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THE ABUNDANT 31-KILODALTON BANANA PULP PROTEIN IS HOMOLOGOUS TO CLASS-III ACIDIC CHITINASES

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Key Word Index—Banana; *Musa acuminata* cv. Grand Nain; Musaceae; fruit ripening; 31 kDa pulp protein; chitinase; storage protein.

Abstract—We have identified and characterized the abundant protein from the pulp of banana fruit (*Musa acuminata* cv. Grand Nain), and have isolated a cDNA clone encoding this protein. Comparison of the amino terminal sequence of the purified 31 kDa protein (P31) suggests that it is related to plant chitinases. Western analyses utilizing rabbit anti-P31 antiserum demonstrate that this protein is pulp-specific in banana. A full-length cDNA clone homologous to class III acidic chitinase genes has been isolated from a pulp cDNA library by differential screening. The identity of this clone as encoding P31 was verified by comparisons between the amino-terminal peptide sequence and the cDNA sequence and cross-hybridization of the translation product of the cDNA clone with P31 antiserum. Northern and western blot analyses of RNA and protein isolated from banana pulp at different stages of ripening indicate that the cDNA and protein are expressed at high levels in the pulp of unripe fruit, and that their abundance decreases as the fruit ripens. Based on its expression pattern and deduced amino acid sequence and composition, we hypothesize that the physiological role of P31 is not for plant protection, but as a storage protein in banana pulp.

INTRODUCTION

Fruit ripening is a complex metabolic process involving changes in colour, flavour, texture and aroma that are catalysed by highly regulated specific enzyme activities. The onset of ripening involves the expression of specific genes. It has become apparent over the last few years that the ripening of fruit is a complex process that involves several catabolic and anabolic pathways. This is reflected by the number of genes isolated recently from various fruit during development and ripening. Many encode enzymes not previously suspected to be associated with ripening such as some proteinase inhibitors [1], stress-related enzymes [2], β-oxidation pathway enzymes [3], metabolic-detoxifying enzymes [4] and chitinases [1]. We have used differential screening of a banana pulp cDNA library to isolate of a number of ripening-associated genes, including several stress- and pathogenesis-related proteins including chitinase [5].

Although chitinases are abundant proteins found in a wide variety of plants, the presence of chitin has not been reported in higher plants. Since chitin is the major structural component of fungal cell walls, it has been proposed that chitinases serve as defense proteins with antifungal activity. Chitinases are reported to be induced in higher plants by a number of different types of stress [6–8]. Many plant chitinases are expressed constitutively, although at a low level [8]. Some evidence exists for the developmental regulation of chitinase expression in specific tissues and at defined stages during plant development. The biological significance of these chitinases has yet to be elucidated, and they may have as yet undetermined functions in plant development [8].

Clarence Ryan and his colleagues have pioneered the studies of plant responses to biological stress [9, 10]. His research has stimulated many laboratories to identify genes which are expressed in response to pathogen attack. In our own studies of fruit development and ripening, we unexpectedly returned to this field of interest when we found a pathogenesis-related gene product with an apparent association with banana fruit development. An abundant 31 kDa protein (P31) is present in banana pulp during devel-

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opment and early ripening, and this report details the characterization of P31 and its corresponding cDNA clone, pBAN3-30, and their homology with other plant class III acidic chitinases. While chitinases are generally associated with a pathogenesis-related function in plants, we report the association of this abundant protein with fruit ripening and hypothesize a potential role as a storage protein in banana pulp.

RESULTS AND DISCUSSION

P31 isolation and tissue distribution

SDS-PAGE analysis of total proteins isolated from pulp of banana fruit at seven ripening stages indicated changes in abundance of several proteins (Fig. 1). The most abundant protein during the pre-climacteric stage (Peel Colour Index or PCI 1 and 2) is a 31 kDa protein (P31) which seemed to decrease in abundance as ripening proceeded (Fig. 1). This protein (P31) was partially purified by a combination of ammonium sulphate precipitation and separation by SDS-PAGE. Polyclonal antiserum was raised against the purified protein. The P31 antiserum recognizes a single 31 kDa polypeptide in banana pulp that is not present in banana peel, meristem, corm, or root tissue (Fig. 2). These results indicate that P31 is fruit-specific.

