) the analysis of posttranslational proteolytic processing of the hevein cDNA-encoded proteins in transgenic tomato plants; ii) their localization in transgenic tomato plants; iii) their plausible activity in transgenic tomato plants.

Materials and Methods

Plant Material

Lycopersicon esculentum cv. UC82 (commercial tomato) plants and transgenic

tomato plants were grown in a growth chamber providing an 18 hour day at 26° C and an 8 hour night at 22° C.

Site-directed Mutagenesis and Plasmid Construction

For subcloning, a *Bgl*II restriction enzyme site was introduced into the 3' untranslated region of a hevein cDNA clone (HEV1) by site-directed mutagenesis as described (Kunkel *et al.*, 1987). The *EcoR*I/*Hind*III fragment of the mutagenized cDNA was subcloned into pBluescript SK$^-$ to facilitate the introduction of a *Xba*I site from the polylinker region of the plasmid. The *Xba*I/*Bgl*II fragment was subcloned into the plant expression vector pGA 643.

Plant Transformation

Lycopersicon esculentum cv. UC82 cotyledons were used for transformation. Seeds of the tomato cultivar UC82 were surface-sterilized for 10 min in a 0.05% Sodium hypochlorite solution (commercial bleach), washed 3-4 times with sterile dd H_2O and plated on medium containing Murashige and Skoog (MS) salt mix and 0.8% Bactoagar. Cotyledons grown for 7-10 days were cut and the middle section was preincubated with feeder plates for 24 hr at 25° C. Cotyledon sections were immersed for 30 min in 5 ml of a broth of *Agrobacterium tumefaciens* strain LB4404 containing the HEV1 construct, blotted, and replaced onto the feeder plates. After a 2 day co-incubation, cotyledon segments were transferred to shoot regeneration medium (Shahin, 1985). Regenerated shoots were excised and transferred to MS rooting media (Horsch *et al.*, 1988).

RNA Gel Blot Analysis

Total RNA was isolated from leaves of untransformed and transgenic tomato plants according to Nagy *et al.*(1988). Total RNA (40 μg) was separated on a 2% agarose gel containing 6% formaldehyde, transferred to nitrocellulose, and hybridized to hevein cDNA (HEV1; Broekaert *et al.* 1990) labeled with α-^{32}P-ATP by the random-primer method (Feinberg and Vogelstein, 1983) as described by Raikhel and Wilkins (1987). Filters were exposed to Kodak X-OMAT AR film at -70° C with intensifying screens.

Protein Extraction and Preparation of Apoplastic Fluid

Tomato leaves or fruits were homogenized with 50 mM MES buffer (pH 5.0) containing 1% polyvinyl pyrrolidone 40 (PVP-40). The extract was centrifuged at 5,000*g* for 20 min to remove cellular debris and insoluble material. The supernatant was precipitated with ammonium sulfate and pelleted at 10,000*g* for 10 min. Pellets were dissolved in 1x phosphate-buffered saline (PBS), dialyzed overnight, dried, and dissolved in 1x PBS (1/10 of original volume of plant extracts). Total protein in crude extracts was determined by the Bradford method (1976).

Apoplastic fluid (extracellular fluid; ECF) was prepared by the method of De Wit and Spikman (1982) with the following modifications. Entire leaves were infiltrated with 50 mM MES buffer containing 10 mM NaCl solution *in vacuo*. Surface-dried leaves were centrifuged for 10 min at 3,000*g*. Extruded ECF was collected and the cell extract (intracellular fluid; ICF) from the ECF-depleted leaves

was prepared as described above.

Marker Enzyme Assay

Glucose-6-phosphate dehydrogenase was assayed by method described (Simcox et al., 1977). For the peroxidase assay, samples were incubated with 300 µl of 10% H_2O_2 and 1 ml of 4 mM 2,2-azino bis (3-ethy benzthiazoline sulfonic acid) diammonium salt (ABST). After 10 min, reactions were stopped with 1 ml of stop solution (0.04% Na_4EDTA/ 0.06 N NaOH/ 1.7% HF). Peroxidase activity was measured at 410 nm.

