

Determination of cDNA and genomic DNA sequences of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*

Evert Bokma^{a*}, Marjolein Spiering^a, Keng-See Chow^b, Patty P.M.F.A. Mulder^a, Toto Subroto^c, Jaap J. Beintema^a

^a Biochemisch Laboratorium, Rijksuniversiteit Groningen, Nijenborgh 4, 9747 AG Groningen, the Netherlands

^b Rubber Research Institute of Malaysia, Biotechnology division, P.O. Box 10150, Kuala Lumpur, Malaysia

^c Laboratorium Biokimia, FMIPA, Universitas Padjadjaran, Bandung, Indonesia

Received 9 October 2000; accepted 10 January 2001

Abstract – Hevamine is a chitinase from the rubber tree *Hevea brasiliensis* and belongs to the family 18 glycosyl hydrolases. This paper describes the cloning of hevamine DNA and cDNA sequences. Hevamine contains a signal peptide at the N-terminus and a putative vacuolar targeting sequence at the C-terminus which are not present in the mature protein. The gene encoding hevamine contains no introns. Southern hybridization of *Hevea brasiliensis* DNA with a full-length hevamine cDNA probe showed that there are multiple copies of genes encoding hevamine or hevamine homologues in the *Hevea brasiliensis* genome. In the lutoid body (vacuolar) fraction of rubber latex only three hevamine components were detected with very similar primary structures, which are probably products of one single locus on the genome. © 2001 Éditions scientifiques et médicales Elsevier SAS

5'- and 3'-RACE / family 18 chitinase / phylogeny / Southern hybridization

PR-proteins, pathogenesis related proteins / RACE, rapid amplification of cDNA ends

1. INTRODUCTION

Hevamine is a chitinase/lysozyme located in the vacuoles, so-called lutoid bodies [1], of rubber latex and belongs to the family 18 glycosyl hydrolases [11]. Chitinases are believed to play a role in plant defence against pathogenic fungi [4]. The primary [14] and tertiary structures of hevamine [34] have been elucidated. Proteins homologous to hevamine are found in plants like cucumber [20, 24], grape [2] and tobacco [19], but also in insects [17], yeast [18] and bacteria [36]. In a previous publication [33], we have described the isolation of three basic hevamine components with almost identical chitinase specific activities from the lutoid body (vacuolar) fraction of latex *Hevea brasiliensis*. These are the two major components, hevamine A and B, and the less basic hevamine X. N-Terminal

amino acid sequences of the three components are identical. In lattices of three investigated rubber tree clones, hevamine A and B occur in equal quantities, while in a fourth investigated clone, hevamine B is completely absent [33]. This suggests that the two proteins are allelic gene products.

Plant family 18 glycosyl hydrolases are also classified as class III chitinases or PR-8 (pathogenesis related) proteins. Other 'pathogenesis related' proteins identified in lutoid bodies of rubber latex are the chitin-binding lectin hevein, which is formed from a larger precursor (PR-4), a glucanase (PR-2) and a thaumatin-like protein. No other proteins with chitinase/lysozyme activity could be identified in the lutoid body fraction of *H. brasiliensis*, although others have reported some indirect evidence for the presence of family 19 chitinases (PR-3 proteins) [7, 22].

This paper describes the isolation and characterization of cDNA and DNA sequences of hevamine. The copy number of hevamine in the *H. brasiliensis* genome was determined by DNA hybridization experiments with a full-length hevamine cDNA probe.

*Correspondence and reprints.

E-mail address: evert.bokma@ntlworld.com (E. Bokma).

2. RESULTS

A PCR reaction was carried out on a *Hevea brasiliensis* cDNA bank as described in Methods. The partial cDNA sequence obtained matched well with the corresponding amino acid sequence in hevamine. This sequence was used to synthesize primers for the 3'- and 5'-RACE to obtain the entire hevamine sequence.

The cDNA encoding the hevamine B sequence was identified by PCR. The deduced hevamine sequence shows a number of features. First, the sequence contains a N-terminal signal peptide of 26 amino acids for protein export (figure 1). All the plant family 18 chitinases characterized thus far have this kind of signal peptide. Hevamine also has a C-terminal peptide sequence of 12 amino acids, LFLHSEKCMTVL (figure 1), which is not present in the mature protein [14]. This sequence is most probably necessary for import in luteoid bodies.

The DNA sequence of the gene encoding hevamine was also determined in DNA isolated from a rubber tree of unknown origin. No introns were found in this sequence. The sequence of hevamine A was found, which differs at only two positions from the hevamine B cDNA sequence: a non-silent mutation at position 270 Arg→Leu (CGG→CGT) in agreement with earlier protein sequence studies [14], and a silent one at valine 96 (GTT→GTA). These results indicate that the two sequences probably originate from two alleles at the same gene locus.

The hevamine B cDNA and hevamine A gene sequences were submitted to the EMBL database under accession numbers AJ007701 and AJ010397, respectively. In (figure 1), hevamine was aligned with *Arabidopsis thaliana* class III chitinase [28]. The degree of identity of the mature sequence of hevamine with *Arabidopsis* chitinase at the nucleotide level is 70.9 %. Outside the coding regions and in the N-terminal signal peptide sequence, similarity drops below 40 %.

The result of the Southern blot hybridization is shown in figure 2. Under stringent conditions, a number of bands were visible. There are no cleavage sites for the used restriction enzymes in the hevamine sequence. When digested with *EcoRI/HindIII*, seven bands were visible. This clearly shows that there are several sequences in the *H. brasiliensis* genome that are homologous with hevamine. It would be interesting to know how the hevamine genes are located in the *Hevea* genome. The hybridization pattern of *Hevea* DNA digested with *NcoI* and *NheI* shows a smaller number of bands with lower mobilities, which suggest

that several of the hevamine genes may be clustered without cleavage sites for the enzymes in between them.

