Plant Cell Physiol. 39(3): 349–356 (1998)

Short Communication

cDNA Cloning, Gene Expression and Secretion of Chitinase in Winged Bean

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cDNA for an acidic class III chitinase (ChitW1) was isolated from winged bean cells. The chitinase was abundantly secreted at later stages of cell culture, when levels of ChitW1 mRNA were also high. The gene was strongly expressed in roots, but a class I chitinase was strongly expressed in leaves.

Key words: cDNA cloning — Chitinase (EC 3.2.1.14) — Salicylic acid — Secretion — Senescence — Winged bean (*Psophocarpus tetragonolobus*).

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of β-1,4 linkages in chitin, a polymer of *N*-acetyl-D-glucosamine that is present in the cell walls of many fungi. Some plants produce chitinases to protect themselves from chitin-containing pathogens (Bell 1981). Chitinases have been reported to have antifungal activity against some fungi in vitro, in particular in combination with β-1,3-glucanases (Broglie et al. 1991). Thus, some pathogenesis-related proteins have been identified as chitinases (Legrand et al. 1987). Chitinases are induced in response to viral or fungal infection, fungal elicitors, salicylic acid, ozone, and the stress hormone ethylene (Collinge et al. 1993). In addition to their putative role in plant defense responses, chitinases may also function in the development of somatic embryos, perhaps by releasing endogenous factors that are related to nodulation factors (Nod factors); (De Jong et al. 1992, Vijn et al. 1993). For example, an endochitinase may be involved in the specificity of the interaction between a bacterium and its host plant in the formation of root nodules by Nod factors of *Rhizobium* (Staehelin et al. 1994). Four classes of chitinases have been proposed on the basis on their primary structures (Collinge et al. 1993). Class I chitinases contain an amino-terminal cysteine-rich domain of about 40 amino acids, a chitin-binding domain, and a highly conserved main structure, separated by a variable hinge region. In general, these enzymes are localized in vacuoles. Class II chitinases are similar to class I chitinases but lack an amino-terminal cysteine-rich domain. They seem to be acidic proteins and are located in the apoplast. Class III chitinases have sequences very diiferent from most of class I and class II chitinases. They can be acidic or basic proteins, and may be localized in the apoplast. Class IV chitinases contain a cysteine-rich domain and a conserved main structure, resembling class I chitinases, but they are significantly smaller as a consequence of four major deletions. In a previous paper (Esaka et al. 1994), we reported the specific secretion of a class I chitinase by salt-adapted

