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Short communication

β-1,3-Glucanase is highly-expressed in laticifers of *Hevea brasiliensis*

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Abstract

Clones encoding β-1,3-glucanase have been isolated from a *Hevea* cDNA library prepared from the latex of *Hevea brasiliensis* using a probe *Nicotiana plumbaginifolia* cDNA encoding β-1,3-glucanase, *gnl*. Nucleotide sequence analysis showed that a 1.2 kb *Hevea* cDNA encoding a basic β-1,3-glucanase showed 68% nucleotide homology to *gnl* cDNA. Northern blot analysis using the *Hevea* cDNA as probe detected a mRNA of 1.3 kb which was expressed at higher levels in latex than in leaf. *In situ* hybridization analysis using petiole sections from *Hevea* localized the β-1,3-glucanase mRNA to the laticifer cells. Genomic Southern analysis suggested the presence of a low-copy gene family encoding β-1,3-glucanases in *H. brasiliensis*.

Multiple isoforms of β-1,3-glucanases have been isolated from plants [14]. These various forms can be divided into four different classes based on mRNA expression profile, protein isoelectric point and sequence homology. Class I β-1,3-glucanases are pathogen-inducible, basic, vacuolar proteins that are expressed in roots and older leaves [1, 2, 9, 12, 15, 21]. Class II, Class III and Class IV β-1,3-glucanases are acidic proteins. Class II and Class III isoforms are induced upon pathogen attack but Class IV isoforms are non-responsive [17, 18, 23].

Hevea brasiliensis, which belongs to the family Euphorbiaceae, is commercially grown for the production of natural rubber. This unique isoprenoid compound, *cis*-1,4-polyisoprene (M_r 4×10^6) [24], is present in latex, the milky cytoplasm of specialized plant cells called laticifers which are located adjacent to phloem vessels. The articulated laticifers of *Hevea*, which form a tube-like network through the plant contain all the normal cell constituents plus rubber particles and characteristic organelles (lutoids and Frey-Wyssling particles). During tapping the laticifers are severed and their cytoplasm, the latex, is expelled. It has previously been observed that the mRNAs encoding 3-hydroxy-3-methyglutaryl coenzyme A reductase [3] and the rubber elongation factor [7], two enzymes involved in the biosynthesis of natural rubber, are highly expressed in laticifers. Using heterologous hybridization probes and northern analysis, it has been shown

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession number U22147 (*Hevea brasiliensis* β-1,3-glucanase cDNA).

that plant defence protein genes including chitinase, chalcone synthase and phenylalanine ammonia-lyase show higher expression in laticifer than in leaf [10]. The latex of *Hevea* has also been found to contain high levels of the plant defence proteins, chitinases and chitinases/lysozymes [13]. Since β-1,3-glucanase has also been implicated in plant defence and its expression in *Hevea* has not yet been investigated, we have initiated a study on the expression of β-1,3-glucanases in *Hevea*.

N. plumbaginifolia cDNA encoding β-1,3-glucanase, *gnl* cDNA [5] (a gift of Dr Dirk Inze), was used as a heterologous hybridization probe to isolate the corresponding cDNA clones from *Hevea*. A cDNA library prepared from *Hevea* latex (gift of Drs S. Sivasubramaniam, V. Vanniasingham and Nam-Hai Chua) was screened by *in situ* plaque hybridization at 42 °C in a solution containing 30% formamide. Several putative *Hevea* cDNA clones encoding β-1,3-glucanase were isolated. Nucleotide sequence analysis carried out on two of the longest clones of 1.2 and 1.1 kb showed that they belonged to the same class. The full-length 1.2 kb cDNA consists of a 40 bp 5′-untranslated region, a 1125 bp coding region, a 76 bp 3′-untranslated region and a poly(A) tail. The coding region encodes a 374 amino acid basic protein with a predicted M_r 41305.

The nucleotide sequence of *Hevea* β-1,3-glucanase shows 68% nucleotide sequence identity to that of the *N. plumbaginifolia gnl* cDNA. Comparison of the predicted amino acid sequence of *Hevea* β-1,3-glucanase with that of the Class I β-1,3-glucanase encoded by *gnl* shows 66% amino acid homology (Fig. 1). *Hevea* β-1,3-glucanase has 54%, 60% and 51% amino acid identity to Class II (*N. tabacum* PR-N [11]), Class III (*N. tabacum* ec321391 [18]) and Class IV (*N. tabacum* sp41a [17]) β-1,3-glucanases, respectively (Fig. 1).