Preliminary efforts to clone the gene encoding hevamine by PCR using degenerate primers before the cDNA sequence was available yielded a sequence homologous, but not identical, to that of hevamine. Figure 3 shows the alignment of a part of the hevamine cDNA sequence, encoding amino acids 14 to 178, with this gene sequence. The sequence similarity between the two DNA sequences is 66 %. This partial DNA sequence was submitted to the EMBL database under accession number AJ297260.

Because of the possibility that the minor component hevamine X represents one of the additional homologous gene products, we have investigated this component more thoroughly. Hevamine X was found to be present in a rather constant ratio of about 20 % compared with hevamine A, independent of the identity of the investigated rubber clone (cultivar) and isolation conditions of the protein. Isoelectric points of hevamine B, A and X were found to be 10.5, 9.9 and 9.3, respectively. As hevamine A and B differ in one charge unit, these results indicate that hevamine A and X also differ in one charge unit. To investigate a difference in primary structure of hevamine A and X, fingerprints of tryptic digests of identical quantities of the two proteins were made and investigated by reversed phase HPLC, followed by mass spectrometry of the isolated peptides. In this way, all expected tryptic peptides could be identified by mass spectrometry (data not shown; Subroto, Ph.D. thesis, 2000). Some additional minor peaks in the digest of hevamine X could be accounted for by peptides originating from trypsin, as more enzyme had been used for the digestion as intended.

Our explanation for the failure to identify a difference in primary structure between hevamine X and A is that a specific partial deamidation of an asparagine or glutamine residue in the hevamine A sequence had occurred in planta or in the first isolation steps of the protein. However, it will be difficult to demonstrate such a deamidation with the HPLC technique used, as the peptide isolations have taken place at a pH value in which carboxylate groups are not ionized and therefore will behave similarly to amide groups. So it is very likely that all three hevamine components identified by us are products of one single locus on the genome of *H. brasiliensis*.

Figure 4 shows the evolutionary relationship of various family 18 plant chitinases. In this tree, hevamine belongs to the group containing the sequences of grape

RUBBER TREE ARABIDOPSIS	1	GAGATAGAACTAGTAAGTGTTCATTACTTGCATATATGGCCAAAAGAACCC *A***GACAA***ATCTTTAACCAAAAACATGACCAACATGA*TCCTC*C*AG M A K R T M T N * T L * K	-22
RUBBER TREE ARABIDOPSIS	54	Q A I L L L L L A I S L I M S S S H V D CAAGCCATCCTTCTTCTCTCTAGCAATCTCACTGATTATGTCCAGCTCTCATGTTGAT **T**T**ATAC**T**T**CAT**G**GCTCTC**GCAAACC**CG**CATCC H V * Y F * F F I S C S L S K P * D A S	-2
RUBBER TREE ARABIDOPSIS	104	G G G I A I Y W G Q N G N E G T L T Q T GGTGGTGGCATTGCCATTACTGGGGTCAAAATGGCAACGAAGGAACCTAACACAAACA A*A*****A*****C**T*****C*****C*A*****T*AC**CT*TGCC**G R * * * * * * * * * * * * * * * * N * S A *	19
RUBBER TREE ARABIDOPSIS	164	C S T R K Y S Y V N I A F L N K F G N G TGCTCCACACGCAATATTTCTTACGTGAATATAGCCTTTCTCAATAAATTTGGCAATGGT *TG***CG**CGG**G*****C**CG**C*****TGTC*****C**A*****C * A * G R * A * * * * V * * * * V * * * * *	39
RUBBER TREE ARABIDOPSIS	224	Q T P Q I N L A G H C N P A A G G C T I CAAGCCCAACCAATCAACCTTGGCGGCATTGTAACCCGCGTGTGAGGTTGACCATTT *****GG*GC*****C**C*****T**G**GAACAC*****CA* * * * E L * * * * * * * * * * * N T * * * H	59
RUBBER TREE ARABIDOPSIS	284	V S N G I R S C Q I Q G I K V M L S L G GTAGCAATGGCATCAGGAGTGGCAATCCAGGAATTAAGGTGATGCTTTCTCTTGGC T*TG**TC*GAGG**AAGA**T**GTCT*GT**C**C*****T**T**G*****C** F G S Q V K D * * * S R * * * * * * * * * *	79
RUBBER TREE ARABIDOPSIS	344	G G I G S Y T L A S Q A D A K N V A D Y GGTGGGATTGGAAGCTACACCTGGCCTCTCAAGCTGATGCAAAAACGTAGCAGACATAT T **C*****A***T*GA**T*GG**GAGG*AG**C*****GTTA*C**T**T*** * * * * N * S I G * R E * * * * V I * * *	99
RUBBER TREE ARABIDOPSIS	404	L W N N F L G G K S S S R P L G D A V L TTGTGGGAATATTTCTTGGGTGGGAATCTTCTTCCCGCCTTAGGTGATGCTGTATTG C*****C**C*****A**A*****A**A*****T**C*A *	119
RUBBER TREE ARABIDOPSIS	464	D G I D F D I E H G S T L Y W D D L A R GATGGTATTGATTTTACATAGAGCATGGTTCAACCCCTGACTGGGACGATCTTGACGT *****C*****CA**T**C**T**C**T**A**C*****T*****C**CA*G * * * * * N * * * L * * P Q H * * * * * *	139
RUBBER TREE ARABIDOPSIS	524	Y L S A Y S K Q G K K V Y L T A A P Q C TACTTATCTGCATATAGCAAGCAAGGCAAGGTGTATTAACTGCGAGCTCCTCAATGT ACTC**A**AAG**T***C*GAG**A*GA**AA**A*****A*G***** T * * K F * H R * R * I * * * * G * * * *	159
RUBBER TREE ARABIDOPSIS	584	P F P D R Y L G T A L N T G L F D Y V W CCATCTCCCTGATAGATATTTAGGAGCTGCGCTTAATCTGCTGCTTTTGAATGATGATGG *****A**C*****TAA*G**A*G**T**C**C**AA*G*****T*** * * * * * L M * S * * * * K R * * * * *	179
RUBBER TREE ARABIDOPSIS	644	V Q F Y N N P P C Q Y S S G N I N N I I GTTCAATTCATAACAATCCACCATGCCAGTATAGCTCAGGTAAACATTAAACAACATCATT A*****C**T*****G**TTC**C**T*****T**C**C**A**C**T**C I * * * * * * * * * * * * * * * * * L F	199
RUBBER TREE ARABIDOPSIS	704	N S W N R W T T S I N A G K I F L G L P AATCTGGAATCGATGGACCATCTATAAATGCAGGGAATATTTTGGGGTTCGGG C**T**A*****CAAG*****T**A**CGCA**CCAA**T**C**C**C**TC**A*** D * * * K * * * * * A * Q * F * * * * *	219
RUBBER TREE ARABIDOPSIS	764	A A P E A A G S G Y V P P D V L I S R I GCAGCTCCTGAGGACGCGGAGCGGATATGTTCCACCGGATGCTGATTTCTCGGATT *****A**T*****T**C**A**C*****A*****A*****C**A**A*** * * * * * * * * * * * * * * * * * T * Q *	239
RUBBER TREE ARABIDOPSIS	824	L P E I K K S P K Y G G V M L W S K F Y CTTCTGAAATAAGAGAGTACCTAAGTACGGAGGTGTATGCTTTGGTCAAGTTCTACG ***GACTT*****TAGG*****T*****A*****T**A*****GG * * T L * * * R * * * * * * * * * * * W	259
RUBBER TREE ARABIDOPSIS	884	D D K N G Y S S S I R D S V L F L H S E GATGATAAGAAATGGCTATAGTCTCCATTCGGACAGTGTATGTTCTCTCCATCTGAA G *****A*****A**C*****A**T**AGT**CT*****G**GAAGAATCG*ATGG * * * * * * * * * * V A *	279
RUBBER TREE ARABIDOPSIS	944	K C M T V L AAGTGTATGACAGTACTTTGAAATTGGGAGAAATATTCGCTGCACCGGTGCATATACAC CCA**GTATGTAT*AA**TCTC**T**TGTG*ATCAATCTCT*GTC*TTGGT*TT*G	285