Chitin-binding Assay

Chitin was prepared by reacetylation of chitosan (Sigma) as described by Molano *et al.* (1977). Protein samples in 1x PBS were applied to a chitin microcolumn. After an incubation of 15-30 min with gentle shaking, flow-through fractions containing unbound proteins were collected. The column was washed with 1x PBS and bound proteins were eluted with 6 M Guanidine-HCl/0.1 N NaOH. The fractions were dialyzed against double-distilled water and lyophilized. The samples were analyzed by immunoblotting as described below.

SDS-PAGE, Two-D PAGE and Immunoblotting

Protein samples were separated on 15% polyacrylamide gels according to Laemmli (1970) or by two-dimensional PAGE as earlier described (O'Farrell, 1975). Proteins were transferred to Immobilon P (Millipore, Bedford, MA), and the blots were

blocked for 2 hr with TBS (5 mM Tris-HCl, pH 7.4, 136 mM NaCl and 26 mM KCl) containing 5% non-fat dry milk. The blots were probed with "domain-specific" antibodies (Lee *et al.*, 1991) or potato chitinase antisera kindly provided by Dr. Erich Kombrink (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany). Protein bands were visualized with alkaline phosphatase-conjugated secondary antibodies as described elsewhere (Black *et al.*, 1984).

Spore Preparation and Inoculation of *Trichoderma hamatum*

T. hamatum was kindly provided by Ing. A. Vanachter (Laboratorium voor Plantenbescherming, Katholieke Universiteit Leuven, Belgium) and was maintained on potato dextrose agar (Merck, Darmstadt, F.R.G.). Spores were collected from 8-day old potato dextrose agar culture by washing the agar surface with sterile ddH_2O. The suspension was filtered and filtrates were centrifuged at 10,000*g* for 30 min. Pellets were resuspended in sterile ddH_2O. For inoculation of fungal spores, tomato fruits were sliced in a sterile hood and each slice was transferred onto a separate petri dish (100mmx15mm). Aliquots of the appropriate density of fungal spores were spread on sliced tomatoes.

RESULTS

Construction of pLHEV5 and its Expression in Transgenic Tomato Plants

Tomato plants were used as a heterologous transgenic system to express the hevein cDNA. The hevein cDNA (HEV1) was inserted into the plant expression vector pGA643 under the control of the 35S Cauliflower Mosaic Virus promoter, and the resulting plasmid was designated pLHV5 (Figure 1A). Tomato plants were transformed by co-cultivating cotyledons of tomato plants with *Agrobacterium* strains harboring the pLHV5. Eighteen kanamycin-resistant plants were regenerated. Transgenic plants expressing hevein mRNA were examined by RNA gel blot analysis. Examination of nine individual kanamycin-resistant plants (Figure 1B, lanes 1 to 4) revealed similar levels of hevein mRNA accumulation. No hybridization was observed in mRNA isolated from untransformed control plants (Figure 1B, lane 5).

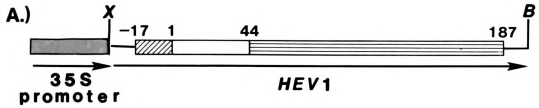
Identification of Hevein Proprotein and its Cleavage Product in Transgenic Tomato Plants

In rubber tree latex, the 20-kD hevein proprotein is posttranslationally processed to the 5-kD hevein and the 14-kD C-terminal polypeptide (Lee *et al.*, 1991). Immunoblot analysis using the antibody against prohevein domain (Figure 2) revealed that leaves (lanes 2, 3, 4 and 5) and fruits (lane 7) from transgenic plants contain a 20-kD protein that comigrates with the 20-kD hevein proprotein from rubber tree lutoid bodies (lane 1). This protein was not detected in leaves and fruits

Figure 1. Hevein cDNA construct and RNA gel blot analysis of HEV1 transcript.