The N-terminus of the partially purified protein was sequenced and the resultant 20-amino acid sequence is: GRNSCIGVYWGQKTDEGSLA (data also appears in Fig. 5). A search of the amino acid sequence database (GenBank) revealed that the N-terminus of P31 shares some homology to amino-terminal peptide sequences from purified acidic chitinases of Mongolian snake-gourd (*Trichosanthes kirilowii* [11]) and chick pea (*Cicer arietinum* [12]).

P31 expression in ripening pulp

P31 expression in banana pulp during ripening was investigated at the protein and transcript levels. Western blot analysis of banana pulp proteins isolated at each of seven chronological stages of ripening (Fig. 3, top panel) indicates that P31 decreases in relative abundance during ripening, consistent with the observations of P31 abundance after separation by SDS-PAGE and staining with CooMassie Blue. Using differential screening, several ripening-associated genes were isolated from a banana pulp cDNA library, including clones with significant homology to chitinases [5]. For determination of relative chitinase transcript abundance during ripening, total RNA was isolated from banana pulp during ripening, at PCI 1 through 7, and probed with labelled pBAN3-30. Northern blot analysis of (Fig. 3, bottom panel) shows that the P31 message is strongly expressed during the first few ripening stages (PCI 1–3) after which the putative chitinase transcript declines in banana pulp through the later stages of ripening. This observation is consistent with the result obtained through western analysis. Northern and western blot analysis together suggest that expression of P31 is both fruit-specific and developmentally regulated in banana. While both the P31 protein and the putative chitinase transcript are abundant during the pre-climacteric stages of fruit ripening (PCI 1–3), their relative levels decrease as ripening progresses.

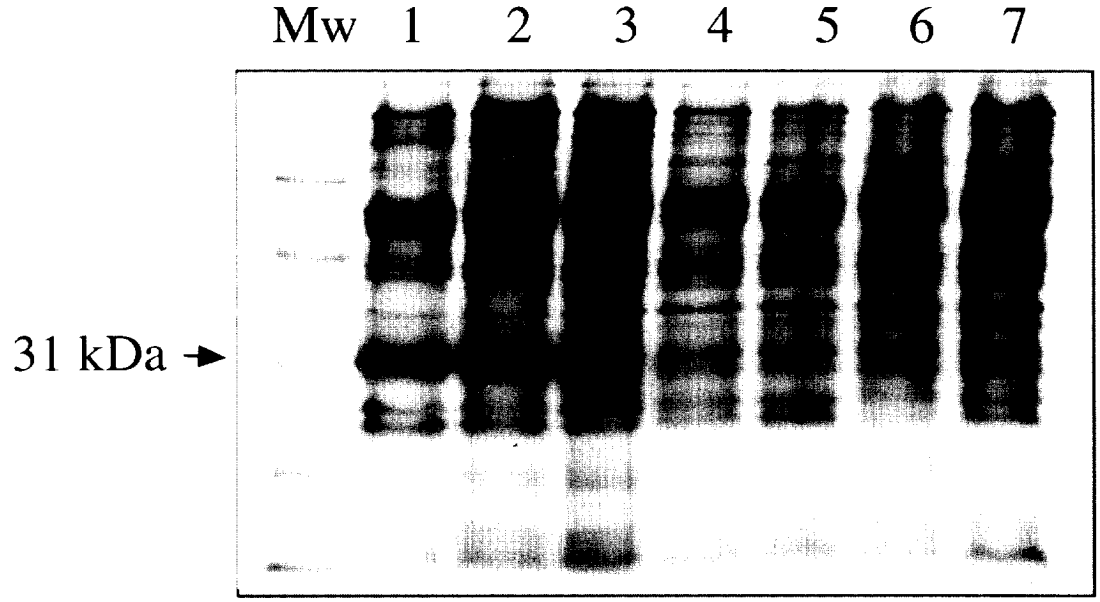

Fig. 1. Total banana pulp protein extract at different stages of ripening, separated by SDS-PAGE and stained with CooMassie Blue. Protein profiles during ripening show the presence of an abundant protein of 31 kDa that decreases in relative abundance during ripening.