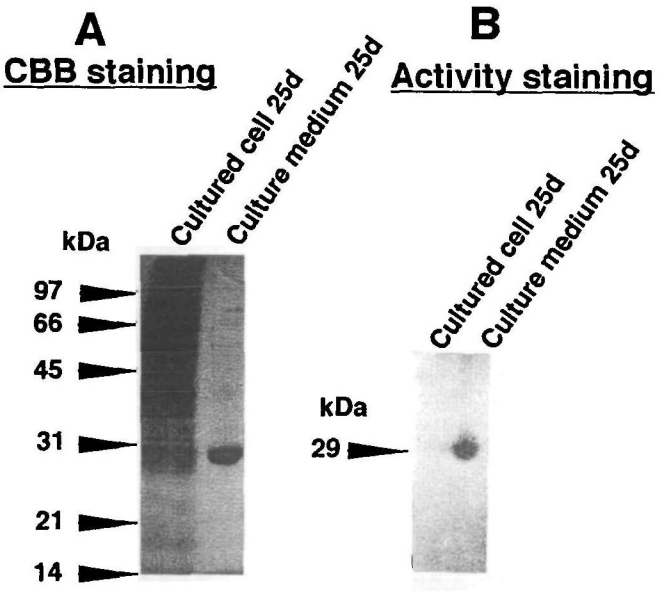
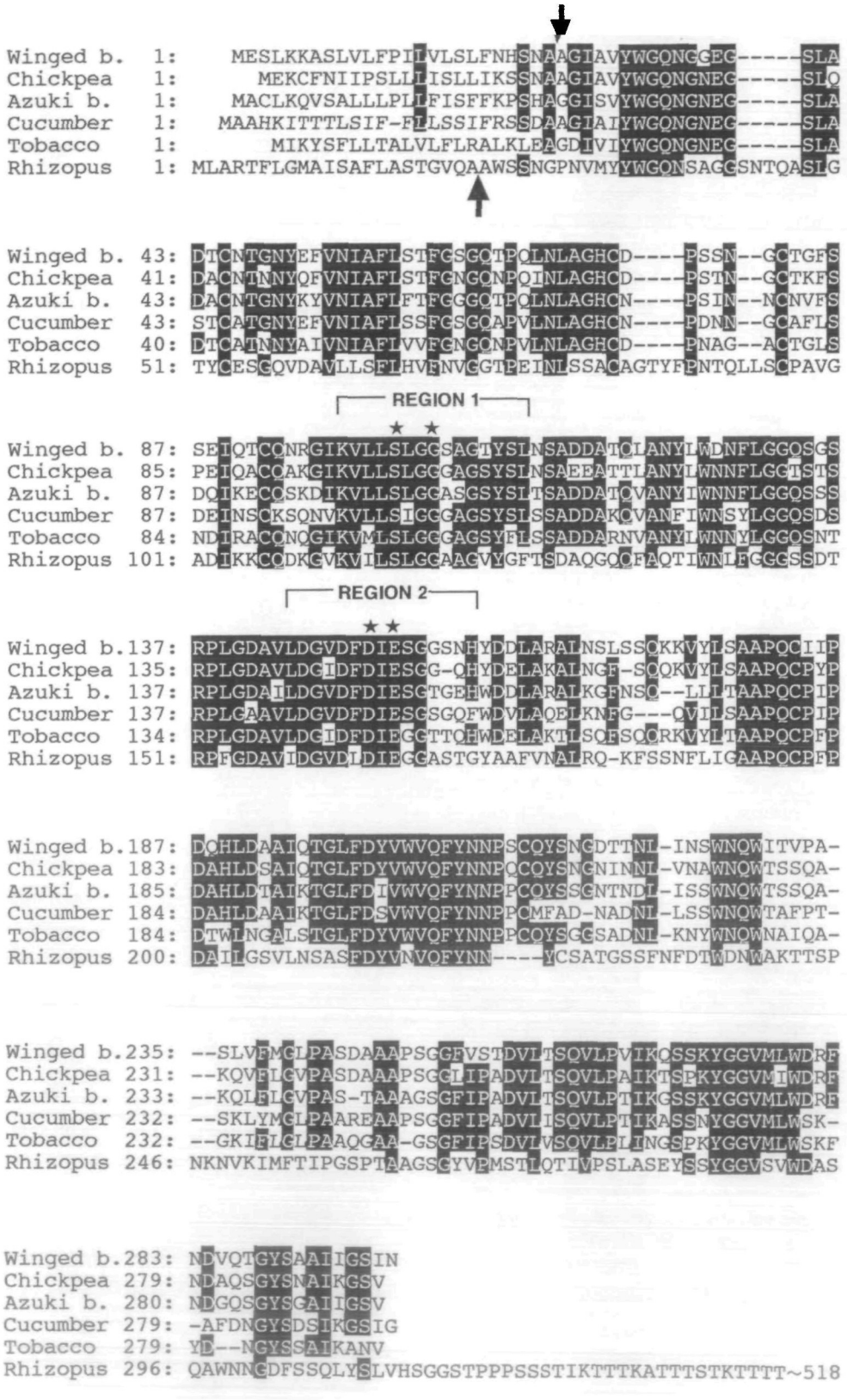


Fig. 1 Analysis by SDS-PAGE and activity staining for chitinase of an extract of cultured cells and the culture medium from a suspension culture of winged bean cells. (A) SDS-PAGE. Winged bean cells were cultured for 25 d. The cultured cells and culture medium were separated by filtration. The cultured cells were ground to a powder in liquid nitrogen. Twenty μg of protein from cultured cells (Cultured cell 25d) and culture medium (Culture medium 25d) were heated at 100°C for 3 min, and subjected to electrophoresis on a 12% (w/v) polyacrylamide gel that contained 0.1% SDS. The gel was then stained with 0.1% (w/v) Coomassie brilliant blue R (CBB staining). (B) Activity staining for chitinase. Twenty μg of protein from cultured cells (Cultured cell 25d) and culture medium (Culture medium 25d) after suspension culture for 25 d were heated at 100°C for 40 s, and subjected to electrophoresis on a 12% polyacrylamide gel that contained 0.1% SDS. The gel was then incubated in 0.1 M sodium acetate (pH 5.2) that contained 1% Triton X-100 at 37°C for 18 h. A 7.5% polyacrylamide gel containing 0.01% glycolchitin was placed on the gel at 37°C for 3 h and was then stained with 0.01% Fluorescent Brightener 28 (Sigma, St. Louis, MO, U.S.A.). After washing with water, the band of the gel corresponding to active chitinase was detected under UV light.



cells of winged bean (*Psophocarpus tetragonolobus*). We now report the isolation of a cDNA clone for an acidic class III chitinase and the differential expression of genes for class III and class I chitinases in winged bean.