Figure 1. Comparison of cDNA and translated amino acid sequence of hevamine B, with the cDNA and translated amino acid sequence of *Arabidopsis thaliana* class III chitinase [28]. The nucleotide and amino acid residues are numbered in the left and right margins, respectively. Dashes indicate gaps introduced for maximal alignment and asterisks replace identical nucleotides and amino acids. The N-terminal signal and C-terminal putative vacuolar targeting sequences, which are not present in the mature protein, are underlined. The DNA sequences that were used for PCR amplification of the genomic hevamine DNA sequence are also underlined. The differences between hevamine A and B (a silent mutation at amino acid position 96 and a non-silent mutation at amino acid position 270) are denoted in bold symbols. The stop codons are denoted in bold symbols and underlined.

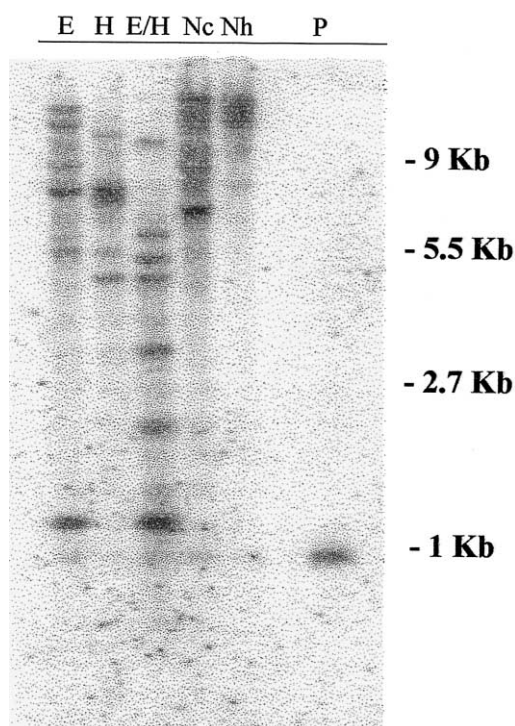


Figure 2. Hybridization of genomic *Hevea* DNA with a full-length hevamine cDNA probe. E, *Eco*RI; H, *Hind*III; Nc, *Nco*I; Nh, *Nhe*I; P, positive control (full-length hevamine cDNA sequence).

(*Vitis vinifera*), cowpea (*Vigna unguiculata*) and thale cress (*A. thaliana*). Despite its extension of 30 amino acids, cucumber ORF1 groups with the cucumber ORF2 and is not closely related to *Sesbania* and soybean, which are sequences with C-terminal extensions. Cucumber ORF2 and ORF3 differ at only 7 % of the nucleotide positions. Therefore, ORF3 gave no additional information and was omitted from the evolutionary analysis.

3. DISCUSSION

The hevamine sequence contains a C-terminal extension of 12 amino acids that most probably functions as a targeting signal for luteoid bodies. Cleaved off C-terminal sequences of similar length but with unrelated sequences have been identified in cDNA and mature protein sequences of the hevein precursor [30] and β -1,3-glucanase [3]. While the targeting signal sequence of β -1,3-glucanase contains a putative N-glycosylation site [3], such sites are absent in the hevein precursor and hevamine. C-Terminal exten-

sions of approximately 30 amino acids were found in cucumber CHI1 [19], *Sesbania rostrata* [8] and soybean [39] chitinases. It may be that these extensions are not all vacuolar targeting signals, since these signals in plants are usually much smaller as shown in tobacco chitinases [12, 23, 32] and barley lectin [6]. The C-terminal extensions of soybean and *Sesbania* chitinases are homologous, but there is no significant similarity between these two and those of cucumber CHI1 and hevamine. In addition, CHI1 may be a pseudogene, because no expression was found of this sequence in cucumber under various conditions [20].