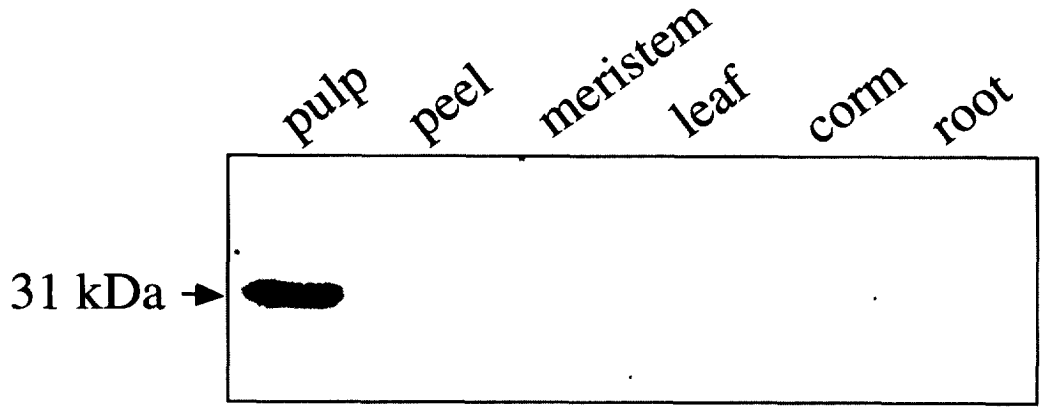

Fig. 2. Western blot analysis of total soluble protein extracted from different banana tissues and hybridized with polyclonal antiserum against purified P31. The antiserum detects a 31 kDa protein in pulp which is not present in peel, meristem, leaf, corm, or root tissue.

pBAN3-30 encodes P31

Three lines of evidence lead us to conclude that pBAN3-30 may encode the abundant 31 kDa pulp protein. First, the expression pattern of the pBAN3-30 transcript during ripening matches very closely with the profile of P31 abundance during ripening as determined by western blot analysis using the P31 antiserum, as seen in Fig. 3. Second, the P31 antiserum recognizes the translation product of the chitinase cDNA insert. The translation products of the cDNA clones pBAN3-36 and pBAN3-45, which are homologous to pBAN3-30 but have been determined to be in-frame with respect to the β-galactosidase gene in the pBluescript cloning vector (Stratagene), were expressed as fusion proteins with β-galactosidase. These fusion proteins were analysed by western blotting and incubation with the P31 antiserum. The P31 antiserum recognizes a 35 kDa polypeptide produced in the IPTG-induced bacterial cells containing an in-frame chitinase cDNA (pBAN3-36 and pBAN3-45) that is not present in cell extracts from bacteria containing only the pBluescript plasmid (no insert) or out-of-frame chitinase cDNA inserts (pBAN3-30 and pBAN3-31) (Fig. 4). Finally, the N-terminal amino acid sequence obtained from the purified protein, which is underlined in Fig. 5, reveals some homology to the deduced amino acid sequence of pBAN3-30 [i.e. 17 of 20 residues are identical]. This match is improved when the first amino acid residue, which is usually considered to be uncertain, is discounted. Despite the high sequence homology, the amino acid sequence from the partially purified protein is not identical to the amino acid sequence deduced from the cDNA clone pBAN3-30. Although is possible that a contaminating polypeptide co-migrated with p31 and influenced the amino acid sequence results, it cannot be discounted that P31 is encoded by a gene family

