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Isolation and Characterization of the Genes Encoding Basic and Acidic Chitinase in *Arabidopsis thaliana*

Deborah A. Samac*, Cathy M. Hironaka, Peter E. Yallaly, and Dilip M. Shah

Monsanto Agricultural Company, Plant Sciences, 700 Chesterfield Village Parkway, Chesterfield, Missouri 63198

ABSTRACT

Plants synthesize a number of antimicrobial proteins in response to pathogen invasion and environmental stresses. These proteins include two classes of chitinases that have either basic or acidic isoelectric points and that are capable of degrading fungal cell wall chitin. We have cloned and determined the nucleotide sequence of the genes encoding the acidic and basic chitinases from *Arabidopsis thaliana* (L.) Heynh. Columbia wild type. Both chitinases are encoded by single copy genes that contain introns, a novel feature in chitinase genes. The basic chitinase has 73% amino acid sequence similarity to the basic chitinase from tobacco, and the acidic chitinase has 60% amino acid sequence similarity to the acidic chitinase from cucumber. Expression of the basic chitinase is organ-specific and age-dependent in *Arabidopsis*. A high constitutive level of expression was observed in roots with lower levels in leaves and flowering shoots. Exposure of plants to ethylene induced high levels of systemic expression of basic chitinase with expression increasing with plant age. Constitutive expression of basic chitinase was observed in roots of the ethylene insensitive mutant (*etr*) of *Arabidopsis*, demonstrating that root-specific expression is ethylene independent. Expression of the acidic chitinase gene was not observed in normal, untreated *Arabidopsis* plants or in plants treated with ethylene or salicylate. However, a transient expression assay indicated that the acidic chitinase promoter is active in *Arabidopsis* leaf tissue.

Plants have developed several biochemical defense mechanisms in response to pathogens and abiotic stresses. Following pathogen attack, plants synthesize phenylpropanoid products such as lignin, low mol wt antimicrobial compounds known as phytoalexins, and several defense-related proteins. Among these proteins are the 'pathogenesis-related proteins' which include the fungal cell wall degrading enzymes chitinase and β-1,3-glucanase (29).

Endochitinases from higher plants catalyze the hydrolysis of chitin, a β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine. The level of chitinase activity increases dramatically after invasion by fungal (13, 16), bacterial (3), or viral pathogens (15). Although chitin does not exist in plant cells, it is a major component of the cell walls of many fungi (1). Purified plant endochitinases have antifungal activity against some fungi *in vitro* (23) and can act synergistically with β-1,3-glucanases purified from plants to inhibit fungal growth (18). Furthermore, the presence of pathogenesis-related proteins is associated with the hypersensitive response (15) and induced resistance (28). These observations suggest that chitinases are part of a general disease resistance mechanism.

In response to pathogens, plants synthesize two classes of chitinases with either basic or acidic isoelectric points. These two classes appear to be targeted to different parts of the cell, and there is evidence that they are differentialay regulated. Acidic chitinase is found in the intercellular space of infected plants and plants treated with salicylic acid or necrotizing salt solutions (20). Basic chitinase accumulates in the central vacuole (19) and is systemically induced by ethylene, a stress-related hormone (5). Most plants synthesize a number of related chitinase isozymes encoded by a multigene family (5, 13, 15). In some cases, these isozymes are differentially regulated during plant growth (27) and pathogen invasion (17). The mechanism of tissue specificity and differential induction of chitinases is only beginning to be elucidated. Recently, two basic chitinase genes from bean were shown to be transcriptionally regulated by ethylene, and the 5′ flanking sequences sufficient for ethylene-dependent expression of one of these genes were defined (4).

We have initiated an investigation into the regulation of the chitinase gene family from *Arabidopsis thaliana* (L.) Heynh. Columbia wild type. This plant is widely used as a model in plant molecular genetic studies because of its small genome size and simple genomic organization (21). Here we report the isolation, sequencing, and expression analysis of single copy genes encoding acidic and basic chitinases in *A. thaliana*. These genes share little sequence homology and are interrupted by introns. The basic chitinase gene displays age-dependent and tissue-specific expression and is induced by treating plants with ethylene. Expression of the acidic chitinase gene was not detected in normal, untreated plants nor in plants treated with ethylene or salicylic acid.

MATERIALS AND METHODS

Isolation and Characterization of the Basic Chitinase Gene

An *Arabidopsis thaliana* (L.) Heynh. Columbia wild-type genomic library (11) was screened by plaque hybridization using a nick-translated maize cDNA encoding a basic chitinase (C Hironaka, D Shah, manuscript in preparation). Hybridization was carried out at 37 °C in 35% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 0.2% (w/v) SDS, and 100 μg/mL tRNA. Filters were washed sequentially in 3 × SSC, 0.2% (w/v) SDS for 30 min at room temperature, 37 °C and 50 °C. Restriction mapping of a positive clone was carried out

using LambdaMap (Promega Biotec). A 3.1 kbp[1] *Eco*RI-*Bgl*II fragment containing the entire chitinase coding sequence was cloned into pBluescript KS (Stratagene) to create pMON8783.