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                                                                    47
Hb    MAISSSTSGTS-SSFPSRTTVMLLLFFFAASVGITDAQ--VGVCYGMQGN
Np          MD..HKHIALQMAAII..GLLVS.TE.VG..S-.......L..
CII       M.LCIK-NG.L--AAALV.VGLLIC.IQMIG..S-I.....KHA.
CIII            MAHLIV.LL..SVLTL.TLDF.G..--A.....R...
CIV     MALWYLFNKR.LGA--A.LI.VGLLMCNIQM.G..SNI.....KIA.
                                                                    97
Hb    NLPPVSEVIALYKKSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDLQ
Np    ....A.Q.VQ...SK..R...L....Q.A.Q........VM.........
CII   ...SDQD..N..NANG.RK....N.DTN.FN.........I..D..LQ...
CIII  G..SPAD.VS.CNRN..R.......DQPT...........M.....P..E
CIV   ...SEQD..N...ANG.RK....NSDTNIFKS.N.....I..D...Q..E
                                                                   146
Hb    SL-TNPSNAKSWVQKNVRGFWSSVLFRYIAVGNEISPVNRGTAWLAQFVL
Np    NIAA.....NN...R...N..PA.K.........V...T-..SS.TRYL.
CII   ..-.D..R.NG...D.IINHFPD.K.K.......V..G.N.Q--Y.P..A
CIII  NVAASQA..DT...N...NY-GN.K.........V..L.ENSKYVP-VL.
CIV   A.-A.S.I.NG...D.I.SHFPY.K.K..SI...V..S.N.Q---YS..L.
                                                                   196
Hb    PAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLD
Np    ......RN..S.....NN....SSV.M..I...F...Q.S..N....FI.
CII   ...Q.VYN.LAA.............TYSGILA.T...KDSI..GEFN.FIN
CIII  N.....QT..SG...GN........ETG.TTDTS...N.R.K....QFIE
CIV   H..E.VYN.LAA.....K...T..TYSG.LA.T...KDSI..EEFK.FIN
                                                                   246
Hb    PIIGFLSSIRSPLLANIYPYFTYAYNPRDISLPYALFTSPSVVVWDGQRG
Np    .....VRR.N....V......S..G.............A.N...Q..SL.
CII   ...Q..VQHNL.....V....GHIF.TA.VP.S.....QQE----ANPA.
CIII  ...N..VTN.A...V.L....AI.N.A-..K.E......SE...N.NG..
CIV   ...E..ARNNL..........GHI..TV.VP.S....NQQG----TNST.
                                                                   295
Hb    YKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAAT-FDNGRTY
Np    .R.....MS..V.A..S..G...I.I.................-TN.AA..
CII   .Q.....L..SM.F.V.K.G.QNV.II........E.NS..T-IE.AQ..
CIII  .R.....I...T.....K...S...I.............GQL.SI..A...
CIV   .Q.....L..SI.F.V.K.G.PNV.II........E.NS...-IE.AQ..
                                                                   342
Hb    LSNLIQHVK--GGTPKRPNRAIETYLFAMFDENKKQPEV-EKHFGLFFPD
Np    YK........--R.S.R...KV.............N.N..L-.......S.N
CII   YE...N...SGA....K.GK..............N.EGDIT.......S..
CIII  NN...S...--..S....SGP....V..L...DQ.D..I-.......SAN
CIV   YR..VN...GGA....K.G.IV............E.NG..T.......Y.N
                                                                   374
Hb    KRPKYNLNFG-AEKNWDISTEHNATILFLKSDM
Np    .Q...P.S..FSDRY....A.N...AAS.I.E.
CII   Q.A..Q...N
CIII  MQ...QIS.N
CIV   RTA..Q...MYS
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Fig. 1. Comparison of the deduced amino acid sequences of *Hevea* (Hb) β-1,3-glucanase and that of *N. plumbaginifolia* (Np) *gnl* [5], Class II (CII [11]), Class III (CIII [18]) and Class IV (CIV [17]) β-1,3-glucanases. Positions of identity are denoted by dots. The putative N-glycosylation sites in *Hevea* β-1,3-glucanase are marked with asterisks. The predicted N-terminal extension and C-terminal extension of *Hevea* β-1,3-glucanase are overlined and underlined, respectively.

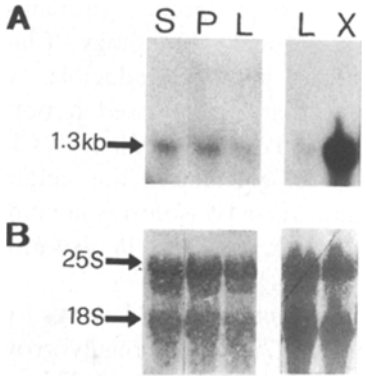

Fig. 2. A. Northern blot analysis. Total RNA (20 μg) from stem (lane S), petiole (lane P), leaf (lane L) and latex (lane X) were hybridized to the 1.2 kb *Hevea* β-1,3-glucanase cDNA. B. The same RNA blot were stained with methylene blue following the procedure of Sambrook *et al.* [19]. 25S and 18S ribosomal RNA bands are marked.