A) The hevein cDNA was subcloned into the *XbaI*/*BglII* site of plant expression vector pGA 643. Signal sequence is indicated by diagonal box. The N-(mature hevein) and C-terminal domains are shown in open and horizontally lined boxes, respectively.

B) Each lane contains 40 µg of total RNA from wild-type (lane 5) and kanamycin-resistant tomato plants (lanes 1-4). The size standard markers are indicated on the left.


A.)
X
B
-17
1
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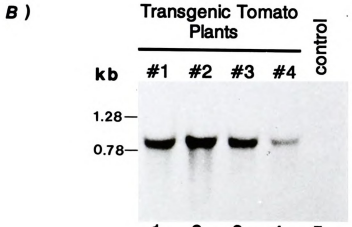
187
35S
promoter
HEV1


B)
Transgenic Tomato
Plants
control
kb
#1
#2
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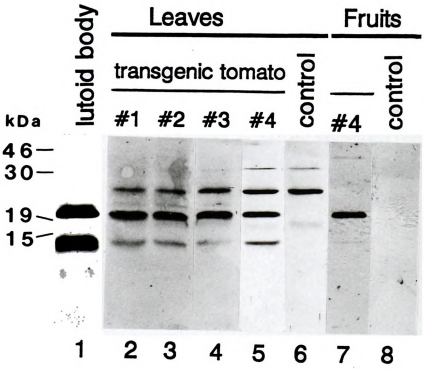
1.28
0.78
1
2
3
4
5

Figure 2. Immunoblot analysis of HEV1-encoded proteins.

Proteins were prepared from lutoid body-enriched fractions (lane 1), leaves of wild-type (lane 6) or transgenic (lanes 2-5) plants, and fruit of wild-type (lane 8) or transgenic (lane 7) plants. After SDS-PAGE, protein bands were immunoblotted with antibodies against prohevein domain (Lee *et al.*, 1991).


lutoid body
Leaves
Fruits
transgenic tomato
control
control
kDa
#1
#2
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#4
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46
30
19
15
1
2
3
4
5
6
7
8

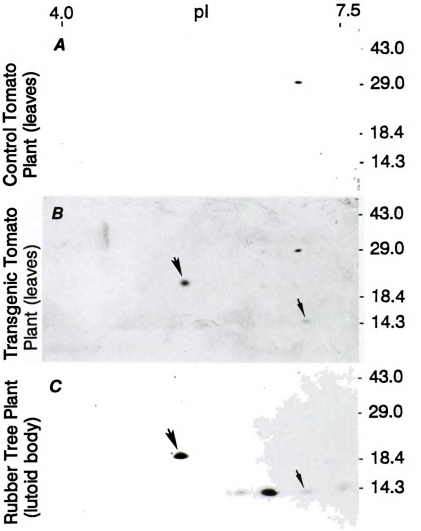
from an untransformed plant (lanes 6 and 8). In addition to the 20-kD band, transgenic plants (lanes 2, 3, 4, 5 and 7) also contain a 14-kD polypeptide that is identical in size to the 14-kD carboxyl-terminal cleavage product of the hevein proprotein from lutoid bodies (lane 1). However, the relative abundance of the 14-kD polypeptide in fruits (lane 7) is much lower than in leaves (lanes 2, 3, 4 and 5). Again, no corresponding protein was found in leaves and fruits of untransformed plants (lanes 6 and 8). Mature hevein (the 5-kD polypeptide), however, could not be detected on the immunoblots. It is possible that this protein is unstable in tomato plants. Endogenous cross-reactive bands were detected in transformed and untransformed plants, specifically in the leaves (lanes 2 to 6), but not in the fruits (lanes 7 and 8).