The gene encoding the hevamine sequence contains no introns. This is different from the thale cress (*A. thaliana*) family 18 chitinase [28]. The *Arabidopsis* gene contains two small introns of 169 and 269 bp [16]. Soybean chitinases Chib1-1 and Chib1-2 contain one intron while soybean chitinase Chib2 is intronless [37]. The cucumber (*Cucumis sativus*) genome contains three tandemly arranged family 18 chitinase genes, CHI1–CHI3. Like hevamine, these genes do not contain introns [20]. It is evident from these observations that there is a lot of variability in the exon/intron structure of the family 18 plant chitinases.

Hybridization studies showed that there are a number of sequences in the *Hevea* genome that are homologous to hevamine. Using PCR on *Hevea* DNA with degenerate primers, we found a partial sequence that is homologous to hevamine. In rice, several family 18 cDNA chitinase isoforms were identified [26]. As already mentioned, in *Cucumis sativus* three isoforms occur at one locus [20], while in *Arabidopsis* only a single gene is present [16, 28]. It is rather certain that the partial sequence of a hevamine homologue shown in figure 3 represents one of the hybridizing genes visible in figure 2. Although a partial sequence of only 164 amino acids was obtained, several tentative conclusions can be drawn. Residues essential for structure and enzymatic activity of hevamine are present in this partial sequence, e.g. five of the six half-cystine residues and the highly conserved amino acid sequence DGIDFDIE, equivalent to Asp120 to Glu127 in hevamine. This sequence contains amino acid residues Asp125 and Glu127 that are essential for catalysis [34]. Residue Gly149 is deleted in this sequence, but this residue occurs in a surface loop of the hevamine structure and is not strictly conserved in other family 18 plant chitinases. So the structure may represent a gene product which is expressed at some location during the life of the rubber tree. In potato and tobacco for instance, chitinases accumulate in the flower organs during the flowering stage [21, 38]. An interesting

HEVHOMOL HEVAMINE	1	* * * A E * * A S G N * Q * * * V * * *	20
		GGCACTTGGCTGAACTTGTGCTCGGGAACATCAATATGTGAACGTAGCTTTCTTG	
		GGAACTCTAACACAAACATGCTCCACACGCAATATTCTTACGTGAATATAGCCTTTCTC	
		G T L T Q T C S T R K Y S Y V N I A F L	
HEVHOMOL HEVAMINE	61	S N * * * * * * * V L * * * * * V * S	40
		TCTAATTTGGGAATGGCCAACTCCTGTGCTGAACCTAGCCGGTCATTGTGTTCTTAGT	
		AATAAATTTGGCAATGGTCAAACCCACAAATCAACCTTGCCGGCCATTGTAACCCGGCT	
		N K F G N G Q T P Q I N L A G H C N P A	
HEVHOMOL HEVAMINE	121	S N * * * G L * S D * Q A * * G * * * *	60
		TCTAATGGTTCACCTGGTTTAAGCTCTGATATTCAAGCTTGCCAAGGTCAAGGCATCAAA	
		GCTGGAGGTTCACCATTTGTCAGCAATGGCATCAGGAGTTGCCAAATCCAAGGAATTAAG	
		A G G C T I V S N G I R S C Q I Q G I K	
HEVHOMOL HEVAMINE	181	* L * * I * * * G * * * S * S * A D * *	80
		GTCCATTATGCCATTGGAGGAGGTGGAGGCAGCTACTCATTTCATCGGCCGATGATGCA	
		GTGATGCTTTCTCTTGGCGGTGGGATGGAAGCTACACCTGGCCTCTCAAGCTGATGCA	
		V M L S L G G G I G S Y T L A S Q A D A	
HEVHOMOL HEVAMINE	241	T Q * * N * * * * * * * T * N * * *	100
		ACCCAAGTTGCTAACTATCTTTGGAATAACTTCCTTGGTGGTACGTCCAATTCACGACCA	
		AAAAACGTAGCAGACTATTGTGGAATAATTCTTGGGTGGGAATCTTCTCCCGTCCC	
		K N V A D Y L W N N F L G G K S S S R P	
HEVHOMOL HEVAMINE	301	* * * * I * * * * * * * A * * G Q F	120
		CTAGGCGATGCCATTTGGATGGTATTGATTTGATATTGAGGCAGGTTCAAGGCCAATTC	
		TTAGGTGATGCTGATTGGATGGTATTGATTTGACATAGAGCATGGTTCAACCTGTGAC	
		L G D A V L D G I D F D I E H G S T L Y	
HEVHOMOL HEVAMINE	361	* * * * * A * N G F * Q * - R * * * *	140
		TGGGACGATCTTGCCAGGGCCCTTAATGGTTTAGCCAACAA--AGGAAGGTGTACTTTA	
		TGGGACGATCTTGACGTTACTTATCTGCATATAGCAAGCAAGCAAGGTTATTTA	
		W D D L A R Y L S A Y S K Q G K K V Y L	
HEVHOMOL HEVAMINE	421	S * * * * * * * A H * S * * I * * *	160
		TCGGCAGCTCCACAGTGTCCCTTCCCTGATGCTCATCTAAGCACTGCAATTAACTAGGC	
		ACTGCAGCTCCTCAATGTCCATTCCCTGATAGATATTTAGGGACTGCGCTTAATACCTGGT	
		T A A P Q C P F P D R Y L G T A L N T G	
HEVHOMOL HEVAMINE	481	* * * * * 165	
		CTATTCGATTACGTG	
		CTTTTGAATATGTA	
		L F D Y V	

Figure 3. Alignment of a *Hevea* DNA sequence homologous with the hevamine cDNA sequence encoding amino acids 14 to 178. The nucleotide and amino acid residues are numbered in the left and right margins, respectively. Dashes indicate gaps introduced for maximal alignment and asterisks replace identical amino acids.

feature is the much lower positive charge, eight charge units, of this sequence fragment than that of the homologous one of hevamine. In the family 19 chitinases, the vacuolar enzymes (class I) generally are basic, while the extracellular pathogenesis related proteins (class II) are acidic. So it will be interesting to know if this chitinase homologue is also an extracellular PR protein, synthesized under stress conditions. The presence of extracellular PR proteins have not yet been investigated in the rubber tree.