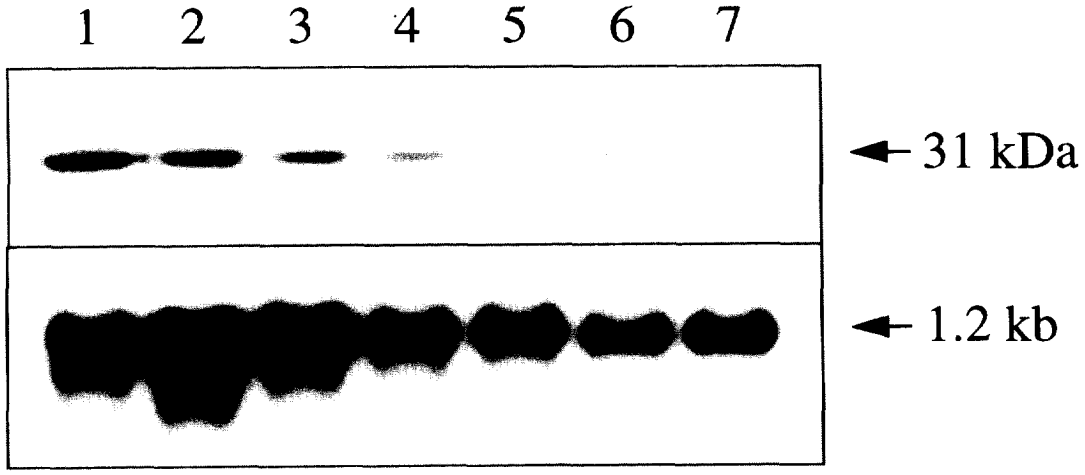

Fig. 3. Expression of P31 (top panel) and pBAN3-30 (bottom panel) in banana pulp during ripening. Total protein and RNA were isolated from banana pulp at each of seven stages of banana fruit ripening (PCI 1 through 7, numbered at top of figure). Pulp proteins (20 μg per lane) were separated by SDS-PAGE and hybridized with the P31 antiserum. Total RNA (10 μg per lane) was separated by agarose gel electrophoresis and transferred to nylon membrane, and hybridized with a ^{32}P-labelled banana chitinase cDNA probe (pBAN3-30). Both the P31 protein and the corresponding chitinase transcript at 1.2 kilobases are abundant in pulp during the early stages of ripening but decrease as ripening progresses.