Two-D Gel Analysis of Hevein-encoded Proteins in Transgenic Tomato Plants

To determine whether HEV1-encoded proteins are modified in transgenic tomato plants, we investigated the correlation between protein isoelectric point (pI) profiles of prohevein and the C-terminal polypeptide in rubber tree and transgenic tomato plants. Lutoid body proteins from rubber tree and leaf extracts from transgenic and untransformed tomato plants were separated by two-dimensional gel electrophoresis and were subsequently immunoblotted using carboxyl domain-specific antibody (Figure 3). The 20-kD hevein proprotein in transgenic tomato plants (Figure 3B, large arrow) is apparently identical in size and pI to that found in lutoid bodies (Figure 3C, large arrow). Based upon pI and molecular mass, the 14-kD C-terminal cleavage product detected in transgenic tomato plants (Figure 3B, small arrow)

Figure 3. Two-D gel analysis of HEV1-encoded proteins.

Protein extracts from lutoid body (C) and leaves of wild-type (A) and transgenic plants (B) were separated by two-D gel electrophoresis. After transfer, blots were probed with a domain-specific antibody against the C-terminus of prohevein (Lee *et al.*, 1991). Large arrows indicate the 20-kD prohevein polypeptides. The 14-kD C-terminal polypeptides are indicated by small arrows.


4.0
pI
7.5
A
Control Tomato Plant (leaves)
43.0
29.0
18.4
14.3
B
Transgenic Tomato Plant (leaves)
43.0
29.0
18.4
14.3
C
Rubber Tree Plant (lutoid body)
43.0
29.0
18.4
14.3

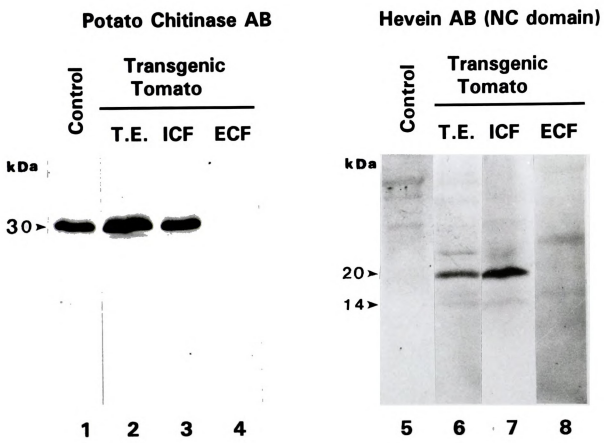
corresponds to one of multiple 14-kD immunoreactive spots in rubber tree (Figure 3C, small arrow). These results indicate that both prohevein and the C-terminal cleavage polypeptides in transgenic tomato plants and in rubber trees are likely to be structurally similar.

Localization of Prohevein and the C-terminal Polypeptide in Transgenic Tomato Plants

Hevein was isolated from the lutoid body-enriched fraction of rubber tree latex. Since the lutoid bodies are believed to be derived from vacuoles, hevein gene products might be localized in vacuoles of transgenic tomato plants. To identify their location, proteins were prepared from apoplastic fluid (extracellular fluid; ECF) and ECF-depleted leaf extracts (intracellular fluid; ICF) from transgenic tomato plants. Purity of the ECF and ICF was monitored by measurement of glucose-6-phosphate dehydrogenase activity and peroxidase activity, and by immunoblot analysis using potato chitinase antiserum (Figure 4, lanes 1 to 4). Thus, glucose-6-phosphate dehydrogenase was used as a cytoplasmic marker, basic chitinase as a vacuolar marker, and peroxidase served as an extracellular marker. Less than 5% of glucose-6-phosphate dehydrogenase activity (nmol/min/mg protein) was detected in the ECF. In addition, the potato chitinase antisera reacted with protein bands of the same molecular weight isolated from the ICF (lane 3) and total leaf extracts (lane 2) of transgenic tomato plants. Similar results were obtained from untransformed control plants (lane 1). Furthermore, greater than 95% of peroxidase activity (units /μg protein) was detected in the ECF. These data indicated that the ECF was

Figure 4. Localization of HEV1-encoded proteins.