Hevamine is a family 18 chitinase. Family 19 chitinases are more often encountered in plants [9]. There are no sequence similarities between the two chitinase families and their three-dimensional structures are also completely different: while family 18 chitinases have a $(\beta/\alpha)_8$ -barrel structure, that of family 19 chitinases contains predominantly α helices [10] and is similar to the structure of animal and phage lysozymes [13]. Antibodies against the family 19 chitinase from bean, *Phaseolus vulgaris* [22], were used to demonstrate the presence of chitinases and a

cDNA probe from this chitinase to demonstrate its expression in latex of *Hevea brasiliensis* [7]. Probably the authors of these two studies have not been aware that there are two chitinase families, which are structurally unrelated. These studies, however, led us to investigate very carefully by structural analyses the presence of family 18 and 19 chitinases in the lutoid body fraction of rubber latex. Hevamine X, A and B are the only proteins with chitinase/lysozyme activity which could be identified in the lutoid body fraction of rubber latex [33]. Investigation of their primary structures (see [14] and this paper) indicates that hevamine A and B are most likely two alleles of the same gene product and hevamine X a chemically modified form of hevamine A, although we cannot exclude the possibility that hevamine X represents the product of a recent gene duplication which is expressed at low level and was not found in our PCR experiments on cDNA and genomic DNA.

Figure 4 shows an evolutionary tree of nucleotide sequences of plant family 18 chitinases. Previous

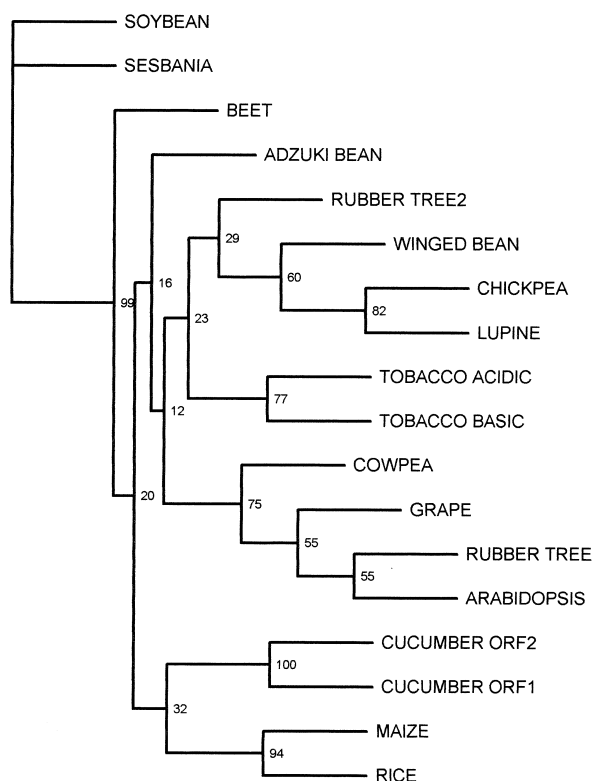


Figure 4. DNA parsimony tree of class III chitinases derived from nucleotide sequences in PAUP version 4b4a. Bootstrap values are indicated in the branches. Before alignment, non-coding regions and N-terminal signal peptides were removed for maximal matching. (AB003195), rice; (S82314), maize; (X88801), cowpea; (AJ007701), rubber tree 1; (Z68123), rubber tree 2 (partial sequence); (AJ297260), grape; (AB006055), *Arabidopsis*; (Z11563), tobacco acidic; (Z11564), tobacco basic; (A23398), beet; (M24365), *Cucumis* ORF2; (M84214), *Cucumis* ORF1; (AB000097), soybean '*Glycine max*'; (Y12706), *Sesbania*; (D49953), winged bean; (X70660), chickpea; (Y16415), *Lupinus*; (D11335), Adzuki bean.

evolutionary analyses of these chitinases have been published by Hamel et al. [9] and Watanabe et al. [37]. However, the tree is rather erratic, has low bootstrap values and is of little value for deriving evolutionary relationships between the taxa investigated. On DNA level, rice and maize group together with a high bootstrap value. Phylogenetic analysis of the protein sequences, however, groups maize together with the basic tobacco sequence and rice together with *Hevea*, *Arabidopsis* and grape vine (data not shown). This difference is due to the fact that the monocots have a codon usage that is different from the dicots. The partial DNA sequence obtained from *H. brasiliensis* groups together with other acidic family 18 chitinases from the *Fabaceae* winged bean, chickpea and lupine

albeit with a very low bootstrap value. Most family 18 chitinases of *Fabaceae* are in one monophyletic group. Soybean and sesbania, which have an already discussed putative C-terminal vacuolar targeting signal form a separate group. In addition, the sequence of a basic chitinase from cowpea (*Vigna unguiculata*) is outside the other *Fabaceae* and groups with sequences from *Hevea*, *Arabidopsis* and grape vine. A similar result was found before [9] and suggests that there are several classes of family 18 chitinases in plants. Although *Hevea* chitinase differs from the *Arabidopsis* chitinase and in such features as the presence of a C-terminal vacuolar targeting signal, the absence of introns in the DNA sequence and the larger number of cross-hybridizing genes on Southern blotting, the sequence of these two plant species group together in the evolutionary tree. Additional plant family 18 chitinase sequences may clarify some features of the evolution of this family of chitinases. But the general impression is that gene duplications, gene losses and perhaps also translocations have resulted in rather unreliable relationships for deriving phylogenetic conclusions.