For sequencing, a series of clones containing overlapping unidirectional deletions were made with exonuclease III and S1 nuclease (9). Template DNA from the unidirectionally deleted clones was sequenced by the dideoxy-chain termination method with Sequenase (U.S. Biochemical Corp.). Sequence analyses were performed with the Wisconsin Genetic Computer Group software.

Cloning of Basic Chitinase cDNA

Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography from 18-d-old plants treated with ethephon. cDNA was synthesized and ligated into the *Eco*RI site of λgt10. Plaques were screened by the same method as for the screening of basic chitinase genomic clones. The probe used was a 1.5-kbp *Bgl*II-*Eco*RI fragment from pMON8817 (see below) containing the entire coding region of the basic chitinase gene. Six positive clones were identified from approximately 24,000 plaques screened. The two largest cDNAs (1.05 kbp and 1.15 kbp) were cloned into pUC119 (30) and sequenced.

Isolation and Characterization of the Acidic Chitinase Gene

Two 16-fold degenerate 17 bp oligonucleotides were synthesized (Achit1: $^{5'}CC^{A}/_{G}TT^{C}/_{T}TG^{C}/_{G}CCCCA^{A}/_{G}TA^{3'}$; Achit2: $^{5'}CC^{A}/_{G}TT^{T}/_{C}TG^{A}/_{T}CCCCA^{A}/_{G}TA^{3'}$) that correspond to the N-terminal amino acid sequence (Tyr-Trp-Gly-Gln-Asn-Gly) that is conserved in the acidic chitinases of rubber, cucumber, and *Parthenocissus quinquifolia* (20). The *Arabidopsis* genomic library was screened with a mixture of these probes as described previously (26). Hybridizations were carried out in 6 × SSC, 10 × Denhardt's solution, 0.5% (v/v) Nonidet P40, and 200 μg/mL tRNA at 42 °C. Filters were washed sequentially in 6 × SSC for 20 min at room temperature and 42 °C and for 5 min at 45 °C. Restriction digests of the positive clones were blotted to nitrocellulose, hybridized to the oligonucleotide probes, and washed under high stringency. A 4.2-kbp *Sal*I-*Hin*dIII fragment of one clone with strong homology to the oligonucleotide probes was subcloned into pUC118 and pUC119 (30) to create pMON8814 and pMON8815, respectively. An oligonucleotide complementary to Achit1 was used as a primer to sequence subclones. The complete sequence of the gene was obtained using oligonucleotide primers to generate overlapping sequences of the coding sequence.

Genomic Southern Hybridization

Samples of total DNA (5 μg) were digested with restriction enzymes, electrophoresed in a 0.8% (w/v) agarose gel, and transferred to Zeta-Probe (Bio-Rad). The filter was hybridized in 30% (v/v) formamide, 0.25 M $NaHPO_4$, 1 mM EDTA, and 7% (w/v) SDS at 42 °C with probes for the basic or acidic chitinase genes. The filter was washed under non-stringent conditions with 3 × SSC and 0.1% (w/v) SDS sequentially at 25 °, 37 ° and 50 °C for 15 min each. To isolate a DNA fragment containing only the basic chitinase gene, site-directed mutagenesis (14) was carried out on pMON8783 to introduce a unique *Bgl*II site just upstream of the first ATG and an *Eco*RI site just downstream of the TAG stop codon. The 1.5-kbp *Bgl*II-*Eco*RI fragment of the resulting plasmid, pMON8817, was nick-translated and used as a probe in DNA filter hybridization analyses. Similarly, a *Bgl*II site was engineered upstream of the first ATG of the acidic chitinase gene (pMON10829). A 1.6 kbp *Bgl*II-*Eco*RV fragment containing the protein coding region and 271 bp of 3′ flanking DNA was used as a probe for the acidic chitinase gene.

Analysis of RNA

RNA was extracted from whole plants as described previously (24). Total *Arabidopsis* RNA (20 μg) was denatured and electrophoresed in a 1.2% (w/v) formaldehyde-agarose gel. The gel was blotted to Zeta-Probe in 10 × SSC. The filter was hybridized and washed as described above for DNA filter hybridizations. Autoradiographs were scanned with an LKB Ultroscan XL Laser Densitometer to estimate the level of gene expression.

Plant Culture and Treatment with Ethephon or Salicylic Acid

Seeds of *A. thaliana* Columbia wild type were sown into Metromix Terralite 200 (Grace Horticultural Products, Cambridge, MA) and subirrigated daily with 14:15:16 (N:P:K) Peat-lite fertilizing solution. Plants were maintained at approximately 350 μE s^{-1} m^{-2} light for 16 h/d at 21 °C, 19 °C at night, and 50% relative humidity. At 18, 26, and 38 d after planting, plants were sprayed to runoff with 1 mg/mL ethephon (2-chloroethylphosphonic acid, Sigma Chemical Co.). Control plants were sprayed with water and pots were then enclosed in plastic bags. Plants were harvested for RNA extraction 48 h after treatment. At 18 d after planting, plants had four to five sets of true leaves; at 26 d, plants were initiating a flowering shoot; and at 38 d, plants were setting seed and beginning to senesce.