Winged bean (*Psophocarpus tetragonolobus* L. DC. Nigeria TPT-2) seeds were supplied by the Okinawa Branch of the Tropical Agriculture Research Center, Ministry of Agriculture, Forestry and Fisheries, Japan. Callus was induced from seedlings, as described previously (Esaka et al. 1992). The callus was grown on 50 ml of Murashige and Skoog's basal medium with 1.0 mg liter^{-1} 2,4-D, 0.1 mg liter^{-1} kinetin, 3% (w/v) sucrose and 0.8% (w/v) agar at 28°C in darkness, and the cultures were maintained by transferring about 1 g of callus to fresh medium at 4-week intervals. Suspension cultures were established as described previously (Esaka et al. 1992), and incubated with agitation on a rotary shaker at 90 rpm at 28°C. The culture medium was separated from cells by filtration. When we analyzed culture medium from a 25-d-old culture by SDS-PAGE, we observed a major band at a position that corresponded to a molecular mass of 29 kDa (Fig. 1A). This secreted protein was purified by extraction from the gel and determination of its amino-terminal amino acid sequence (AGIAVYWG-QNGGEGSLA) suggested that it was a class III chitinase. Indeed, activity staining for chitinase (Dumas-Gaudot et al. 1994) confirmed the presence of such a protein in the culture medium (Fig. 1B).

Poly(A)-rich RNA was prepared from winged bean cells that had been cultured for 7 d and was used to construct a cDNA library, with λgt10 as the vector. The cDNA library was screened by plaque hybridization with a mixture of 32-mer and 29-mer oligonucleotides that were based on the amino-terminal amino acid sequence. Approximately 230,000 independent recombinants were screened and one positive clone (ChitW1) was isolated. The cDNA insert of this clone (about 1.1 kbp) contained a sequence that encoded the amino-terminal amino acid sequence of the class III chitinase from winged bean. The nucleotide sequence of the cDNA insert included a 897-bp open reading frame that encoded a putative polypeptide (ChitW1) of 298 amino acids, a 16-bp 5′-untranslated region, a 100-bp 3′-untranslated region and 45-bp poly A tail (data not shown). We found the sequence AATAA, which is the conserved sequence of a polyadenylation signal, 53 bp upstream of the poly A tail. The deduced amino acid sequence contained

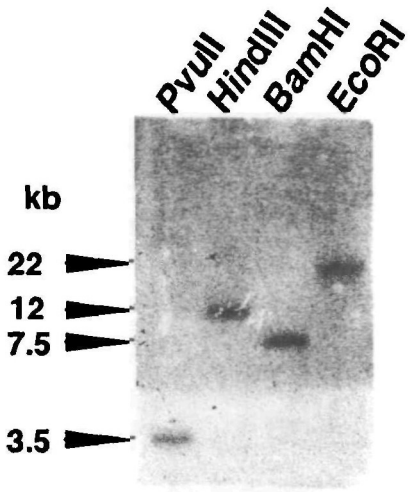


Fig. 3 Genomic Southern analysis with ChitW1 cDNA as the probe. Genomic DNA (20 μg) from winged bean cells was digested separately with *Pvu*II, *Hin*dIII, *Bam*HI and *Eco*RI, subjected to electrophoresis on a 0.7% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Japan, Tokyo, Japan). The DNA on the filter was hybridized to the probe at 42°C in 6× SSC, 5× Denhardt's solution, 1% (w/v) SDS, 0.05 M phosphate buffer (pH 7.0), 50% (w/v) formamide and 0.5 mg ml^{-1} denatured salmon sperm DNA. The filter was washed with 2× SSC plus 0.1% (w/v) SDS at 42°C.

an amino-terminal extension of 25 amino acid residues beyond the amino terminus of the mature enzyme (Fig. 2). This additional amino-terminal sequence was rich in hydrophobic residues and probably represented a signal peptide. The nucleotide sequence did not encode any putative sites of N-glycosylation (Asn-X-Ser/Thr, where X can be any amino acid). Thus, the protein might not be N-glycosylated, even though glycosylation of some chitinases has been reported (De Jong et al. 1992).