4. METHODS

4.1. Isolation of total RNA from rubber latex of *Hevea brasiliensis*

This protocol is adapted from the method of Prescott and Martin [27]. Twenty millilitres latex from clone RRIM600 was collected in sterile Greiner tubes containing 20 mL RNA extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 10 mM EDTA, 10 % SDS, pH 9.5). After collection, the latex was sent from Malaysia to the Netherlands in dry ice. The latex was centrifuged at $8\,000 \times g$ for 10 min at 4 °C. The supernatant was transferred into a new tube and blended with a polytron while keeping the samples on ice and extracted with an equal volume phenol/chloroform. The pellet and the top rubber layer were discarded. Phases were separated by centrifuging at $5\,000 \times g$ at 4 °C. The aqueous phase was transferred to a new tube and the phenol/chloroform extraction was repeated. The supernatant was again transferred to a new tube and extracted with chloroform/isoamylalcohol, followed by centrifugation at 4 °C. To the aqueous phase, a stock of 8 M LiCl was added to a final concentration of 2 M. After overnight incubation at –20 °C, the sample was centrifuged at $8\,000 \times g$ for 15 min at 4 °C. The pellet was resuspended in 1 mL TE per 40 mL latex

sample. The sample was again precipitated with 8 M LiCl as before, but incubated for 2 h at -20°C . After centrifugation ($8\,000 \times g$, 15 min, 4°C), the pellet was resuspended in approximately 100 μL TE depending on the pellet size.

4.2. Isolation of DNA from *Hevea brasiliensis* leaves

H. brasiliensis leaves of an unknown clone were obtained from a tree in a greenhouse of Hogeschool Larenstein (Deventer, the Netherlands). After harvesting, young leaves were frozen in liquid nitrogen. If not used immediately, the leaves were stored at -80°C .

For the isolation of DNA from *H. brasiliensis* leaves, the method of Murray and Thompson [25] was used.

4.3. Determination of the cDNA sequence using 5'- and 3'-RACE

The polymerase used for the PCR reactions was the high fidelity mix from Boehringer Mannheim (2 U per reaction mixture). First, a PCR reaction was carried out on a *H. brasiliensis* latex cDNA bank prepared in the Rubber Research Institute of Malaysia (Dr F.C. Low and Dr E. Vogt, unpubl.) with a degenerate primer pair: 5'-TAT TGG GG(C/T) CAA AA(C/T) GG(A/G/C/T) AA(C/T) GA-3' and 5'-(G/A)TT (G/A)TT (G/A)TA (G/A)AA (C/T)TG (G/A)AC CCA-3'. These primers encode amino acids 6 to 13 and 179 to 185 of heveamine, respectively [14]. The following PCR conditions were used: 1 min 94°C , 30 s 50°C , 45 s 72°C , 35 cycles. After each cycle the synthesis step was extended for 5 s. The PCR product was ligated in the PCR2.1 T-vector (Invitrogen) and sequenced [29]. Based on this sequence information, primers were synthesized for the 5'- and 3'-RACE to obtain the entire cDNA sequence encoding heveamine. For the RACE procedures, the 5'/3' RACE kit of Boehringer Mannheim was used. For first strand DNA synthesis in the 3'-RACE, 2 μg total RNA was incubated with AMV reverse transcriptase and the oligo dT-anchor primer: 5'-ACCACGCGTATCGATGTC-GACTTTTTTTTTTTTTTTT-3' according to the manufacturers specifications. The PCR reaction was carried out with a specific primer encoding amino acids 43 to 50: 5'-AAA TCA ACC TTG CCG GCC ATT G-3' and the PCR anchor primer: 5'-GACCA-CGCGTATCGATGTCGAC-3'. The conditions used were the same as described above. The product was ligated in PCR2.1 T-vector (Invitrogen) and sequenced [29].

For the first strand cDNA synthesis in the 5'-RACE, 2 μg total RNA was transcribed with AMV reverse transcriptase and the following antisense primer: 5'-TAT TCC ACA AAT AGT CTG CTA GG-3' encoding amino-acids 116 to 123. The resulting antisense cDNA strand was tailed with a poly-A tail according to the manufacturers specifications. PCR was carried out as described above with the PCR anchor primer and a specific antisense primer encoding amino acids 85 to 92, with the following sequence: 5'-TGT AGC TTC CAA TCC CAC CGC C-3'. The product was ligated in the PCR2.1 (Invitrogen) T-vector and sequenced according to the dideoxy method [29].

4.4. Determination of the DNA sequence encoding heveamine

Based on the cDNA sequence obtained with 5'- and 3'-RACE, primers were designed to obtain the full-length DNA sequence of heveamine. The sense primer, 5'-CAT TTA CTT GCA ATA ATG GCC-3', contains the start and the second codon at the 3'-end (underlined). The antisense primer: 5'-TGC ACC GAA TAA TTT CTC CC-3' is located 23 bases behind the stop codon. PCR was performed as described above. The products were ligated as described above. After transformation and miniprep, an aliquot of the samples was digested with *EcoRI* and run on an agarose gel. Plasmids containing an insert were sequenced. Before the mRNA sequence was available, efforts were made to identify the genomic sequence of heveamine by PCR with genomic DNA using degenerated primers coding for amino acids 6–13 (coding strand) and 179–185 (non-coding strand).

4.5. Southern analysis

For the Southern analysis all aqueous solutions were prepared with three times distilled water or Milli-Q water. Approximately 20 to 30 μg genomic DNA was digested overnight with 40 U restriction enzyme or restriction enzyme mix. The digested DNA was electrophoresed overnight on a 0.8 % agarose gel with $0.5\times$ TBE as running buffer. The applied voltage was 1 to $1.5\text{ mV}\cdot\text{cm}^{-1}$. After staining with ethidium bromide, a gel photo was taken. The gel was incubated for 5 min in 0.25 M HCl, followed by 10 min in 0.4 M sodium hydroxide solution. Blotting was performed with Nylon positive charge membranes (Boehringer Mannheim) for 6 h with 0.4 M sodium hydroxide and using capillary transfer. After blotting the membrane was incubated for 5 min in neutralization solution (1 M NaCl, 0.5 M Tris-HCl, pH 7.0), followed by 5 min incubation in $2\times$ SSC and air-dried.