Two methods of salicylic acid induction were performed. In one test, the leaves of 26-d-old plants were sprayed with 2 mM salicylic acid, pH 6.5, to runoff. In a second test, detached leaves from 18-d-old and 38-d-old plants were floated on the 2 mM salicylate solution. In both tests, leaves were harvested 48 h after treatment for RNA extraction.

Particle Bombardment Transient Assay

The construct pMON8846 consists of the 1.2 kbp *Sal*I-*Bgl*II fragment containing the 5′ untranslated region of the acidic chitinase gene ligated to the β-glucuronidase (GUS) coding region and the nopaline synthase polyadenylation region (12) inserted into pUC119. This construct was precipitated onto 1.2 μM tungsten particles (12), and the DNA-coated microprojectiles were introduced into 26-d-old *Arabidopsis* leaves with the Biolistics (DuPont) particle gun (12).

[1] Abbreviations: kbp, kilobase pair; bp, base pair.

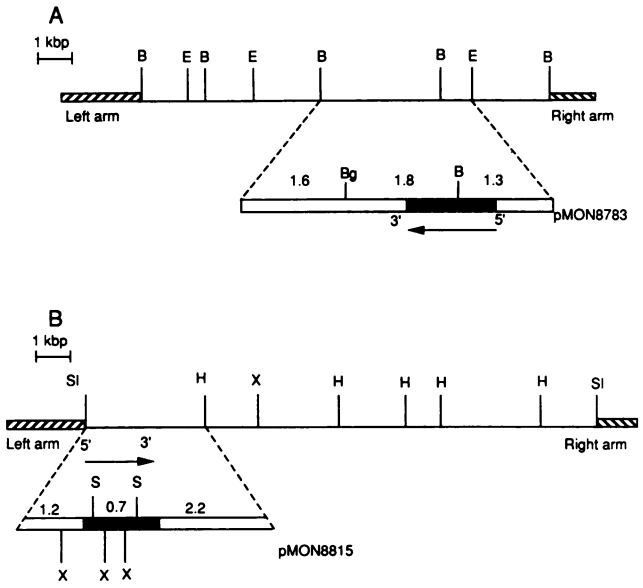

Figure 1. Restriction map of the genomic clones encoding the basic and acidic chitinases in *Arabidopsis*. The solid bars indicate the coding region. A, Restriction map of λA2 encoding the basic chitinase; B = *Bam*HI, E = *Eco*RI, Bg = *Bgl*II. B, Restriction map of λACh5 encoding the acidic chitinase; Sl = *Sal*I, H = *Hin*dIII, X = *Xba*I, S = *Sac*I.

After 24 h, the leaves were stained with the GUS substrate (12) and examined for blue GUS-positive spots.

RESULTS

Characterization of the Basic Chitinase Gene

Approximately 60,000 plaques of an *Arabidopsis* genomic library were screened with a maize basic chitinase cDNA. Four chitinase-positive phages were purified and analyzed by DNA filter hybridization. All contained the same 3.5-kbp *Bam*HI fragment hybridizing to the maize probe. The basic chitinase coding region from one clone, λA2, was mapped to a 3.1-kb *Eco*RI-*Bgl*II fragment (Fig. 1A) by DNA filter hybridization. A total of 2640 bp were sequenced from this fragment, including 695 bp of the 5′ nontranslated region and 458 bp of 3′ flanking DNA (Fig. 2). The basic chitinase coding region is divided into two exons of 459 and 551 bp by an intron of 476 bp. The position of the intron was deduced by comparing the sequence to the basic chitinase genes from tobacco (27), bean (5), and potato (7). The intron is 74% A + T while the coding regions are 49% A + T. The consensus intron-donor sequence, GGT, and the acceptor sequence, AGG (22), are present at the intron-exon borders. This is the first report of an intron in a chitinase gene.

Two cDNA clones that hybridized to the basic chitinase gene probe were isolated from a library made from RNA of plants treated with ethylene. The sequences of the cDNAs exactly matched the sequence of the coding region of the genomic clones, confirming the position of the intron. Interestingly, the two cDNAs use different polyadenylation sites located at 85 bp and 214 bp from the termination codon (Fig. 2).

The protein deduced from the nucleotide sequence is 335 amino acids long and is very similar to other plant basic endochitinases, particularly at the carboxyl terminus (Fig. 3). By comparison with the amino acid sequences of basic chitinase from tobacco (27), a hydrophobic signal peptide of 33 amino acids is predicted at the amino terminus of the *Arabidopsis* basic chitinase. The mature protein is highly hydrophilic and has a net basic charge. The *Arabidopsis* chitinase is most similar to the tobacco chitinase with 73% amino acid sequence identity between the two mature enzymes (27). The bean and *Arabidopsis* chitinases have 71% identity, and the potato and *Arabidopsis* amino acid sequences are 69% identical.