Apoplastic (ECF; lanes 4 and 8) or cell extracts (ICF; lanes 3 and 7) from transgenic tomato leaves were separated by SDS-PAGE and immunoblotted with a potato chitinase antibody (lanes 1-4) or prohevein domain-specific antibody (lanes 5-8). Whole leaf crude extracts (total extract: T.E.) from wild-type (lanes 1 and 5) or transgenic plants (lanes 2 and 6) were used as controls.


Potato Chitinase AB
Control
Transgenic Tomato
T.E. ICF ECF
kDa
30
1 2 3 4
Hevein AB (NC domain)
Control
Transgenic Tomato
T.E. ICF ECF
kDa
20
14
5 6 7 8

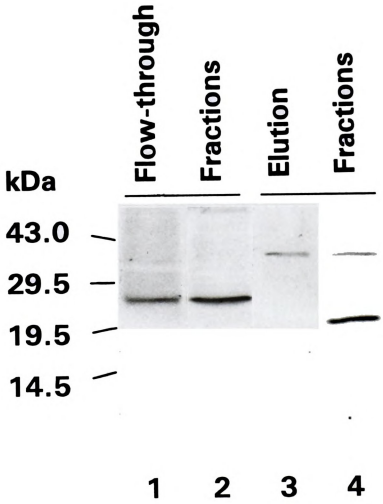
essentially uncontaminated with the ICF. Immunoblot analysis revealed that the hevein proprotein was detected in the ICF (lane 7) and total leaf extracts (lane 6), but not in the ECF (lane 8) and total untransformed leaf extracts (lane 5). These data demonstrated that the 20-kD and 14-kD hevein-derived proteins were localized intracellularly in tomato plants, presumably in vacuoles. These results are in agreement with localization of the 20-kD proprotein, 14-kD C-terminal polypeptide and 5-kD mature hevein in lutoid bodies of rubber trees.

Chitin-binding Affinity of HEV1-encoded Proteins in Transgenic Tomato Plants

It has been shown that mature hevein (5-kD) and prohevein (20-kD) of rubber tree have the ability to bind chitin (Lee *et al.*, 1991). To determine whether or not HEV1-encoded proteins in transgenic tomato plants were also capable of binding to chitin. The C-terminal cleavage product (14-kD) was detected in the flow-through fraction containing unbound proteins from a chitin affinity column (Figure 5, lane 2). The prohevein (20-kD) was found in the fraction containing bound proteins after elution with 6 M guanidine-HCl/ 0.1 N NaOH (lane 4). This result was consistent with the chitin-binding ability of prohevein in rubber tree. Again, neither prohevein nor the C-terminal polypeptide was detected in flow-through (lane 1) and eluted (lane 3) fractions of untransformed plants. In addition, one of the endogenous immuno-reactive proteins (28-kD) was found only in the flow-through fractions (lanes 1 and 2). The other one (40-kD) was detected exclusively in the elution fractions (lanes 3 and 4).

Figure 5. Chitin-binding property of HEV1-encoded proteins.

Proteins from untransformed (lanes 1 and 3) and transgenic (lanes 2 and 4) tomato plants were subjected to chitin column chromatography. Flow-through (lanes 1 and 2) and elution (lanes 3 and 4) fractions were separated by SDS-PAGE and immunoblotted with NC domain-specific antibody.