For the labelling reaction, approximately 100 ng full-length hevamine sequence was labelled with (α - ^{32}P) dCTP using the random prime labelling kit (Boehringer Mannheim) according to the manufacturers specifications. Non-incorporated (α - ^{32}P) dCTP was removed by a Sephadex G-50 (fine) column. The membrane was hydrated, followed by incubation for 6 h in 12 mL hybridization mix (0.5 M sodium phosphate, 1 mM EDTA 6.5 % SDS, pH 7.2, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ herring sperm DNA) at 65 °C. The labelled DNA was heat denatured. After cooling on ice, the probe was added to the hybridization mix and incubated overnight at 65 °C. The next day, the membrane was washed at 65 °C with wash buffer 1 (2 $\times$ SSC, 0.5 % SDS) for 15 min, followed by several washing steps for 10 min in buffer 2 (1 $\times$ SSC, 0.1 % SDS), until no radioactivity could be detected in the buffer solution. Finally, the membrane was washed three times with buffer 3 (0.3 $\times$ SSC, 0.1 % SDS) for 5 min. For the washing steps, all the buffers were preheated at 65 °C to enhance removal of background radiation from the membrane. After drying, the membrane was incubated in the phosphor imager (Molecular Dynamics type 425) to visualize hybridizing DNA restriction fragments.

4.6. Structural studies of hevamine components

Hevamine components were isolated as described by Subroto et al. [33]. Isoelectric points of the components were determined by IEF using the PHAST system (Pharmacia). Disulfide bridges of hevamine components were cleaved with tributylphosphine followed by S-pyridylethylation with 4-vinylpyridine [15]. Reaction products were removed from the protein by repeated dialysis against 0.2 M ammonium bicarbonate and the reduced proteins were digested with trypsin (treated with L-tosylamide-2-phenylalanylchloromethane, TPCK) in 0.2 M ammonium bicarbonate at 37 °C for 4 h at substrate to enzyme ratios of 100:1 (by mass). Tryptic peptides were isolated by reversed phase HPLC on a nucleosyl 10 C18 column (30 $\times$ 0.45 cm) with a 0–70 % acetonitrile gradient in 0.1 % trifluoroacetic acid for 60 min at a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$. The effluent was monitored at 214 nm and the peaks were collected manually and dried.

MALDI-TOF mass spectrometry was used to identify peptides present in the collected peaks from the known amino acid sequence of hevamine A [14]. Peptide quantities corresponding to about 1 μg hevamine used for the tryptic digestions dissolved in 70 % acetonitrile in 0.1 % trifluoroacetic acid (1 μL)

were mixed with equal volumes sinapic acid dissolved in the same solvent (10 $\text{mg}\cdot\text{mL}^{-1}$; decantation of a clear solution) and were dried on a target. The spectra were recorded on a ToFSpec E & SE Micromass mass spectrometer.

4.7. Alignment and phylogeny of chitinase sequences

For phylogenetic analysis, nucleotide sequences were aligned in ClustalX [35]. Tree building was performed in PAUP version 4b4a [31] using maximum parsimony [5]. One hundred replicates were used for the determination of the bootstrap values in the branches.

Acknowledgments. We thank Mr Piet Wietzes for help with the mass spectrometry analysis. The Foundation ‘Max Gruber Fonds’ for supporting the stay of T.S. in Groningen. Dr H.Y. Yeang (Rubber Research Institute of Malaysia) for supplying us the latex material and Dr Jean-Yves Dubois for help and advice with the phylogenetic analysis. This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for scientific Research (NWO).

REFERENCES

- [1] Archer B.L., Hevamine: a crystalline basic protein from *Hevea brasiliensis* latex, *Phytochemistry* 15 (1976) 297–300.
- [2] Busam G., Kassemeyer H.H., Matern U., Differential expression of chitinases in *Vitis vinifera* L. responding to systematic acquired resistance activators or fungal challenge, *Plant Physiol.* 115 (1997) 1029–1037.
- [3] Chye M.L., Cheung K.Y., β -1,3 glucanase is highly expressed in laticifers of *Hevea brasiliensis*, *Plant Mol. Biol.* 29 (1995) 397–402.
- [4] Collinge D.B., Kragh K.M., Mikkelsen J.D., Nielsen K.K., Rasmussen U., Vad K., Plant chitinases, *Plant J.* 3 (1993) 31–40.
- [5] Fitch W.M., Towards defining the course of evolution, minimum change from a specific tree topology, *Syst. Zool.* 20 (1971) 406–415.
- [6] Gal S., Raikhel N.V., A carboxy-terminal plant vacuolar targeting signal is not recognized by yeast, *Plant J.* 6 (1994) 235–240.
- [7] Gidrol X., Chrestin H., Tan H.L., Kush A., Hevein a lectin-like protein from *Hevea brasiliensis* (rubber tree) is involved in the coagulation of latex, *J. Biol. Chem.* 269 (1994) 9278–9283.
- [8] Goormachtig S., Valerio-Lepiniec M., Szczy-