Characterization of the Acidic Chitinase Gene

Degenerate oligonucleotides corresponding to a conserved six amino acid sequence from the N terminus of the acidic

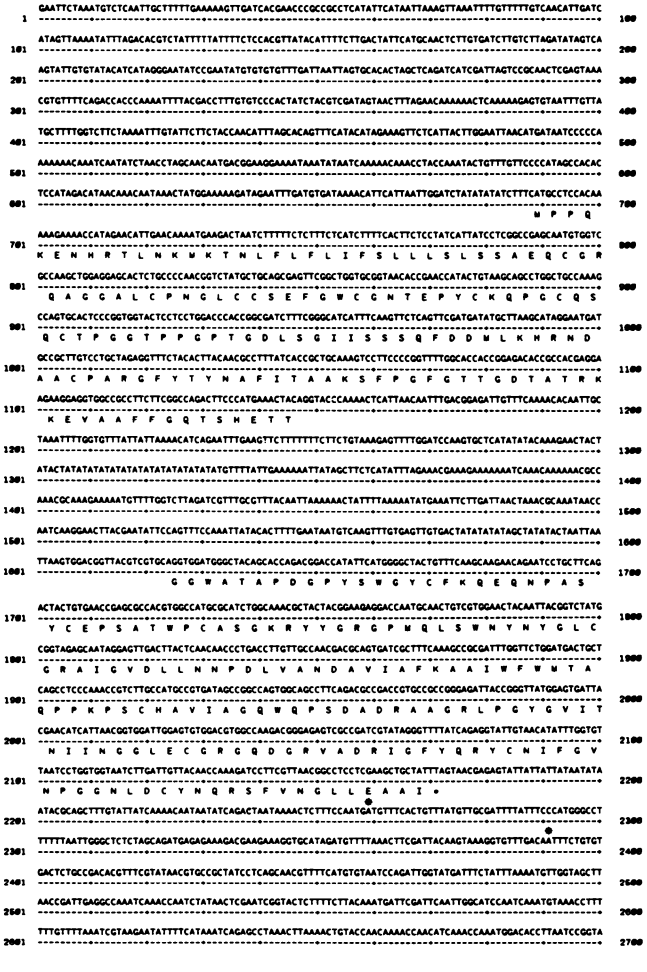

Figure 2. Complete nucleotide sequence of the basic chitinase gene isolated from *Arabidopsis*. The deduced amino acid sequence is shown below the DNA sequence. An intron of 476 bp interrupts the gene from position 1149 to 1625. The two putative polyadenylation sites identified from the sequence of cDNA clones are indicated by stars.

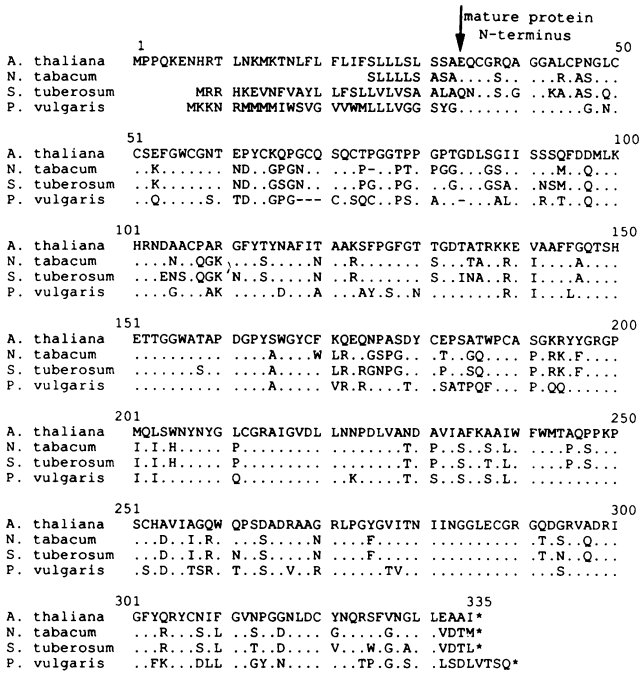

Figure 3. Comparison of the amino acid sequences of basic plant chitinases. The complete amino acid sequence of the *Arabidopsis* gene is given. Only the amino acids differing from the *Arabidopsis* sequence in the tobacco, potato, and bean sequences are shown.

chitinase from cucumber, rubber, and *Parthenocissus quinquifolia* (20) were used as probes in plaque hybridizations to identify acidic chitinase-positive genomic clones. One clone, λAC5, showed strong hybridization even when the filter was washed in 6 × SSC at 54 °C. The acidic chitinase gene was mapped to a 4.2 kbp *Sal*I-*Hin*dIII fragment (Fig. 1B). From this fragment, a total of 3000 bp were sequenced, 1151 bp of 5′ flanking DNA and 500 bp of 3′ flanking DNA (Fig. 4). Two introns of 169 bp and 269 bp interrupt the protein coding sequence. The gene organization was deduced by comparing the DNA sequence to the sequence of the acidic chitinase from cucumber. The extensive amino acid sequence similarity between the two genes enabled us to define the exon-intron borders in the *Arabidopsis* gene unambiguously. The intron-exon splice junctions of both introns conform to the GT/AG rule (22). The predicted protein product of the gene is 302 amino acids with a putative 30 amino acid hydrophobic signal peptide. The mature protein sequence, 272 amino acids, has a net acidic charge. The *Arabidopsis* acidic chitinase is seven amino acids longer than the mature protein sequence of the cucumber acidic chitinase but overall the *Arabidopsis* and cucumber acidic chitinases have 60% amino acid sequence identity (Fig. 5). The amino acid sequence similarity increases to 74% if conserved amino acid changes are included. When the amino acid sequences of the basic and acidic chitinases from *Arabidopsis* were compared, the two proteins were found to have only 31% overall amino acid sequence identity.