Flow-through
Fractions
Elution
Fractions
kDa
43.0
29.5
19.5
14.5
1
2
3
4

Activity of HEV1-encoded Polypeptides in Transgenic Tomato

It has been reported that hevein (5-kD) and barley proteins homologous to the C-terminal polypeptide (14-kD) have an inhibitory effect on growth of several chitin-containing fungi *in vitro* (Van Parijs *et al.*, 1991; Hejgaard *et al.,* 1992). We also found that 5-kD (hevein) and 20-kD (proprotein) polypeptides have an ability to bind chitin (Lee *et al.,* 1991). These data suggest that the HEV1-encoded polypeptides are possibly involved in the plant defense response. This possibility was examined by monitoring whether transgenic tomatoes expressing the HEV1-encoded polypeptides enhanced resistance to certain type(s) of chitin-containing fungi. Since our transgenic tomato plants express the HEV1-encoded polypeptides constitutively under the control of 35S promoter, it is possible to inoculate fungi on different types of tissues (Figure 6) at any stages. We chose *Trichoderma hamatum*, a saprophytic fungus. We tested tomato fruits rather than leaves, because tomato fruit (Figure 3, lanes 7 and 8) has no immuno-reactive bands (HEV1-related proteins) that were found in leaves (Figure 3, lanes 2 to 6). Approximately 10^5 spores of *T. hamatum* were inoculated on slices of transgenic and untransformed control tomatoes. After 3-4 days, fungal growth on transgenic tomatoes was reduced (Figure 6, D and F), compared to that on control tomato (Figure 6E). Wild-type and transgenic tomatoes treated with ddH_2O (A, B and C) were not contaminated by other microorganisms. This result suggests that function of the HEV1-encoded polypeptides may relate to plant defense.

Figure 6. Inhibitory effect on fungal growth in transgenic tomato fruits.

Slices of untransformed (B and E) and transgenic (A, C, D and F) tomato fruits were treated with ddH_2O (A, B and C) or were inoculated with *T. hamatum* (D, E and F). The tomato slices were incubated for 3-4 days at room temperature.

Transgenic Tomato #1
Control Tomato
Transgenic Tomato #2
A
B
C
D
E
F
Treatment with dd H_2O
Infection with T. hamatum

Discussion

In this chapter, transgenic tomato plants have been used to develop an experimental system to study posttranslational processing, targeting and possible function of the HEV1-encoded proteins. Immunoblot analysis showed that all of the kanamycin-resistant plants expressed HEV1-encoded proteins at a comparable level. These results are consistent with RNA blot data showing that similar levels of hevein mRNA are detected in all transgenic plants. Together, these data suggested that the low level of protein expression observed is due to a positional effect of the hevein construct rather than to other control mechanisms such as posttranscriptional control. In transgenic tomato plants, prohevein is posttranslationally cleaved by processing mechanisms similar to those found in rubber tree, suggesting that the processing machinery is conserved among different plant species. The major difference between the two systems, however, is the absence of mature hevein in tomato which implies that a chitin-binding domain (mature hevein) alone is unstable after cleavage of prohevein. Instability of one or more chitin-binding domains was also observed in a tobacco system expressing stinging nettle lectin (B. Iseli and N. Raikhel, unpublished results).

The chitin-binding proteins studied have been shown to be secretory proteins (Raikhel *et al.*, 1993); most of them are vacuolar. In rubber tree, hevein was also found in the lutoid body (of vacuolar origin). Moreover, localization of prohevein and the C-terminal polypeptide in the lutoid body was determined immunologically (Lee *et al.*, 1991). To investigate the subcellular location of these proteins in

transgenic plants, immunohistochemistry and organellar fractionation studies were attempted but failed, presumably because of low abundance of these proteins in transgenic plants. However, immunoblot analysis of ICF and ECF proteins showed that the location of prohevein and the C-terminal polypeptide in transgenic tomato plants were intracellular (Figure 4). These results and localization of hevein-derived proteins in lutoid bodies of rubber tree suggest that prohevein and the C-terminal cleavage product are probably in the vacuoles of transgenic tomato plants.

