Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7633–7637, October 1990
Botany

Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*)

(polymerase chain reaction/amino acid sequence/cDNA clone/latex/plant hormones abscisic acid and ethylene)

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Communicated by Anton Lang, July 13, 1990 (received for review May 14, 1990)

ABSTRACT **Hevein is a chitin-binding protein that is present in laticifers of the rubber tree (*Hevea brasiliensis*). A cDNA clone (HEV1) encoding hevein was isolated by using the polymerase chain reaction with mixed oligonucleotides corresponding to two regions of hevein as primers and a *Hevea* latex cDNA library as a template. HEV1 is 1018 base pairs 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% homologous 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.**

Latex of the rubber tree (*Hevea brasiliensis*) is produced by highly specialized cells known as laticifers (1). 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 (2). 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 (3). Hevein is a small, single-chain protein of 43 amino acids unusually rich in cysteine and glycine (4). Recently, hevein has been shown to bind chitin and to inhibit the growth of several chitin-containing fungi (5). Therefore, it has been suggested that hevein plays a role in the protection of wound sites from fungal attack (5).

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 (6–9), whereas a related lectin from the dicotyledonous *Urtica dioica* is thought to be composed of two such domains arranged in tandem (10). Basic chitinases from bean (11, 12), tobacco (13), and poplar (14) 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 (15).

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 20-year-old rubber trees (*H. brasiliensis* RRIM600). These trees were regularly tapped for latex and were treated with 0.1% ethephon (2-chloroethylphosphonic acid) 2 weeks prior to tapping, a procedure that enhances latex production (16). For studying the regulation of hevein expression by RNA blot-hybridization (Northern blot) analysis, 4-month-old seedlings of *H. brasiliensis* RRIM600, grown in a growth chamber, were used.

RNA Isolation and Construction of the Latex-Specific cDNA Library. Total RNA from the latex was extracted as described by Kush *et al.* (17), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose affinity chromatography as described by Silflow *et al.* (18). Double-stranded cDNAs were prepared from 10 μg of poly(A)$^+$ RNA as described by Gubler and Hoffman (19) with the cDNA synthesis system of Amersham. The cDNA was ligated into phage λgt10 (Stratagene) with *Eco*RI linkers (New England Biolabs) and was packaged *in vitro* by using Gigapack II Gold (Stratagene). The host strain used for screening plaques was *Escherichia coli* C600hfl.

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 volume containing 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 1 μM of each primer, 200 μM each dNTP, 2.5 units of *Thermus aquaticus* polymerase (Cetus), and 1 μg of phage λgt10 cDNA library. Twenty-five cycles of amplification were performed (96°C for 1 min, 47°C for 2 min, 72°C for 3 min, and 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 (20).

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Abbreviations: ABA, abscisic acid; PCR, polymerase chain reaction.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M36986).

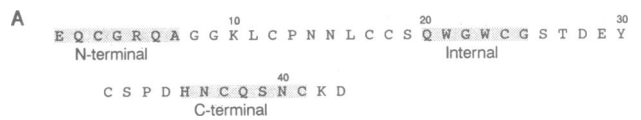
Isolation of the HEV1 Clone and DNA Sequencing. The amplified PCR fragment was labeled with [^{32}P]dATP by the random primer technique (21). Approximately 200,000 plaque-forming units of the λgt10 cDNA library were screened with the ^{32}P-labeled PCR product as the probe by *in situ* plaque hybridization at high-stringency conditions (22). Plaques producing positive signals were selected and rescreened with 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 (23) using deoxyadenosine 5′-[α-(^{35}S)thio]triphosphate and 7-deaza-dGTP in place of dGTP (24). The complete sequence of the clones was obtained by sequencing overlapping deletions generated by phage T4 DNA polymerase (25). 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 4-month-old seedlings was prepared by the method of Kirk and Kirk (26) with 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 (21).

Wounding of *Hevea* Stems and Ethephon and Abscisic Acid (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 hr later. Ethephon (0.1%; Sigma) was sprayed on young seedlings to produce ethylene, and RNA was isolated from stem, leaf, and latex after 24 hr. ABA (50 μM) dissolved in sterile water containing 0.01% ethanol was sprayed on the plant, and RNA was extracted from stems, leaf, and latex after 24 hr as described above. Control plants were sprayed with water/ethanol solution alone. Autoradiograms were scanned with a Gilford (Oberlin, OH) densitometer.