- glowski K., Van Montagu M., Holsters M., De Bruijn F.J., Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection, *Mol. Plant-Microbe Interact.* 8 (1995) 816–824.
- [9] Hamel F., Boivin R., Tremblay C., Bellemare G., Structural and evolutionary relationships among chitinases of flowering plants, *J. Mol. Evol.* 44 (1997) 614–624.
- [10] Hart P.J., Monzingo A.F., Ready M.P., Ernst S.R., Robertus J.D., Crystal structure of an endochitinase from *Hordeum vulgare* L. seeds, *J. Mol. Biol.* 229 (1993) 189–193.
- [11] Henrissat B., Bairoch A., New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities, *Biochem. J.* 293 (1993) 781–788.
- [12] Heitz T., Segond S., Kaufmann S., Geoffroy P., Prasad V., Brunner F., Fritig B., Legrand M., Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme, *Mol. Gen. Genet.* 245 (1994) 246–254.
- [13] Holm L., Sander C., Structural similarity of plant chitinase and lysozymes from animals and phage. An evolutionary connection, *FEBS Lett.* 340 (1994) 129–132.
- [14] Jekel P.A., Hartmann B.H., Beintema J.J., The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex, *Eur. J. Biochem.* 200 (1991) 123–130.
- [15] Jost W., Bak H., Glund K., Terpstra P., Beintema J.J., Amino acid sequence of an extracellular phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells, *Eur. J. Biochem.* 98 (1991) 1–6.
- [16] Kawabe A., Innan H., Terauchi R., Miyashita N.T., Nucleotide polymorphism in the acidic chitinase locus (ChiA) region of the wild plant *Arabidopsis thaliana*, *Mol. Biol. Evol.* 14 (1997) 1303–1315.
- [17] Kramer K.J., Corpuz L., Choi H.K., Muthukrishnan S., Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*, *Insect Biochem. Mol. Biol.* 23 (1993) 691–701.
- [18] Kuranda M.J., Robbins P.W., Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*, *J. Biol. Chem.* 266 (1991) 19758–19767.
- [19] Lawton K., Ward E., Payne G., Moyer M., Ryals J., Acidic and basic Class III chitinase mRNA accumulation in response to TMV infection of tobacco, *Plant Mol. Biol.* 19 (1992) 735–743.
- [20] Lawton K.A., Beck J., Potter S., Ward E., Ryals J., Regulation of cucumber class III chitinase gene expression, *Mol. Plant Microbe Int.* 7 (1994) 48–57.
- [21] Lotan T., Ori N., Fluhr R., Pathogenesis-related proteins are developmentally regulated in tobacco flowers, *Plant Cell.* 1 (1989) 881–887.
- [22] Martin M.N., The latex of *Hevea brasiliensis* contains high levels of both chitinases and chitinases/lysozymes, *Plant Physiol.* 95 (1991) 469–476.
- [23] Melchers L.S., Sela-Buurlage M.B., Vloemans S.A., Woloshuk C.P., Van Roekel J.S., Pen J., Van den Elzen P.J., Cornelissen B.J., Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β -1,3-glucanase in transgenic plants, *Plant Mol. Biol.* 21 (1993) 583–593.
- [24] Métraux J.P., Burkhart W., Moyer M., Dincher S., Middlesteadt W., Williams S., Payne G., Carnes M., Ryals J., Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase, *Proc. Natl. Acad. Sci. USA* 86 (1989) 896–900.
- [25] Murray M.G., Thompson W.F., Rapid isolation of high molecular weight plant DNA, *Nucleic Acids Res.* 8 (1980) 4321–4325.
- [26] Nagasaki H., Yamamoto K., Shomura A., Koga-Ban Y., Takasuga A., Yano M., Minobe Y., Sasaki T., Rice class III chitinase homologues isolated by random cloning of rice cDNAs, *DNA Res.* 4 (1997) 379–385.
- [27] Prescott A., Martin C., A rapid method for the quantitative assessment of levels of specific mRNAs in plants, *Plant Mol. Biol. Rep.* 4 (1987) 219–224.
- [28] Samac D.A., Hironaka C.M., Yalla P.E., Shah D.M., Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*, *Plant Physiol.* 93 (1990) 907–914.
- [29] Sanger F., Nicklen S., Coulson A.R., DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. USA* 74 (1977) 5463–5467.
- [30] Soedjanaatmadja U.M., Subroto T., Beintema J.J., Processed products of the hevamin precursor in the latex of the rubber tree (*Hevea brasiliensis*), *FEBS Lett.* 363 (1995) 211–213.
- [31] Swofford D.L., PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), version 4, Sinauer Associates, Sunderland, Massachusetts, 1998.
- [32] Sticher L., Hofsteenge J., Neuhaus J.M., Boller T., Meins F., Posttranslational processing of a new class of hydroxyproline-containing proteins. Prolyl hydroxylation and C-terminal cleavage of tobacco (*Nicotiana tabacum*) vacuolar chitinase, *Plant Physiol.* 101 (1993) 1239–1247.
- [33] Subroto T., Koningsveld G.A., Schreuder H.A., Soedjanaatmadja U.M.S., Beintema J.J., Chitinase and β -1,3-glucanase in the luteoid-body fraction of *Hevea* latex, *Phytochemistry* 43 (1996) 29–37.
- [34] Terwisscha van Scheltinga A.C., Kalk K.H., Beintema J.J., Dijkstra B.W., Crystal structure of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor, *Structure* 2 (1994) 1181–1189.
- [35] Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acid Res.* 25 (1997) 4876–4882.
- [36] Tsujibo H., Orikoshi H., Imada C., Okami Y., Miyamoto K., Inamori Y., Site-directed mutagenesis of chitinase from *Alteromonas* sp. Strain O-7, *Biosci. Biotechnol. Biochem.* 57 (1993) 1396–1397.
- [37] Watanabe A., Nong V.H., Zhang D., Arahira A.,

- Yeboah N.A., Udaka K., Fukazawa C., Molecular cloning and ethylene-inducible expression of *Chib1* chitinase from soybean (*Glycine max* (L.) Merr.), Biosci. Biotechnol. Biochem. 63 (1999) 251–256.
- [38] Wemmer T., Kaufmann H., Kirch H.H., Schneider K., Lottspeich F., Thompson R.D., The most abundant soluble basic protein of the stylar transmitting tract in potato (*Solanum tuberosum*) is an endochitinase, Planta 194 (1994) 264–273.
- [39] Yeboah N.A., Arahira M., Nong V.H., Zhang D., Kadokura K., Watanabe A., Fukazawa C., A class III acidic endochitinase is specifically expressed in the developing seeds of soybean (*Glycine max* [L.] Merr.), Plant Mol. Biol. 36 (1998) 407–415.