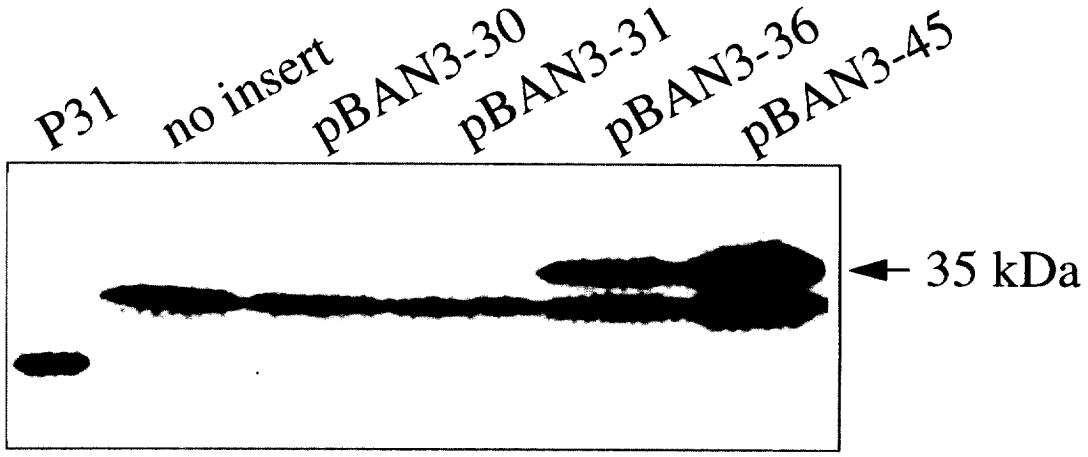

Fig. 4. Western blot analysis of the translation products of four banana chitinase cDNA clones homologous to pBAN3-30 expressed as fusion proteins with β-galactosidase in pBluescript and hybridized with P31 antiserum. The polyclonal antiserum recognizes a 35 kDa polypeptide in bacterial cultures containing in-frame cDNA inserts (pBAN3-36 and pBAN3-45) that is not present in bacterial cells containing either the pBluescript cloning vector without an insert (no insert) in chitinase cDNA inserts that are not in-frame with the β-galactosidase gene (pBAN3-30 and pBAN3-31).

```
pBAN3-30

TTTGGTTGTGCCTAACAGAGAGAGAGAGACAGACCGATAGCCTCCTCATT  50
CACTATGGCGATCCGATCGCCAGCTTCGCTGCTGTTATTTGCGTTCCTGA 100
    M  A  I  R  S  P  A  S  L  L  L  F  A  F  L
TGCTTGCGCTCACGGGAAGACTGCAGGCCCGGCGCAGCTCATGCATTGGC 150
M  L  A  L  T  G  R  L  Q  A  R  R  S  S  C  I  G
                        G  R  N  S  C  I  G
GTCTACTGGGGACAAAACACCGACGAGGGAAGCTTAGCAGATGCTTGTGC 200
 V  Y  W  G  Q  N  T  D  E  G  S  L  A  D  A  C  A
 V  Y  W  G  Q  K  T  D  E  G  S  L  A
CACAGGCAACTACGAATACGTGAACATCGCCACCCTTTTCAAGTTTGGCA 250
  T  G  N  Y  E  Y  V  N  I  A  T  L  F  K  F  G
TGGGCCAAACTCCAGAGATCAACCTCGCCGGCCACTGTGACCCTCGGAAC 300
M  G  Q  T  P  E  I  N  L  A  G  H  C  D  P  R  N
AACGGCTGCGCGCGCTTGAGCAGCGAAATCCAGTCCTGCCAGGAGCGTGG 350
 N  G  C  A  R  L  S  S  E  I  Q  S  C  Q  E  R  G
CGTCAAGGTGATGCTCTCCATCGGAGGTGGCGGGTCTTATGGCCTGAGTT 400
  V  K  V  M  L  S  I  G  G  G  G  S  Y  G  L  S
CCACCGAAGACGCCAAGGACGTAGCGTCATACCTCTGGCACAGTTTCTTG 450
S  T  E  D  A  K  D  V  A  S  Y  L  W  H  S  F  L
GGTGGTTCTGCTGCTCGCTACTCGAGACCCCTCGGGGATGCGGTTCTGGA 500
 G  G  S  A  A  R  Y  S  R  P  L  G  D  A  V  L  D
TGGCATAGACTTCAACATCGCCGGAGGGAGCACAGAACACTATGATGAAC 550
  G  I  D  F  N  I  A  G  G  S  T  E  H  Y  D  E
TTGCCGCTTTCCTCAAGGCCTACAACGAGCAGGAGGCCGGAACGAAGAAA 600
L  A  A  F  L  K  A  Y  N  E  Q  E  A  G  T  K  K
GTTCACTTGAGTGCTCGTCCGCAGTGTCCTTTCCCGGATTACTGGCTTGG 650
 V  H  L  S  A  R  P  Q  C  P  F  P  D  Y  W  L  G
CAACGCACTCAGAACAGATCTCTTCGACTTCGTGTGGGTGCAGTTCTTCA 700
  N  A  L  R  T  D  L  F  D  F  V  W  V  Q  F  F
ACAACCCTTCGTGCCATTTCTCCCAGAACGCTATCAATCTTGCAAATGCG 750
N  N  P  S  C  H  F  S  Q  N  A  I  N  L  A  N  A
TTCAACAATTGGGTCATGTCCATCCCTGCGCAAAAGCTGTTCCTTGGGCT 800
 F  N  N  W  V  M  S  I  P  A  Q  K  L  F  L  G  L
TCCTGCTGCTCCTGAGGCTGCTCCAACTGGTGGCTACATTCCACCCCATG 850
  P  A  A  P  E  A  A  P  T  G  G  Y  I  P  P  H
ATCTCATATCTAAAGTTCTTCCGATCCTAAAGGATTCCGACAAGTACGCA 900
D  L  I  S  K  V  L  P  I  L  K  D  S  D  K  Y  A
GGAATCATGCTGTGGACTAGATACCACGACAGAAACTCCGGCTACAGTTC 950
 G  I  M  L  W  T  R  Y  H  D  R  N  S  G  Y  S  S
TCAAGTCAAGTCCCACGTGTGTCCAGCGCGTCGGTTCTCCAACATCTTAT 1000
  Q  V  K  S  H  V  C  P  A  R  R  F  S  N  I  L
CTATGCCGGTGAAGTCTTCCAAGTAAACCTGAACGGCGTAGATGATCGGT 1050
S  M  P  V  K  S  S  K  *
GGTCGAAAACTCCGATCATCATGGGTCCCCATCCGTATCCGTGCGTTGCT 1100
ACGTTATGGTGTTTCCCTTGTATGTTGGTCTTTTCAATAATATAATAAGG 1150
GGTTAGTTTTACGTTTCCAAAAAAAAAAAAAAAAA                 1186
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Fig. 5. Complete nucleotide sequence of the cDNA clone pBAN3-30 and deduced amino acid sequence of the pBAN3-30 translation product. The N-terminal amino acid sequence obtained from purified P31 is aligned with the translation product and underlined, and is identical to the deduced amino acid sequence of pBAN3-30 at 17 of 20 residues. The translation initiation codon ATG starting at position 55 of pBAN3-30 is underlined as well as the in-frame stop codon at position 1024. Other features of the cDNA sequence include several putative polyadenylation signals between positions 1136 and 1148 (underlined).

in banana, members of which are high homologous, though not identical, and cannot be distinguished from one another by northern or western analyses.

Sequence analysis of pBAN3-30

The complete nucleotide sequence of pBAN3-30 and the deduced amino acid sequence of the translation product are shown in Fig. 5. The cDNA insert is 1186 bp long and includes the entire chitinase coding region. The ATG beginning at position 55 is likely to be the translation initiation codon because the nucleotide sequence flanking the first ATG codon matches 8 of the 12 bases in the consensus for translation start sites in plants [13] whereas the sequences flanking another potential in-frame downstream start site (at position 100) is identical only at 5 of the 12 bases. There is an in-frame termination codon at position 1024 and several putative polyadenylation signals between positions 1136 and 1148.