Class I β-1,3-glucanases are synthesized as preproteins and the N-terminal extension and C-terminal extension are cleaved during or after transport of the protein to the vacuole [20]. The presence of an N-terminal extension (amino acid residues 1 to 36) and a C-terminal extension (amino acid residues 353 to 374) on the deduced amino acid sequence of *Hevea* β-1,3-glucanase further suggests that it belongs to Class I β-1,3-glucanases (Fig. 1). The N-terminal extension of *Hevea* β-1,3-glucanase consists of a region (amino acid residues 4 to 19) enriched in serine and

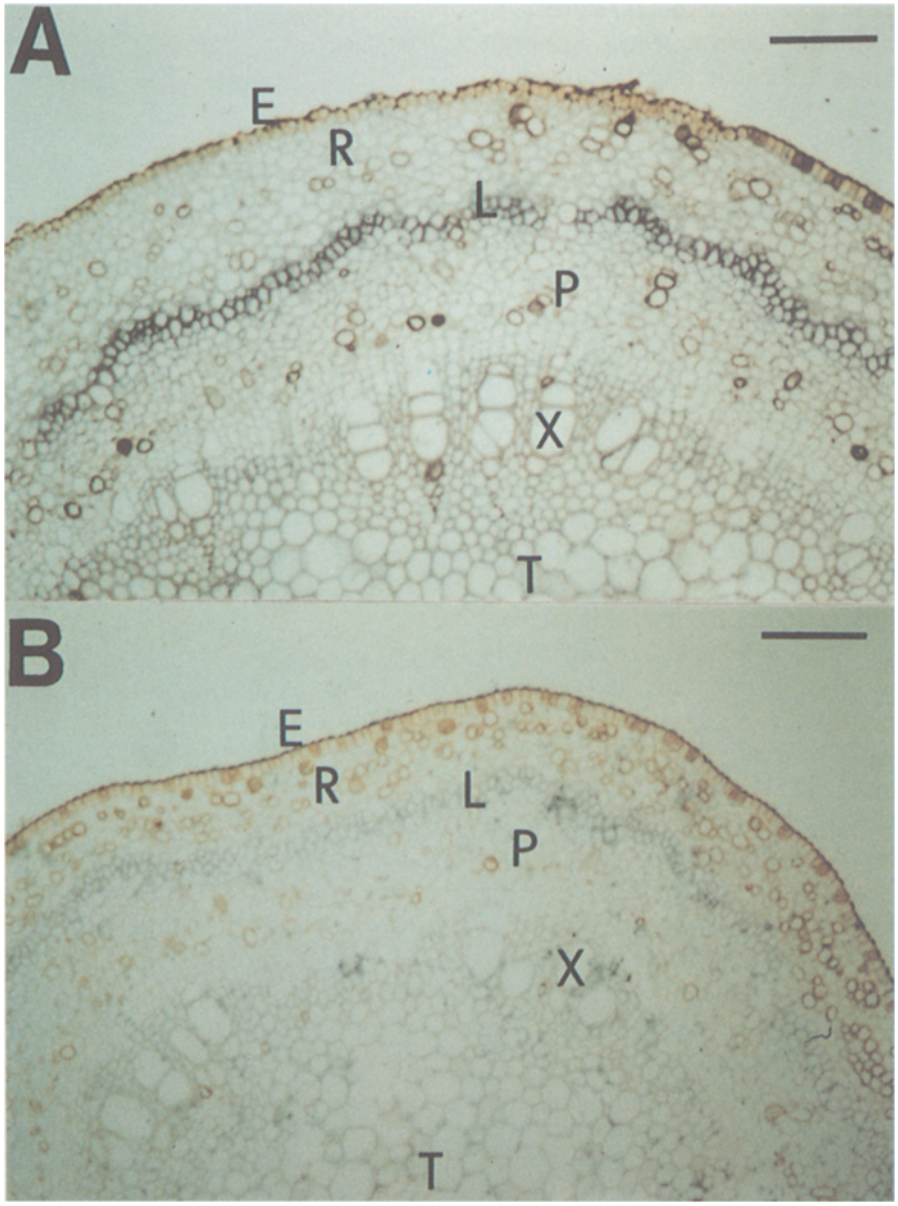

Fig. 3. Localization of β-1,3-glucanase mRNA in transverse sections of *Hevea* petiole. A. Light micrograph of section hybridized with the β-1,3-glucanase antisense probe. B. Light micrograph of section hybridized with the β-1,3-glucanase sense probe. E, epidermis; R, cortex; L, laticifer; P, phloem; X, xylem; T, pith. Bar represents 200 μm.