The amino acid sequence deduced from the nucleotide sequence of ChitW1 cDNA was very similar to those of the class III chitinases reported previously (Fig. 2), and it contained the six cysteine residues that are characteristic of class III chitinases (Lawton et al. 1992). In tobacco, two cDNAs for acidic (putative pI, 4.42) and basic (putative pI, 8.32) class III chitinases have been isolated (Lawton et al. 1992). The putative pI of the mature protein encoded by ChitW1 cDNA was 4.0, which is the lower than the previ-

Fig. 2 Comparison of amino acid sequences of class III chitinases from winged bean, chickpea (Vogelsang and Barz 1993), azuki (Ishige et al. 1993), cucumber (Metraux et al. 1989), tobacco (Lawton et al. 1992), and *Rhizopus oligosporus* (Yanai et al. 1992). Amino acid sequences were aligned to give maximum homolog. Amino acid residues conserved in more than four sequences are shown in black boxes. REGION 1 and REGION 2 indicate two small regions in the putative catalytic domain. Stars indicate amino acid residues conserved also in chitinase from *Streptomyces erythraeus* (Kamei et al. 1989), chitinase A from *Serratia marcescens* (Jones et al. 1986), chitinase A1 from *Bacillus circulans* (Watanabe et al. 1992), endo-H from *Streptomyces plicatus* (Robbins et al. 1984) and the α subunit of the killer toxin of *Kluyveromyces lactis* (Stark et al. 1990). Arrows indicate sites of cleavage of signal peptides. Dashes indicate gaps introduced to give the best alignment. The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number D49953 (chitinase).

ously reported pIs of class III chitinases. Thus, the cDNA appeared to encode an acidic class III chitinase. Indeed, the amino acid sequence was more similar to those of acidic class III chitinases from chickpea (75.7%; Vogelsang and Barz 1993), azuki bean (69.7%; Ishige et al. 1993), cucumber (63.6%; Metraux et al. 1989) and tobacco (62.2%;