The open reading frame spans 323 amino acids from which a translation product of 35 232 Da is predicted. A GenBank search using the full cDNA sequence reveals significant homology between pBAN3-30 and chitinase genes characterized from winged bean (*Psophocarpus tetragonolobus*, M. Esaka and T. Teramoto, unpublished), cow pea (*Vigna unguiculata*, L.T.T. Vo *et al.* unpublished), azuki bean (*Vigna angularis* [14]), maize (*Zea mays* [15]), and chick pea (*Cicer arietinum* [16]). The deduced amino acid sequence of pBAN3-30 encoding P31 in banana shares sequence homology with other plant chitinases, especially with class III acidic chitinases that have been characterized from various dicots. At the amino acid level, the banana acidic chitinase amino acid sequence shows significant homology, 47–53% identity, to acidic chitinases from

A.

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banana        MAIRSPASLLLFAFLMLALTGRLQARRSSCIGVYWGQNTDEGSL
chick pea           MEKCFNIIPSL.LISLLIKSSNAAG.A......GN....
grape             MARTPQSTPLLIS.SVLAL..TSYAGG.AI.....GN..T.
Arabidopsis   MTNMTLRKHVIY.L.FISCSLSKPSDASRGG.AI.....GN..N.
tobacco             MIKYS.LLTA.V.FL.ALKLEAGD.VI.....GN....
sugar beet        MAAKIVSVLFLIS.LIFASFESSHG.Q.VI.....G.....
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B.

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banana        SDKYAGIMLWTRYHDRNSGYSSQVKSHVCPARRFSNILSMPVKSSK
chick pea     .P..G.V.I.D.FN.AQ....NAI.GS.
grape         .P..G.V...SK.Y.DQ.....SI..S.
Arabidopsis   .R..G.V...SKFW.DKN....SILAS.
tobacco       .P..G.V...SKFY.--N....AI.AN.
sugar beet    .A..G.V...SKAY.--.....AI..S.
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Fig. 6. Amino acid alignments of (A) amino- and (B) carboxy-terminal regions of banana P31 with class III acidic chitinase sequences from chickpea (*Cicer arietinum* [16]), grape (*Vitis vinifera*, Busam *et al.* unpublished). *Arabidopsis thaliana* [17], tobacco (*Nicotiana tabacum* [18]), sugar beet (*Beta vulgaris* [19]). Dots indicates an amino acid residues identical to the banana P31 amino acid sequence on the top line. Dashes indicate a gaps introduced to aid the alignment. (A) Amino-terminal alignment illustrates the lack of sequence homology of the signal peptide sequence of plant chitinases. (B) The carboxy-terminal region indicates the 18 residue C-terminal extension unique to the banana P31 sequence.

Arabidopsis thaliana [17], wine grape (*Vitis vinifera*, Busam *et al.* unpublished), tobacco (*Nicotiana tabacum* [18]), chickpea, sugar beet (*Beta vulgaris* [19]), winged bean, and cucumber (*Cucumis sativus* [20]).

An amino acid sequence alignment of the amino-terminal and carboxy-terminal regions of several plant acidic chitinases with P31 from banana appears in Fig. 6. Hydrophilicity analysis of the deduced protein sequence of P31 reveals a hydrophobic region from amino acid 1 to 25 [underlined in Fig. 6(A)]. This region may represent a signal sequence that would direct targeting to the ER. If this putative signal peptide is removed, the remaining sequence closely matches the N-terminal sequence obtained from the purified protein, which suggests that P31 is post-translationally processed. Importantly, this signal peptide does not share significant homology with the signal peptide sequences of other plant class III acidic chitinases [see Fig. 6(A)], which are typically localized to the extracellular space [7, 8, 18, 21, 22].

In addition to the N-terminal signal peptide, the banana P31 sequence is further distinguished from other chitinase sequences by the presence of an 18 amino acid C-terminal extension [underlined in Fig. 6(B)]. C-terminal propeptides (CTPPs) have been identified in a number of monocot and dicot polypeptides that direct proteins to the plant vacuole. Among others, CTPPs have been characterized in vacuolar lectins from barley and rice, and from vacuolar β-1,3-glucanase and chitinase from tobacco (reviewed in [23]). In general there is little sequence homology among plant vacuolar targeting sequences. However, weak homology can be detected between the C-terminal extension of P31 (SNILSMP) and vacuolar targeting sequences that have been characterized in the sweet potato storage protein sporamin (NPIRLP) [24] and in a 2S albumin from Brazil nut (NLSPMRCP) [25].