Basic and Acidic Chitinases are Encoded by Single Copy Genes

Genomic DNA filter hybridization analyses were carried out to establish the copy number of the basic and acidic chitinase genes and to test for the presence of other, related chitinase genes. The basic chitinase probe was a nick-translated 1.5 kbp *Bgl*II-*Eco*RI fragment from pMON8817 consisting of the protein coding region plus the intron. With this probe under non-stringent conditions, single strongly hybridizing bands were seen for each restriction digest (Fig. 6). After long exposures, several weak bands were seen which may correspond to sequences distantly related (less than 50% nucleotide similarity) to basic chitinase.

Genomic filter hybridization analyses with the 1.6 kbp *Bgl*II-*Eco*RV fragment from pMON10829 containing the acidic chitinase gene also revealed only single strongly hybrid-

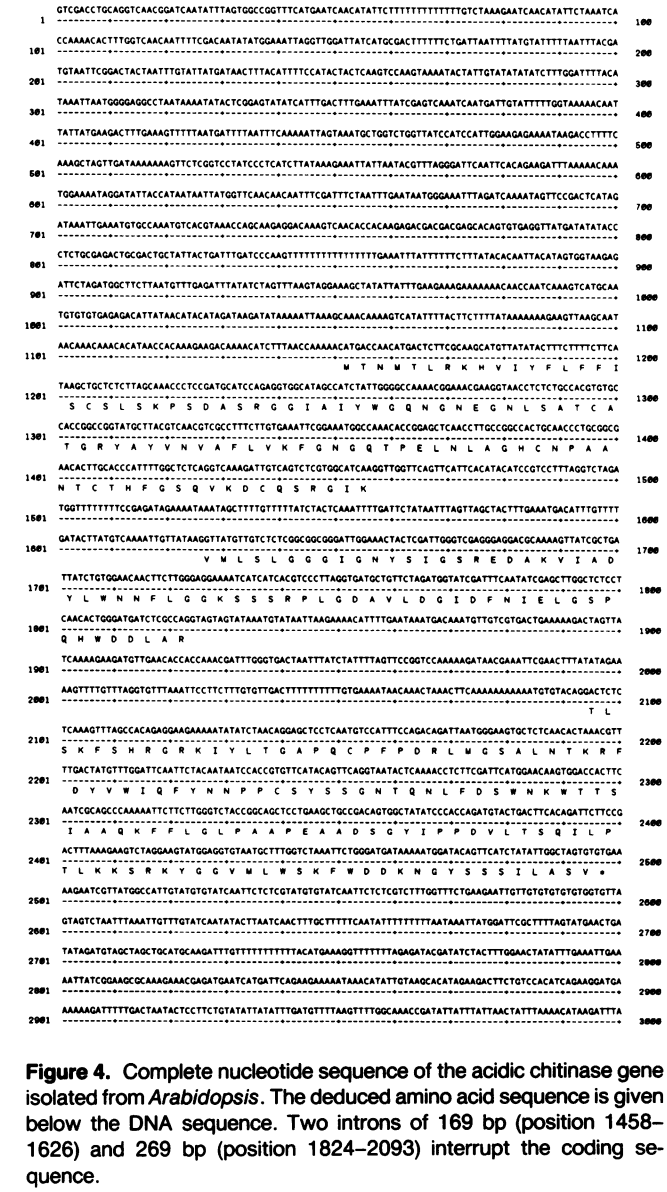

Figure 4. Complete nucleotide sequence of the acidic chitinase gene isolated from *Arabidopsis*. The deduced amino acid sequence is given below the DNA sequence. Two introns of 169 bp (position 1458–1626) and 269 bp (position 1824–2093) interrupt the coding sequence.

```
                                          mature protein
                                            N-terminus
            1                                                    50
A. thaliana  MTNMTLRKHV IYFLFFISCS LSKPSDASRG GIAIYWGQNG NEGNLSATCA
C. sativus        MAAHKI TTTLSIFFLL SSIFRSSDAA ..........  ...S.AS...

           51                                                   100
A. thaliana  TGRYAYVNVA FLVKFGNGQT PELNLAGHCN PAANTCTHFG SQVKDCQSRG
C. sativus    ..NYEF..I. ..SS..S..A .V........ .DN.G.AFLS DEINS.K.QN

          101                                                   150
A. thaliana  IKVMLSLGGG IGNYSIGSRE DAKVIADYLW NNFLGGKSSS RPLGDAVLDG
C. sativus   V..L..I... A.S...S.AD ...QV.NFI. .SY...Q.D. ....A.....

          151                                                   200
A. thaliana  IDFNIELGSP QHWDDLARTL SKFSHRGRKI YLTGAPQCPF PDRLMGSALN
C. sativus   V..D..S..G .F..V..QE. KN.GQ----V I.SA.....I ..AHLDA.IK

          201                                                   250
A. thaliana  TKRFDYVWIQ FYNNPPCSYS SGNTQNLFDS WNKWTTSIAA QKFFLGLPAA
C. sativus    .GL..S..V. .......MF- AD.AD..LS. ..Q.-.AFPT S.LYM.....

          251                                                   300
A. thaliana  PEAADS-GYI PPDVLTSQIL PTLKKSRKYGG VMLWSKFWDD KNGYSSSIL
C. sativus   R...P.G.F. .A...I..V. ..I.A.SN... ......AF.- -....D..K

          301
A. thaliana  ASV*
C. sativus   G.IG*
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Figure 5. Comparison of the amino acid sequences of the *Arabidopsis* and cucumber acidic chitinases. The complete amino acid sequence of the *Arabidopsis* gene is given. Only the amino acids differing from the *Arabidopsis* sequence in the cucumber sequence are shown.

izing bands in each lane (Fig. 6). Weakly hybridizing bands were not observed even after long exposures, indicating that the acidic chitinase gene is also encoded by a single copy gene. The difference in the sizes of restriction fragments hybridizing to the basic and acidic chitinase probes suggests that the two genes are not linked.

Expression of Basic Chitinase