RESULTS

Isolation and Sequence of a Full-Length cDNA Clone. The primary structure of the hevein protein has been reported (Fig. 1*A*; ref. 4). We synthesized three sets of oligonucleotide primers corresponding to the amino-terminal, carboxyl-terminal, and internal regions of the hevein protein (Fig. 1*A*). Each primer was a degenerate mixture of oligonucleotides encoding the shaded amino acids (Fig. 1*B*). The amino-terminal and carboxyl-terminal primers were used in the PCR. The template for the PCR was phage DNA prepared from a *H. brasiliensis* λgt10 cDNA library. The amplified DNA fragment was identified by agarose gel electrophoresis as a band of the expected size of 140 base pairs (bp). To confirm that the amplified DNA was derived from a portion of the hevein cDNA, the 140-bp fragment was gel-purified and reamplified with the amino-terminal and internal primers. The predicted size of the reamplified PCR product was 80 bp. To rescue the 140-bp 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. 1*B*) 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 showed that each contained an open reading frame corresponding to the hevein sequence.



B

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

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 in the single-letter code. Peptide sequences corresponding to the PCR primers are indicated by the shaded boxes. (*B*) Nucleotide sequences of the PCR primers in which M is A or C; N is A, G, C, or T; R is A or G; S is C or G; W is A or T; and Y is 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).

For isolating the full-length hevein cDNA clone, a λgt10 cDNA library was synthesized from poly(A)$^+$ RNA isolated from the latex of *H. brasiliensis*. Approximately 200,000 primary recombinant phages were screened with the 140-bp 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 compose a predicted signal sequence structure (27), and the cleavage site between the −1 and +1 amino acid matches the amino terminus found by protein sequence analysis (4). 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 144 additional amino acids (Fig. 2) extending beyond the hevein sequence.

The 3′-untranslated region contained two consecutive in-frame termination codons (TAATAA; Fig. 2, stars) and a 316-nucleotide untranslated region. A potential polyadenylylation 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 *H. 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 carboxyl-terminal region of the HEV1 cDNA clone, which hybridized to the same 1.0-kb mRNA species (Fig. 3, 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 *WIN1* and *WIN2* of potato (15) demonstrated a high degree of homology in both the amino-terminal and carboxyl-terminal portions (Fig. 4). The overall sequence identity between the HEV1 and the WIN deduced amino acid sequences was as high as 65% (HEV1/WIN1) and 68% (HEV1/WIN2). 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

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                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 AGTAATGGAGGACTAGATTTGGACGTGAATGTTTTCCGTCAACTGGACACAGATGGGAAAGGATAT

    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
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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 polyadenylylation signal is underlined.

analysis. RNA was isolated from intact leaves, stems, roots, and latex from four young seedlings of *H. 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 hr, the RNA was extracted from leaves, stem, and latex of both intact and wounded plants. Northern blot analysis (Fig. 5*A*) 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. 5*A*). A similar increase in hevein mRNA in leaves, stems, and latex was observed 24 hr after spraying rubber tree seedlings with either 0.1% ethephon (Fig. 5*B*) to produce ethylene or 50 μM ABA (Fig. 5*C*). As a control for the specificity of the observed responses, each blot was reprobed with a probe for β ATPase (28) which is known to be constitutively expressed in plants (17). None of the treatments affected the level of β ATPase mRNA in any tissue (Fig. 5*D*). No hevein mRNA could be detected in roots from intact, wounded, or hormone-treated plants (data not shown).

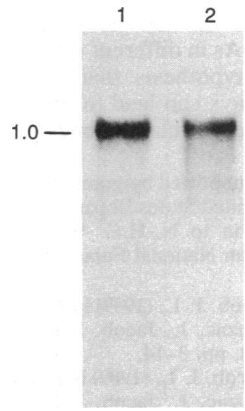

FIG. 3. Northern blot analysis of total RNA from rubber tree latex. Lanes: 1, blot probed with a ^{32}P-labeled DNA fragment corresponding to the amino-terminal region of HEV1 (nucleotides 61–182; see Fig. 2); 2, same blot stripped and reprobed with a ^{32}P-labeled DNA fragment including the carboxyl-terminal portion of HEV1.