Vacuolar proteins possess special amino acid sequences for vacuolar localization. These amino acid sequences are not conserved and are located at different positions within the vacuolar proteins studied. The C-terminal propeptide (CTPP) of barley lectin has been shown to be necessary and sufficient for vacuolar targeting (Bednarek *et al.*, 1990). The C-terminal extension of tobacco vacuolar chitinase is also required for localization to the vacuole, while an extracellular chitinase lacks a C-terminal extension. In the case of hevein and its related proteins, a sequence comparison (Figure 7) shows that the hevein proprotein and *win2*-encoded protein contain a C-terminal extension of >10 amino acids. The same sequence is not found in the C-terminal homologous proteins such as tobacco PR-4 and tomato P2, which are extracellular (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991). As described above, a similar relationship was also found in vacuolar chitinase and its extracellular counterpart. It is interesting to note that two chitin-binding proteins, barley lectin and tobacco basic chitinase, possess vacuolar targeting signals at the C-terminal ends (Bednarek and Raikhel, 1991; Neuhaus *et al.*, 1991). Based upon these data, we propose that the C-terminal extension sequence of prohevein is

		location
PROHEVEIN	VNYQFVDCGDSFN-PLFSVM-KSSVIN	(lutoid body)
WIN2	|||||N|||NV|V||L||VD|E	(unknown)
WIN1	|||||||||N	(unknown)
PR-4a/b	|||E||N|N|	(extracellular)
PR-P2	|||E||N|	(extracellular)

Figure 7. Comparison of the C-terminal amino acid sequences of prohevein, *win* gene-encoded proteins, PR-4a/b and PR-P2 proteins.

Amino acids identical to prohevein are denoted with a vertical line. Gaps in the aligned sequences are indicated by a dash. Partial amino acid sequences were derived from prohevein, residues 180-204 (Broekaert *et al.*, 1990); *win*2, residues 189-211, *win*1 190-200 (Stanford *et al.*,1989); PR-4a/b, residues 113-122, PR-P2, residues 113-120 (Friedrich *et al.*, 1991; Linthorst *et al.*,1991).

involved in sorting to the vacuole.

Our growth inhibition studies showed that transgenic plants expressing HEV1-encoded proteins exhibit enhanced resistance to *T. hamatum*, a chitin-containing fungus. It is unknown whether growth of *T. hamatum* was inhibited by HEV1-encoded proteins alone. We also tested other chitin-containing fungi, *Botrytis cinerea* and *Rhizoctonia solani*. However, no inhibitory effect on growth of these fungi was observed in transgenic tomato fruits. It is possible that the amount of HEV1-encoded proteins expressed in transgenic tomato plants is not sufficient to inhibit growth of these fungi. In a broad sense, these results reflect the idea that individual pathogenesis-related proteins may possess differential inhibitory effects on growth of different fungi. Together, our studies have raised the possibility that the overexpression of HEV1-encoded proteins in transgenic plants may contribute to enhancing resistance to particular fungal pathogens.

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CHAPTER 5

SUMMARY AND PROSPECTS FOR FUTURE RESEARCH

The data presented in this dissertation have demonstrated how mature hevein is formed through the secretory pathway. *In vitro* translocation experiments showed that the hevein signal peptide is cotranslationally removed. Immunoblot analysis indicated that prohevein and cleavage products (hevein and the C-terminal polypeptide) are present in the lutoid body; and in transgenic tomato plants, prohevein and the C-terminal polypeptide are localized intracellularly. Based upon these data, we can predict the fate of these proteins in laticifer cells of rubber tree and in transgenic tomato plants at the cellular level. Preprohevein (22-kD) is synthesized on rough endoplasmic reticulum (RER), cotranslationally processed, and translocated into the lumen of ER. Then, prohevein (20-kD) is passed through the Golgi apparatus. After or during translocation of prohevein to the lutoid bodies, this protein is partially processed to form mature hevein and a C-terminal polypeptide lacking a hinge region of 6 amino acids in the lutoid bodies. Alternatively, when targeted to the vacuole, prohevein is partially processed to yield only the C-terminal polypeptide. The incomplete processing involved in hevein formation is unique among the known posttranslational modifications occurring in other chitin-binding proteins (Raikhel *et al.*, 1993). First, this 'partial' processing has not been identified in other chitin-binding proteins. Second, the precursor form (prohevein) as well as the cleavage products may have their own biological activities, as discussed below. This raises the question as to what mechanism regulates this posttranslational processing. One approach will be to isolate and characterize the respective protease genes involved in hevein biosynthesis. Initially, proteases can be purified from the fraction containing protease activity by