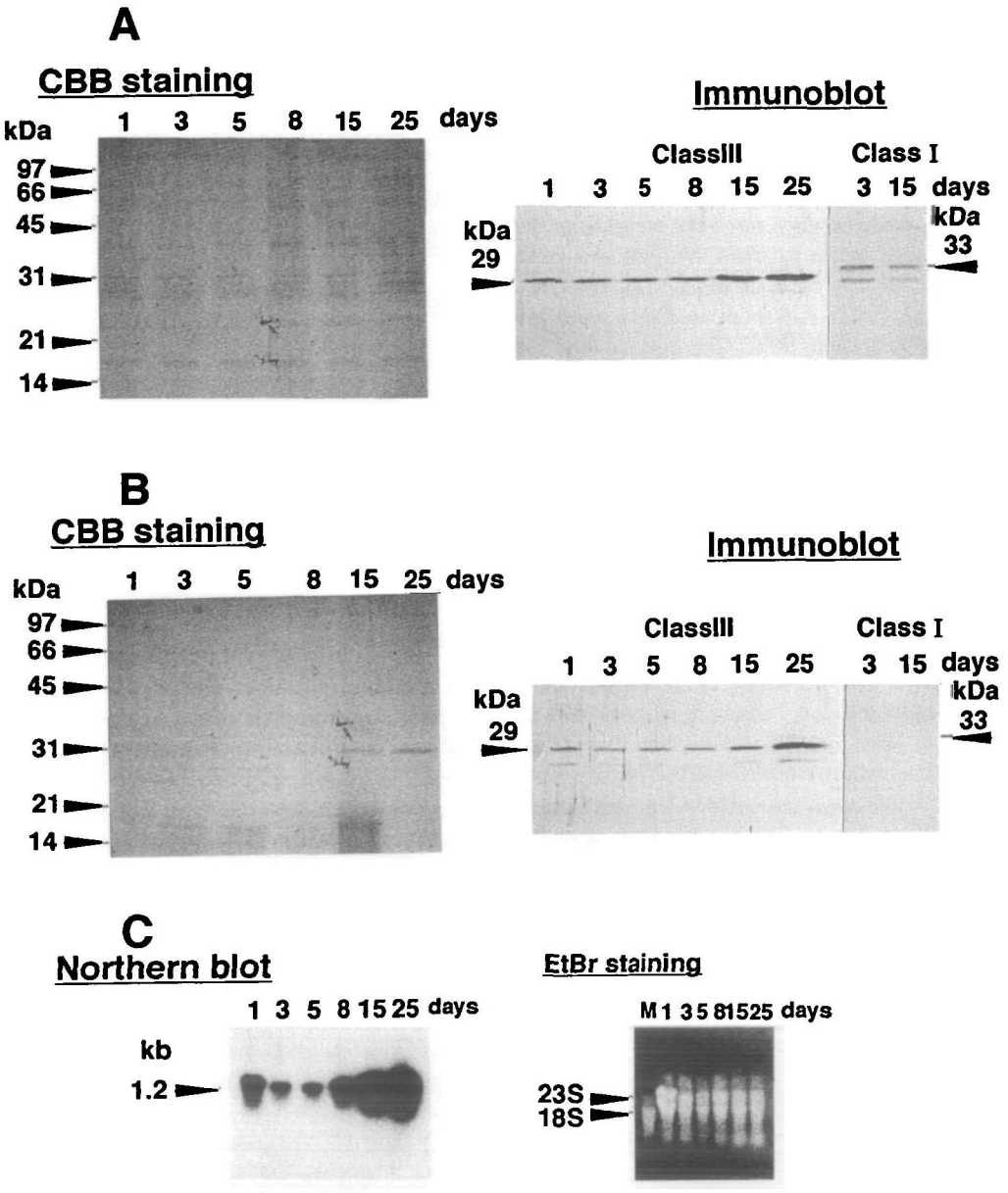


Fig. 4 Gene expression and secretion of ChitW1, an acidic class III chitinase, and of basic class I chitinase in suspension culture of winged bean cells. (A) SDS-PAGE and immunoblotting analyses of cultured winged bean cells. Cells were cultured for 1, 3, 5, 8, 15 and 25 d, collected by filtration and ground to powder in liquid nitrogen. Ten μg of protein isolated from the powder were used for SDS-PAGE as described in legend to Fig. 1 and immunoblotting analyses. Immunoblotting analysis was perfomed with antibodies against ChitW1 and against pumpkin class I chitinase as described previously (Esaka et al. 1990). (B) SDS-PAGE and immunoblotting analyses of 50-μl samples of filtered culture medium from suspension cultures. Analyses were performed as described in (A). (C) Northern blotting analysis of ChitW1 mRNA in cultured winged bean cells. Cultured cells were ground to a powder in liquid nitrogen. Twenty μg of total RNA, isolated from the powder, were fractionated on a 1.5% (w/v) agarose gel that contained 15% (v/v) formaldehyde. The gel was stained with ethidium bromide (EtBr staining). The RNA was transferred to a nylon membrane (Hybond-N; Amersham Japan, Tokyo, Japan) and hybridized to the ChitW1 cDNA probe at 42°C in 50% (w/v) formamide. The membrane was washed with 0.1 × SSC plus 0.1% (w/v) SDS at 65°C. M, RNA size markers (23S and 18S ribosomal RNAs).

Lawton et al. 1992) than to those of basic class III chitinases from rubber tree (61.3%; Jekel et al. 1991) and tobacco (57.8%; Lawton et al. 1992).