Based on amino acid sequences, chitinases can be grouped into four classes. Class I, which include the majority of chitinases described, possess a N-terminal cysteine-rich lectin or 'hevein' (chitin-binding) domain and a highly conserved catalytic domain. Class II chitinases lack the N-terminal cysteine rich domain but have a high amino acid sequence identity to the main structure of class I chitinases. Class III chitinases show little sequence similarity to plant enzymes in class I or II, but may in fact be more similar to bacterial chitinases. The majority of class III chitinases are classified as such on the basis of homology to previously described lysozymes with chitinase activity. Class IV chitinases contain a cysteine-rich domain and conserved main structure which resemble those of class I chitinases but are significantly smaller due to four deletions [7, 8, 21]. Although the banana pulp chitinase shares significant amino acid sequence homology with other plant class III acidic chitinases, the predicted isoelectric point of P31 is 7.63 (neutral). In addition, studies to determine the chitinase active sites in bacterial chitinases appear to be conserved in plant, bacterial and fungal sequences [26]. At least five highly conserved amino acids have been shown to be necessary for chitinase activity, and the deduced amino acid sequence of P31 indicates that only three of the five amino acids necessary for chitinase activity are conserved in banana P31 (not shown) [27, 28].

Role of chitinase in banana pulp

In plants, class III chitinases have been reported to be induced in response to various stresses such as pathogenesis and wounding [14, 18–20, 29]. Recently, it has been reported that the expression of several pathogenesis and stress-related proteins, including chitinases, is associated with fruit ripening. A transcript encoding endochitinase has been detected in avocado during normal fruit ripening [1], and several genes encoding pathogenesis-related proteins such as endochitinase are associated with ripening in banana pulp [5]. High activity of exochitinase activity in pathogen-free unripe kiwifruit suggests that expression is developmentally regulated rather than pathogen-induced [30]. In cherry, it was determined that a very abundant protein in ripening fruit was homologous with thaumatin-like protein [31], and in tomato, a thaumatin-like protein with antifungal activity has been associated with fruit development and ripening [32]. Considering the antifungal activity that they exhibit in other plants, it is possible that these putative chitinases fulfill a protective role during fruit development and ripening. However, in contrast to the ripening-associated PR-proteins studied in cherry, avocado, and tomato, banana P31 decreases in abundance during ripening. Although it is possible

that the putative banana chitinase serves a protective role during fruit development, an alternate hypothesis is that it serves as a storage protein in this tissue.

Storage proteins are a heterogeneous group of proteins for which no defined assay is available. According to a recent review [33], storage proteins generally share the features listed below; we relate traits of P31 to general features of storage proteins.

(1) *Storage proteins are very abundant.* We have found P31 to be very abundant in unripe banana pulp, accounting for approximately 20 to 30% of total soluble pulp protein. (2) *Storage proteins are preferentially degraded during a subsequent developmental stage.* For example, a vegetative storage protein characterized from the bark of poplar trees accumulates during fall and winter and is degraded during shoot growth in the spring. P31 is preferentially degraded during banana fruit ripening. Both the transcript and protein abundance decrease during ripening. If P31 is indeed a storage protein in banana pulp, this preferential degradation implies the existence of a protease specific to the storage protein, and inhibition of the protease would inhibit degradation of the storage protein. (3) *Storage proteins are generally localized in storage vacuoles within the cell.* The sub-cellular localization of P31 has not yet been determined. According to the deduced amino acid sequence of P31, there is a putative signal peptide sequence for P31 that is 25 amino acids long and it is hydrophobic. In addition, the amino acid sequence of P31 from banana pulp is distinguished from other plant class III acidic chitinases by the presence of an 18 amino acid C-terminal extension that shows some homology to previously characterized C-terminal vacuolar targeting signals, suggesting vacuolar localization of P31. (4) *Many storage proteins contain a large proportion of amino acid residues with nitrogen-containing R-groups.