DISCUSSION

In this paper we present the amino acid sequence of hevein as deduced from a cDNA clone designated HEV1. The first 43 deduced amino acids are identical to the known hevein sequence as determined by direct amino acid sequencing (4). 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 posttranslational modification. Thus, cleavage of the 187-amino acid proprotein 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*-acetylglu-

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HEV1    M*NI****FIVVL*LC**LTGVAIAEQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSG
WIN2    MVKLSCGPILLALVLCISLTSVANAQQCGRQRGGALSGNNLCCSQFGWCGSTPEYCSPSQGCQSQCTGSG

HEV1    EGVG*GGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRSKYGWTAFCGPVGAHGQSSCGKC
WIN2    PDPGQGGSAQNVRATYHIYNPQNVGWDLNAVSAYCSTWDANKPYAWRSKYGWTAFCGPVGPRGRDSCGKC

HEV2    LSVTNTGTGAKTTVRIVDQCSNGGLDLDVNVFRQLDTDGKGYERGHITVNYQFVDCGDSFN*PLFSVMKSSVIN
WIN2    LRVTNTRTGAQTTVRIVDQCSNGGLDLDINVFQQIDTDGVGNQQGHLIVNYQFVNCGDNVNVPLLSVV*DK**E
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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.

cosamine-oligomer-specific lectins from wheat (7), barley (8), rice (9), and *U. dioica* (10); to the amino termini of basic chitinases (11–13); and to polypeptides encoded by wound-induced genes (*WIN1* and *WIN2*) of potato (15). The carboxyl-terminal portion of the HEV1-encoded protein shows homology to the carboxyl termini of proteins deduced from *WIN1* and *WIN2* genes but not to those of chitinases or to chitin-binding lectins. The carboxyl terminus of HEV1 shows 74% homology to the deduced amino acid sequences from the *WIN1* gene and 79% to the *WIN2* gene. The amino terminus (minus the putative signal sequence) has 72% and 74% homology to *WIN1* and *WIN2*, 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.

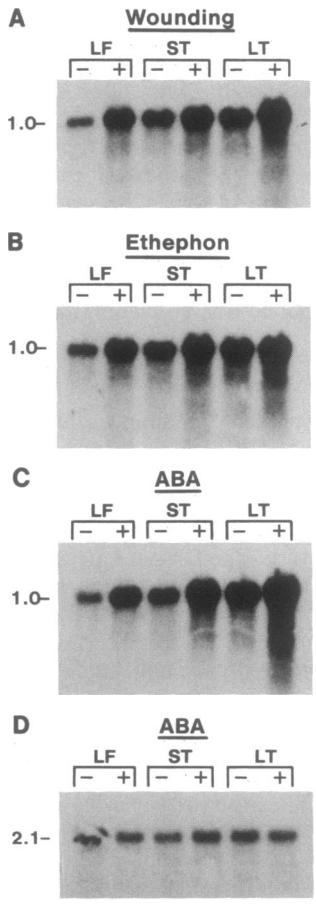

FIG. 5. Effect of stress treatments on the accumulation of hevein transcripts. Total RNA (15 μg per lane) was isolated from leaves (lanes LF), stems (lanes ST), and latex (lanes LT) of either untreated (−) or treated (+) rubber tree plants. (*A*) Treatment: wounding; probe: coding region of HEV1. (*B*) Treatment: ethephon (0.1%); probe: coding region of HEV1. (*C*) Treatment: 50 μM ABA; probe: coding region of HEV1. (*D*) Treatment: 50 μM ABA; probe: β-ATPase cDNA.

Wheat germ agglutinin has been demonstrated to act as an antinutrient factor for insect larvae (29). In addition, mature hevein (5) as well as the stinging nettle lectin (30) and chitinases (31, 32) 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 posttranslational 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 (33).

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 (11, 34) and hydroxyproline-rich glycoproteins in carrots (35). Recently, ABA has been implicated in the wound-induced response of protease inhibitor II in potato and tomato (36). 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 (35, 37).

This research was supported by grants from the National Science Foundation and the United States Department of Energy to N.R. and Rockefeller Foundation to N.-H.C. W.B. is a Senior Research Assistant of the Belgian National Fund for Scientific Research.

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