Chitinases are localized in vacuoles or in the apoplast (Boller and Metraux 1988). Some studies have demonstrated that a short carboxy-terminal extension of about six amino acids, found in precursors to chitinases, is necessary for targeting to the vacuole (Neuhaus et al. 1991). The putative protein encoded by ChitW1 cDNA does not contain a carboxy-terminal extension similar to those found in precursors to vacuolar chitinases. In general, it has been suggested that acidic chitinases are secretory enzymes, while basic chitinases are localized in vacuoles. However, basic class III chitinase from tobacco (Lawton et al. 1992) appeared to be secreted into the extracellular space. By contrast, basic class III chitinase from rubber tree appears to be localized in vacuoles (Jekel et al. 1991) but its cDNA has not yet been cloned. Thus, the localization in cells of class III chitinases has not been established. The deduced amino acid sequence of ChitW1 was 35.9% homologous to that of chitinase from *Rhizopus oligosporus* (Yanai et al. 1992; Fig. 2). Furthermore, the amino acid sequences in the two regions (regions 1 and 2) of the putative catalytic domain were similar to those of chitinases from microorganisms such as *Streptomyces erythraeus* (Kamei et al. 1989), *Serratia marcescens* (Jones et al. 1986) and *Bacillus circulans* (Watanabe et al. 1992), to endo-H of *Streptomyces plicatus* (Robbins et al. 1984) and to the *α* subunit of the killer toxin of *Kluyveromyces lactis* (Stark et al. 1990). Residues corresponding to Ser102, Gly105, Asp150 and Glu152 in ChitW1 are conserved in all these enzymes (Fig. 2), suggesting that these amino acid residues might be important for the catalytic functions of these enzymes. The amino acid sequence in region 2 is also similar to that of chitobiases from rat and human (Kuranda and Robbins 1991).

We performed genomic Southern blotting analysis with ChitW1 cDNA to determine the number of copies of the gene for the acidic class III chitinase in the winged bean genome. Under low-stringency conditions, a single strongly hybridizing band was found in each restriction digest (3.5 kbp with *Pvu*II, 12 kbp with *Hin*dIII, 7.5 kbp with *Bam*HI, 22 kbp with *Eco*RI; Fig. 3), suggesting that ChitW1 is encoded by a single-copy gene in winged bean. In *Arabidopsis thaliana*, an acidic class III chitinase has also been reported to be encoded by a single-copy gene (Samac et al. 1990).

We investigated expression of the gene for ChitW1, an acidic class III chitinase in winged bean cells in suspension culture (Fig. 4). The cells were ground to a powder in liquid nitrogen with a mortar and a pestle. An extract of the powder was analyzed by SDS-PAGE. Total RNA was isolated from the powder as described by Verwoerd et al. (1989). An specific antiserum against purified ChitW1 was raised in a rabbit. Immunoblotting analysis with the antiserum revealed the immunoreactive protein in both cells and the culture medium (Fig. 4A, B). In cells, the immunoreactive protein was detected during the lag phase of culture (1, 3, 5, and 8 d; Fig. 4A), and the level increased at later stages (15 and 25 d). It was unclear whether the immunoreactive chitinase was localized in vacuoles or in the cell wall. In the culture medium, the immunoreactive protein was also detected as a major protein at 15 and 25 d, and it was detectable at a low level at the early stages (1, 3, 5, and 8 d) of culture (Fig. 4B). Thus, secretion of the acidic class III chitinase increased at the later, stationary phase.

Northern blotting analysis with ChitW1 cDNA showed that levels of ChitW1 mRNA increased after 8 d of culture and were high at the later, stationary phase (15 and 25 d). Levels of the mRNA were low at early stages (3 and 5 d; Fig. 4C). Thus, the mRNA for the acidic class III chitinase might be actively transcribed in suspension cultures at later stages. Chitinase is a pathogenesis-related protein (Legrand et al. 1987). No typical inducer for pathogenesis-related proteins was added to our suspension cultures. Its synthesis

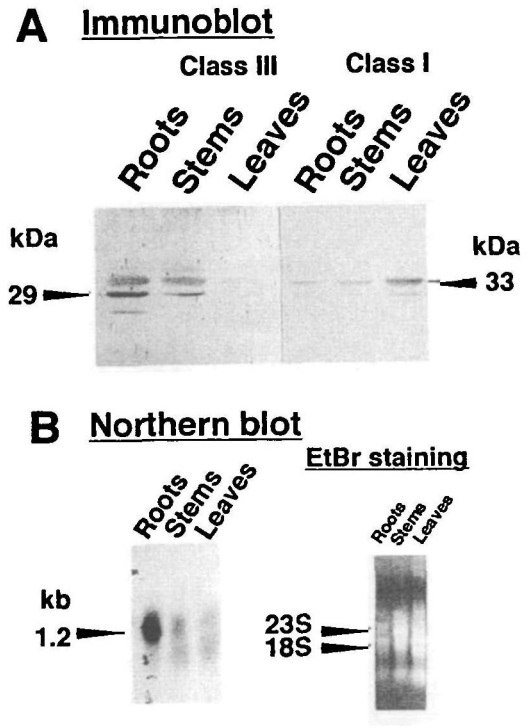


Fig. 5 Expression of gene for ChitW1, an acidic class III chitinase, and for basic class I chitinase in various tissues of winged bean. (A) Immunoblotting analysis of ChitW1 and class I chitinase in roots, stems and leaves. Each tissue was ground to a powder in liquid nitrogen. Twenty μg of protein from each powder were used for analysis, which was performed as described in the legend to Fig. 4. (B) Northern blotting analysis of ChitW1 mRNA in roots, stems and leaves. Twenty μg of total RNA isolated from the powder in (A) were used for Northern blotting analysis, which was performed as described in the legend to Fig. 4.