Total RNA was isolated from *Arabidopsis* plants at three time points: 18-d-old plants with 4 to 5 pairs of true leaves, 26-d-old plants initiating flowering shoots, and 38-d-old plants setting seed and beginning to senesce. RNA was extracted from roots, leaves, and flowering shoots of control plants and plants exposed to ethylene. A number of reports demonstrate that ethylene induces systemic expression of basic chitinase (5). Leaves of *Arabidopsis* plants were also wounded by crushing, an injury which has been reported to induce basic chitinase in bean (8).

We found that expression of the basic chitinase gene in *Arabidopsis* is age- and organ-dependent (Fig. 7). Low levels of chitinase mRNA were detected in whole 18-d-old water control plants. Ethylene increased the level of message approximately 5-fold. In 26-d-old plants, leaves from water-treated plants again contained low levels of the chitinase mRNA. However, roots contained approximately 10 times the level found in leaves. After ethylene treatment, the expression in roots increased approximately 2-fold and the expression in leaves increased approximately 30-fold. The level of chitinase expression in senescing 38-d-old water-treated plants was very high, almost equivalent to the expression in 26-d-old ethylene-treated plants. Chitinase expression was induced to the highest level in 38-d-old plants treated with ethylene and was detected in all parts of the plant. Wounding, either of 18- or 26-d-old plants, did not induce basic chitinase expression in leaves.

The ethylene-insensitive mutant (*etr*) of *Arabidopsis* (2) was used to investigate whether the constitutive root-specific expression is ethylene independent. RNA was extracted from 26-d-old *etr* plants treated with water or ethephon and analyzed by RNA-DNA hybridization. Constitutive expression of basic chitinase was observed in roots of *etr* plants (Fig. 7) demonstrating that the expression is independent of ethylene induction. Systemic expression of basic chitinase was not induced in *etr* plants by ethylene treatment.

Expression of the acidic chitinase gene was not detected in any part of water- or ethylene-treated plants. Several attempts were made to induce acidic chitinase expression by treating leaves with salicylic acid. Although salicylic acid induces acidic chitinase in cucumber (20), the *Arabidopsis* acidic chitinase mRNA could not be detected in total RNA from plants treated with salicylate or after wounding (data not shown). However, the results of a particle bombardment transient assay indicate that the acidic chitinase gene is most

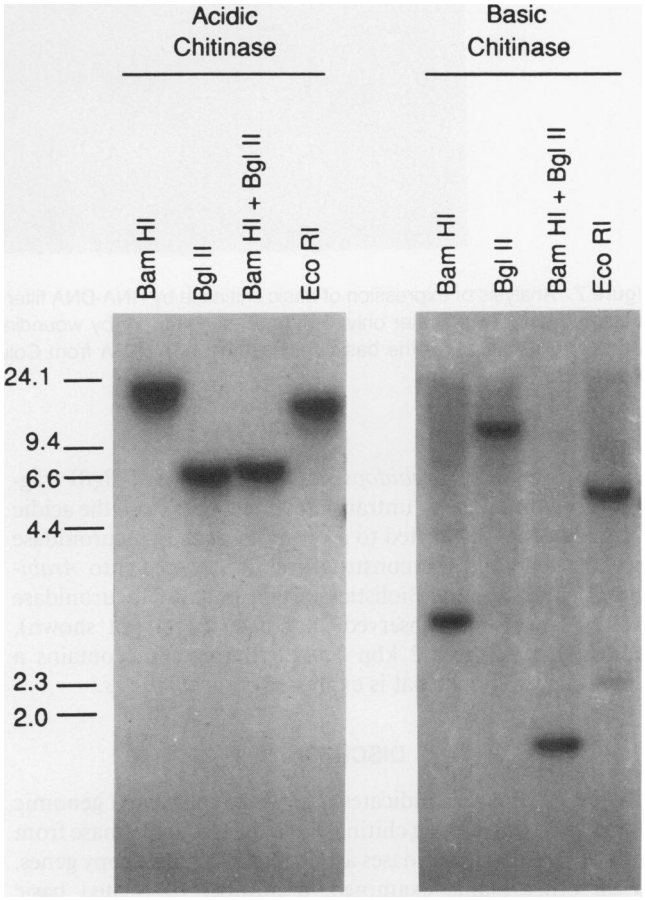

Figure 6. DNA-DNA filter hybridization of *Arabidopsis* genomic DNA probed with the chitinase gene probes. Total DNA (5 μg) was digested with the indicated restriction enzymes, electrophoresed in a 0.8% agarose gel, blotted, and hybridized to the gene specific probes. Washes were carried out under nonstringent conditions. The filter on the left was probed with the coding sequence of the acidic chitinase. The filter on the right was probed with the basic chitinase coding region.

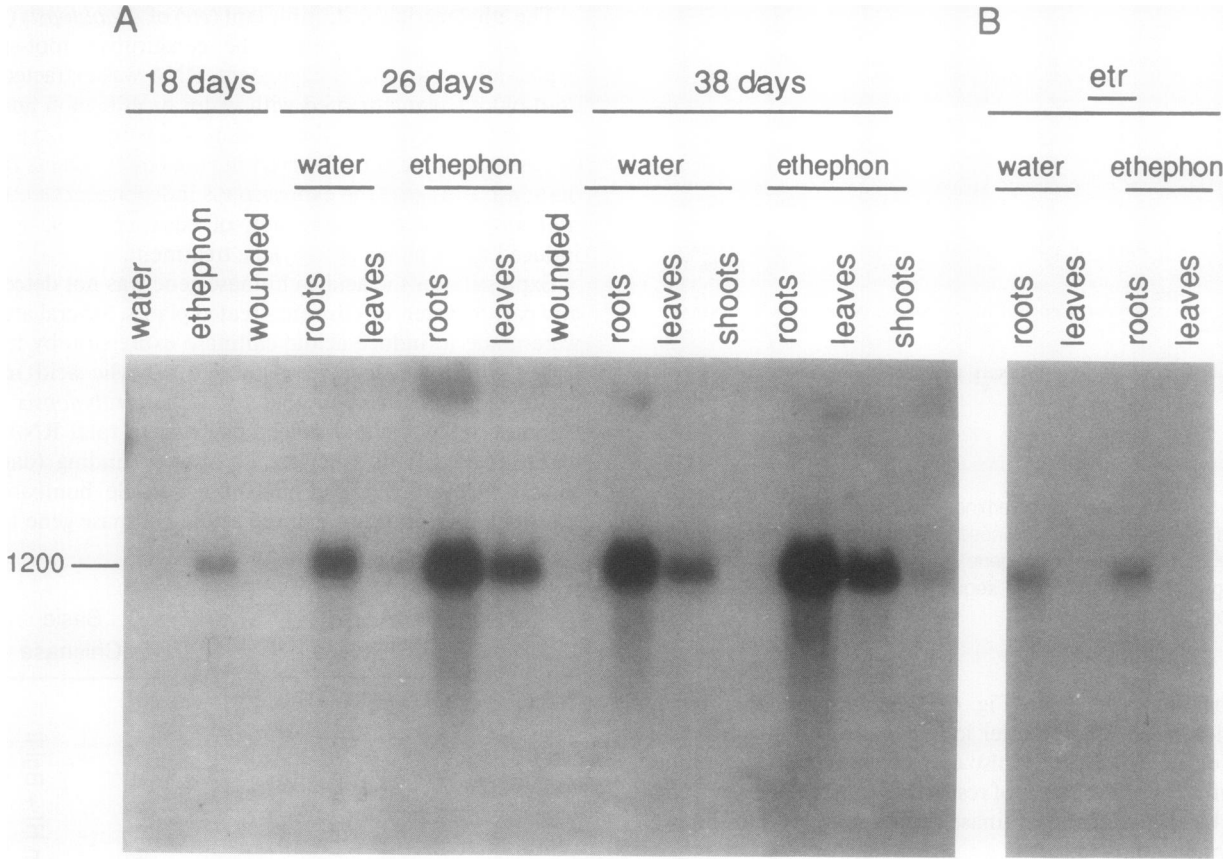