conventional chromatography. Oligonucleotide probes or PCR primers designed from partial peptide sequence of purified protease then can be used to screen a cDNA or genomic library derived from rubber tree latex.

Most chitin-binding proteins are localized in the vacuole or a vacuole-like organelle such as the lutoid body. Studies of sorting signals in barley lectin and tobacco class I chitinase indicate that a C-terminal extension sequence (15 or 7 amino acids, respectively) is necessary and sufficient for vacuolar targeting (Bednarek *et al.*, 1990; Neuhaus *et al.*, 1991). These studies demonstrated that the vacuolar sorting signals in chitin-binding proteins are located at the end of their C-termini. Amino acid comparisons between prohevein and PR-4/P2 (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991) revealed a 17 amino acid extension at the C-terminus of prohevein, suggesting that these sequences are required for vacuolar targeting. Analysis of transgenic plants expressing a hevein construct in the presence or absence of this sequence will answer this question.

It has been proposed that hevein is involved in plant defense, since hevein displays the property of chitin-binding and exhibits *in vitro* antifungal activity (Van Parijs *et al.*, 1991). The data presented here and others' studies (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Hejgaard *et al.*, 1992) not only support this hypothesis, but also suggest that prohevein and two cleavage products, mature hevein and the C-terminal polypeptide, are biologically active *in vivo*. First, hevein mRNA was induced by wounding and plant stress hormones such as ABA and ethylene. Second, homology between the C-terminus of prohevein and the pathogenesis-related proteins (PR) such as PR-4 and PR-P2 (Friedrich *et al.*, 1991; Linthorst *et al.*,

1991) suggests that the C-terminal polypeptide has its own biological activity. Microsequencing showed that the 'mature form' of the C-terminal polypeptide lacks a 6 amino acid hinge region as well as a chitin-binding domain (hevein), indicating that the structure of the C-terminal polypeptide is similar to those of PR-4 and P2 proteins. Most importantly, *in vitro* antifungal activity was demonstrated for the PR proteins from barley whose partial peptide sequences shared the homology with the proteins homologous to the C-terminal domain of prohevein (Hejgaard *et al.*, 1992). Third, not only hevein but also prohevein retained chitin-binding affinity. Furthermore, in a family of chitin-binding proteins, the presence of proteins containing the chitin-binding domain and a number of structurally unrelated domains shows that these fusion proteins acquire novel properties. In this respect, it is interesting to note that class I chitinases from tobacco have higher specific chitinolytic activities than their class II counterparts (Legrand *et al.*, 1987). The presence of the combination of prohevein, hevein and the C-terminal polypeptide may provide stronger protection of plants against pathogens, assuming that all forms are active.

As discussed above, class I chitinase, a natural fusion protein, has been proven to have higher chitolytic activity than class II chitinase that contains only a chitolytic domain. I have also shown that an artificial fusion protein consisting of the 43-residue hevein domain and a bacterial maltose-binding protein exhibits affinity toward chitin, but not toward the maltose-binding protein itself. In addition, both fusion proteins and the maltose-binding protein alone retained the ability to bind maltose. These observations raise the possibility that crop protection against

specific pathogens can be achieved by the design and manipulation of artificial proteins that combine one or more chitin-binding domains with a protein of novel activity.

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