might be induced by the 2,4-D and/or kinetin that is added to the culture medium to maintain cells in suspension cultures. There are reports that expression of chitinase in tobacco cells in cultures is regulated by auxin and cytokinin (Shinshi et al. 1987). However, it is likely that ChitW1, an acidic class III chitinase, was induced by cell senescence and was then secreted at high levels into the culture medium since both expression and secretion were marked at the later stages of suspension culture. By contrast, immunoblotting with antiserum against pumpkin basic class I chitinase (Esaka et al. 1990) showed that an immunoreactive protein of 33 kDa was present almost constantly only in cells (Fig. 4A) and not in extracellular spaces (Fig. 4B). It seems likely that a class I chitinase is expressed constitutively and localized in vacuoles of winged bean cells.

We investigated the expression of ChitW1 in roots, stems and leaves by immunoblotting (Fig. 5A). In roots, we detected an intensely immunoreactive protein of 29 kDa. Thus, it appeared that the acidic class III chitinase was strongly expressed in roots. The immunoreactive protein was slightly detectable in stems and was undetectable in leaves. We do not know whether the protein of about 33 kDa was derived an isozyme of class III chitinase or was another protein that reacted with the antibodies. Northern blotting analysis also showed that roots contained higher levels of ChitW1 mRNA than stems (Fig. 5B). In leaves, ChitW1 mRNA was undetectable. Immunoblotting with antiserum against a class I chitinase from pumpkin revealed that an immunoreactive protein was abundant in leaves, with little of it in roots and stems (Fig. 5A). Thus, the tissue specificity of the expression of ChitW1, an acidic class III chitinase, appeared to be different from that of a class I chitinase.

It is interesting that a large amount of acidic class III chitinase was constitutively expressed in winged bean roots, in which class I chitinase was barely expressed, when we consider the biological functions of chitinases. Expression of chitinase has been reported to be developmentally regulated in tobacco flowers (Lotan et al. 1989) and the enzyme appears to have an important function in the early somatic embryo development of carrot (De Jong et al. 1992, 1993). Thus, ChitW1 might be involved in root devel-