Figure 7. Analysis of expression of basic chitinase by RNA-DNA filter hybridization. RNA was extracted from roots, leaves, and flowering shoots of plants treated with water only, 1 mg/mL ethephon, or by wounding at 18, 26, and 38 d after planting. The filters were probed with the 1.5-kbp coding sequence of the basic chitinase gene. A, RNA from Columbia wild-type plants. B, RNA from 26-d-old ethylene-insensitive mutant (*etr*) plants.

likely expressed in *Arabidopsis*. The 1.2 kbp *Sal*I-*Bgl*II fragment containing the 5′ untranslated sequences from the acidic chitinase gene was ligated to a promoter-less β-glucuronidase reporter gene and the construct was introduced into *Arabidopsis* leaves with the Biolistics particle gun. β-Glucuronidase positive areas were observed 24 h later (data not shown), indicating that the 1.2 kbp *Sal*I-*Bgl*II fragment contains a functional promoter that is expressed in *Arabidopsis*.

DISCUSSION

Sequence analysis indicates that we have isolated genomic clones encoding a basic chitinase and an acidic chitinase from *Arabidopsis*. Both chitinases are encoded by single copy genes. In all other plants examined, a number of related basic chitinase isozymes are encoded in small, multigene families. The function of multiple isozymes may be to allow plants to respond in a tissue-specific or stimulus-specific manner. The isoforms of the plant defense-related proteins chalcone synthase (25) and hydroxyproline-rich glycoprotein (6) show tissue-specific and stimulus-specific expression. Similarly, the chitinase Ch1 in pea is expressed during fungal attack and after ethylene treatment, whereas a different chitinase, Ch2, is expressed primarily in maturing seed pods (17). Because the *Arabidopsis* chitinase genes are each encoded by single copy genes, the regulation and the promoters of these genes may be complex. It may be possible to identify *cis*-acting elements in these promoters that are responsive to different tissue-specific and environmental signals.

In bean, the ethylene-responsive promoter region for induction of chitinase has been identified (4). Expression of at least two of the basic chitinase isoforms is induced by ethylene, and both genes are transcriptionally regulated. No highly conserved regions of sequence similarity were found between the ethylene-responsive element in bean and the 5′ flanking sequences of the basic chitinase gene in *Arabidopsis*. It will be interesting to establish if the *Arabidopsis* basic chitinase is induced by stimuli other than ethylene and to define tissue- or stimulus-specific promoter elements.

The amino acid sequences of basic chitinases are highly conserved among tobacco, potato, bean, and *Arabidopsis*. The cucumber and *Arabidopsis* acidic chitinases also have a high degree of similarity. However, the *Arabidopsis* acidic and basic chitinases are not related. In tobacco, sequence comparison of partial cDNA clones reveals significant amino acid sequence similarity (65%) between the acidic and basic

chitinase in the C-terminal 67 amino acids (10). This suggests that, although basic chitinases in different plants are similar, the acidic chitinases may be quite diverse.