threonine residues, followed by a hydrophobic region (amino acid residues 22 to 29) (Fig. 1). Although there is no significant amino acid sequence homology between the N-termini of *Hevea* β-1,3-glucanase and *gnl*-encoded β-1,3-glucanase, they both consist of a hydrophobic region typical of signal peptides [22] and is believed to be involved in protein targeting to the vacuole. Interestingly the N-terminal sequence of the propeptide of barley aleurain which has been shown be responsible for directing the protein to the vacuole is also rich in serine residues [8]. Comparison of the C-terminal extension of *Hevea* with that of *N. plumbaginifolia* shows that there is some conservation in amino acid sequence and in the putative N-glycosylation site (amino acid 364 in *Hevea* β-1,3-glucanase). The C-terminal extension, particularly amino acid residues 365 to 370 in *Hevea*, is rich in hydrophobic amino acids. It has been suggested that a hydrophobic/acidic motif structure, rather than the specific amino acid sequence forms a sorting signal in carboxy-extension propeptides [16]. It has been established that the C-terminal extension and N-glycan of Class I isoforms of β-1,3-glucanase are removed during processing [20].

The expression of *Hevea* β-1,3-glucanase was examined by northern blot analysis and *in situ* hybridization studies. Total cell RNA was isolated from stems, petioles and leaves of *Hevea* plants following the procedure of Nagy *et al.* [15]. The northern blot was hybridized to the ^{32}P-labelled 1.2 kb *Hevea* β-1,3-glucanase probe at 42 °C in a solution containing 50% formamide. The blot was washed at 65 °C in 0.1 × SSC, 0.1% SDS. A 1.3 kb mRNA was found to hybridize to this probe and it was more highly expressed in stem and petiole than leaf (Fig. 2). In order to investigate whether its relatively lower expression in leaf is related to the presence of fewer laticifer cells in leaf than in stem and petiole we extracted total cell RNA from laticifers and compared the expression of *Hevea* β-1,3-glucanase in laticifer and leaf. We observed that β-1,3-glucanase was expressed abundantly in laticifer compared to leaf (Fig. 2).

In situ hybridization studies were carried out using a digoxigenin-labelled RNA probe to examine the distribution of β-1,3-glucanase mRNA in petiole sections of *Hevea*. These experiments were carried out following the conditions described by Cox and Goldberg [4]. The *Hevea* β-1,3-glucanase cDNA was cloned into pBluescript SK – (Stratagene). The plasmid derivative was cleaved with either *Bam* HI or *Hin*d III to generate antisense or sense RNA probes, which were synthesized *in vitro* using T7 RNA polymerase and T3 RNA polymerase, respectively. The antisense and sense probes were hybridized with 6 μm thick petiole sections overnight at 40 °C. Sections were washed and digoxigenin-labelled RNA probes were detected after hybridization by an

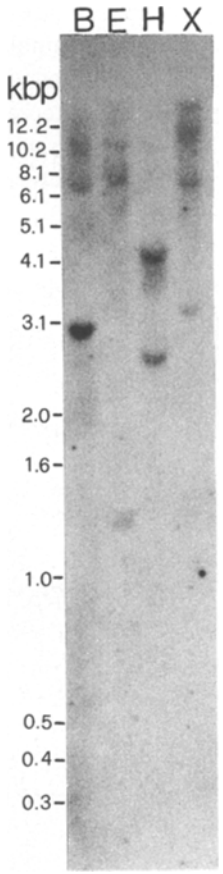

Fig. 4. Genomic Southern analysis. *Hevea* genomic DNA (20 μg) was digested with *Bam* HI (B), *Eco* RI (E), *Hin*d II (H) and *Xba* I (X), separated by gel electrophoresis, blotted onto Hybond N (Amersham) membrane and hybridized with a ^{32}P-labelled *Hevea* β-1,3-glucanase cDNA probe.

alkaline phosphatase-linked immunoassay (Boehringer-Mannheim). This assay uses a colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) which produces a purple precipitate. Slides were mounted with GelTol aqueous mounting medium (Immunon) and were examined with a microscope. We observed that the laticifer cells of the *Hevea* petiole were stained purple and concluded that the expression of β-1,3-glucanase is localized to these cells almost exclusively (Fig. 3).

The laticifer-specific cDNA we have isolated was used in genomic Southern blot analysis to investigate the presence of a β-1,3-glucanase gene family in *Hevea*. Genomic DNA was obtained from young leaves following the procedure of Dellaporta *et al.* [6]. Total genomic DNA was restricted with *Bam* HI, *Eco* RI, *Hind* II and *Xba* I, electrophoresed and blotted. Southern blot analysis using the 1.2 kb *Hevea* β-1,3-glucanase probe showed that there were 2–4 hybridizing bands with each digest (Fig. 4). These results suggest that a low-copy gene family of β-1,3-glucanase is present in *Hevea*.

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