* Amino acid composition of P31 indicates that 22% of residues have N-containing R-groups [Trp, (W), Gln (Q), Asn (N), Lys (K), Arg (R), His (H)]. This is approximately the same proportion of N-containing amino acids in vegetative storage proteins from soybean and poplar (21 to 25%). Interestingly, the amino acid composition of P31 is not significantly higher than the N- of other plant chitinases (17 to 23%). (5) *Storage proteins typically lack any other metabolic or structural role.* However, this is not necessarily true for soybean vegetative storage protein, which has retained a minimal acid phosphatase activity, and patatin, a potato tuber storage protein that exhibits residual lipid acyl hydrolase activity. Preliminary evidence suggests that protein extracts from banana pulp have very low chitinase activity, as measured by soluble chitobiose released from radiolabelled chitin. In addition, only three of the five amino acids which have been determined to be essential for chitinase activity are conserved in P31. Taken together, this evidence lends support to the hypothesis that P31, while sharing sequence homology with plant chitinases, may actually be serving as a storage protein in banana pulp.

EXPERIMENTAL

Plant materials. Ethylene treated and untreated banana fruit (*Musa acuminata* cv. Grand Nain) were obtained from the Northside Banana Company (Houston, TX). The pulp and peel of fruit representing each of the seven stages of ripening (PCI 1 through 7) were sepd and quick-frozen in liquid N_2. Tissues from 10 individual fruit were pooled to obtain a uniform representative sample for each ripening stage and ground to a fine powder under liquid N_2 in a stainless steel Waring blender. Ground samples were stored at -80° until utilized. Other banana tissues were obtained from green house-grown Grand Nain plants.

Protein isolation for antiserum production, N-terminal sequencing, and western blotting. Soluble banana pulp proteins were differentially pptd from pulp extracts with ammonium sulphate. P31 was most abundant in the 40 to 60% ammonium sulphate fr., as determined by SDS-PAGE sepn [34] followed by CooMassie Blue staining [35]. The 31 kDa protein band was excised from the gel, homogenized and used to immunize a rabbit for antiserum production, according to standard protocols. In addition, proteins from the 40 to 60% ammonium sulphate fr. were sepd by SDS-PAGE and transferred PVDF protein sequencing membrance and stained with Coommassie blue. The stained 31 kDa protein band was excised from the membrane and the N-terminal sequence was determined.

Total protein (20 μg per lane) isolated from banana root, corm, leaf, meristem, peel and pulp at different stages of ripening were sepd by SDS-PAGE and electrophoretically transferred to PVDF membrane. The membranes were incubated with the primary antiserum at a 1:500 dilution, and the bound antibodies were visualized using chemiluminescence.

Northern blot analysis. Total RNA was extracted from banana leaf, corm, root, peel, meristem, and floral structures and from banana pulp at PCI 1 through 7 [36]. Agarose gel electrophoresis, and northern blotting and hybridization were performed according to standard protocols [35]. The cDNA clone pBAN3-30 was radiolabelled with ^{32}P-dCTP by random priming and used as a probe.

pBAN3-30 isolation and sequence analysis. pBAN3-30 was isolated from a banana pulp cDNA library by differential screening [5]. The complete sequence of the cDNA insert was determined on both strands, and the open reading frame was translated. Sequence homology of pBAN3-30 and the translation product (P31) were determined using the BLAST search algorithm for searching GenBank [37]. For the amino acid alignments, plant chitinase sequences showing significant homology to P31 were downloaded from GenBank and aligned manually.

Expression of recombinant P31. A total of 10 homologous chitinase clones were isolated from the banana pulp cDNA library by differential screening, including pBAN3-30, pBAN3-31, pBAN3-36, and pBAN3-45

[5]. These four clones were used for the expression of P31 for western blot analysis of the translation products. It was determined that pBAN3-36 and pBAN3-45 contained chitinase coding sequences that were in-frame with respect to β-galactosidase in the pBluescript cloning vector. All four of the cDNA clones, in *Escherichia coli* XL1-blue host cells, were grown to log phase in selective media and then induced by IPTG. Total bacterial proteins were sepd by SDS-PAGE and transferred to PVDF membrane. The western blot was hybridized with P31 antiserum and visualized using chemiluminescence.

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