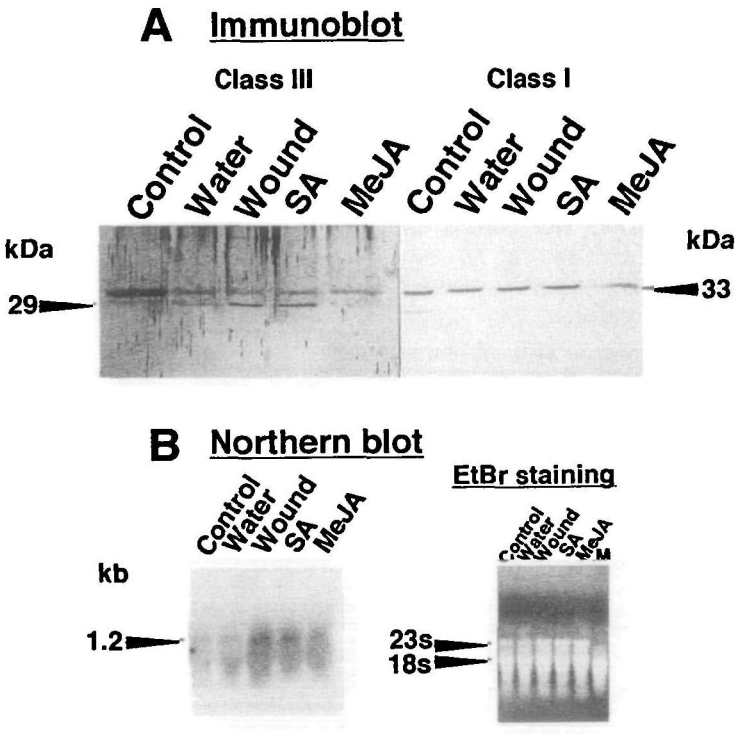


Fig. 6 Effects of wounding, salicylic acid and methyl jasmonate on gene expression of ChitW1, an acidic class III chitinase, and of basic class I chitinase in winged bean cotyledons. (A) Immunoblotting of crude extracts from non-treated (Control) and treated cotyledons. Green cotyledons were placed on discs of filter paper moistened with water (Water), 0.72 mM salicylic acid (SA) or 0.045 mM methyl jasmonate (MeJA) in plastic petri dishes. For wounding treatment, the cotyledons were pricked as described in the text (Wound) and incubated in the light at 28°C for 48 h. They were then ground to a powder in liquid nitrogen. An extract (20 μg of protein) of the powder was used for immunoblotting, performed as described in the legend to Fig. 4. (B) Northern blotting analysis of ChitW1 mRNA in non-treated (Control) or treated cotyledons. Cotyledons were ground to a powder in liquid nitrogen. Twenty μg of total RNA from the powder were used for Northern blotting analysis, performed as described in the legend to Fig. 4. M, RNA size markers (23S and 18S ribosomal RNAs).

opment of winged bean.

Chitinases are regulated by a variety of hormones, various stresses, and other chemical agents. Therefore, we investigated the expression of ChitW1 in winged bean cotyledons in response to wounding and salicylic acid (SA), an inducer of pathogenesis-related proteins (Yalpani et al. 1991; Fig. 6). In addition, we investigated the effect of methyl jasmonate (MeJA) because expression of chitinase in cultured plant cells has been reported to be induced by jasmonate or jasmonate-related compounds (Suzuki et al. 1995; Fig. 6). For the treatment with SA or MeJA, green cotyledons were harvested from plants grown on moist vermiculite in the light at 28°C for 15 d and were placed on discs of filter paper that had been moistened with 0.72 mM SA or 0.045 mM MeJA in plastic petri dishes. The cotyledons were incubated under constant light at 28°C for 48 h. For wounding of cotyledons, green cotyledons were harvested from plants that had been grown on moist vermiculite in the light at 28°C for 15 d. They were placed on moistened discs of filter paper in plastic petri dishes, and each was pricked about one hundred times with a sterile needle. The materials were then incubated at 28°C in the light for 48 h. ChitW1 was barely expressed in intact cotyledons, as noted above. The immunoreactive protein of 29 kDa was barely detectable in the analysis of both intact cotyledons (control) and cotyledons treated with water (Fig. 6A). However, the amount of immunoreactive protein was increased by wounding and SA but not by MeJA, suggesting that ChitW1 in cotyledons was induced by wounding and SA (Fig. 6A). Again, the protein of about 33 kDa was detected at a high level in all cotyledons. There is a possibility that the protein was an isozyme of class III chitinase because both acidic and basic class III chitinases are expressed in tobacco (Lawton et al. 1992). Northern blotting analysis also showed that the expression of ChitW1 mRNA was promoted by wounding and SA, but not by MeJA (Fig. 6B). By contrast, immunoblotting with antibodies against pumpkin class I chitinase showed that cross-reacting polypeptide in winged bean was not induced by these stimuli (Fig. 6A), even though a class I chitinase in pumpkin fruits is markedly induced by wounding (Esaka et al. 1993). The induction of ChitW1 by wounding suggests the involvement of ethylene, synthesized as a result of wounding. However, ethylene has been proposed to induce class I chitinase only, and not class III chitinase. Further studies are required to clarify whether ChitW1 is inducible by ethylene.

Finally, we want to emphasize that the expression of ChitW1, an acidic class III chitinase, was high in our suspension cultures. The reason is unknown. In a previous paper (Esaka et al. 1990), we showed that a basic class I chitinase is abundantly secreted at early stages from pumpkin cells in suspension culture. We suggested that the basic class I chitinase in pumpkin cells might be induced by wounding or by the shear stress that is associated with suspension cultures (Esaka et al. 1993). In winged bean cells, by contrast, the acidic class III chitinase might be induced by the stress that accompanies cell senescence, although it is unclear whether ethylene, a senescence hormone of plants, is involved in the induction. Further studies are needed to clarify the molecular mechanisms of the control of gene expression and the secretion of chitinases by winged bean cells.

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(Received February 12, 1997; Accepted December 25, 1997)