By sequence comparison of the *Arabidopsis* chitinases to published sequences, we discovered the presence of introns in both the basic and acidic chitinase genes. The protein coding region of the acidic chitinase gene contains two introns of 169 bp and 269 bp, while the coding region of the basic chitinase gene is interrupted by one large intron of 476 bp. These are the first introns described in chitinase genes. This finding was surprising because of the tendency of *Arabidopsis* genes to be less complex than homologous genes from other plant species. We are currently testing whether introns are important for the regulation or expression of chitinase in *Arabidopsis*.

The level of expression of basic chitinase in *Arabidopsis* is both organ-specific and age-dependent. A high level of basic chitinase mRNA was detected in roots but not in leaves or flowering shoots of control plants. Exposure to ethylene induced systemic expression of the gene; however, induced expression was greater in older plants. Constitutive expression was also greater in older plants. Possibly, the higher level of expression of chitinase in older plants was due to a higher endogenous production of ethylene by these plants than by younger plants. In tobacco, both chitinase and β-1,3-glucanase are found primarily in roots and in lower leaves of untreated plants. This developmental regulation is most likely controlled by auxin and cytokinin gradients within the plant (27). By examining the expression of basic chitinase in the ethylene insensitive mutant of *Arabidopsis*, we have demonstrated that the constitutive expression in roots is ethylene independent.

We could not detect expression of the acidic chitinase in total RNA of untreated *Arabidopsis* plants or in plants treated with salicylic acid or ethylene. However, activity of the acidic chitinase promoter region was detected when it was fused to the β-glucuronidase reporter gene. Possibly, this activity was induced by the wounding caused by entry of the tungsten micro-projectiles. Because the acidic chitinase mRNA was not detected in total RNA from wounded whole leaves, expression may be highly localized to the cells directly adjacent to the wound or occur for a brief time shortly after wounding. The expression of basic and acidic chitinases is induced by pathogens, but it is not known if both forms of the enzyme are coordinately regulated. We are currently investigating whether infection by pathogens will induce acidic or basic chitinase gene expression in *Arabidopsis*. The availability of molecular probes for acidic and basic chitinases will facilitate experiments to understand the mechanism of regulation and targeting of these enzymes and to evaluate their relative importance in plant defense.

Note added in proof:

The sequence of a tobacco basic chitinase gene was recently published (Plant Mol Biol 14: 357–368, 1990) showing the presence of two introns within the coding sequence. The position of the first intron corresponds exactly to the position of the intron in the *Arabidopsis* basic chitinase gene.

S1 mapping of the 5′ end of the basic chitinase mRNA indicates that transcription initiates at position 705 in the sequence (Fig. 2) which reduces the signal peptide to 20 amino acids.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Bartnicki-Garcia S** (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu Rev Microbiol **22:** 87–108
2. **Bleecker AB, Estelle MA, Somerville C, Kende H** (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241:** 1086–1089
3. **Broekaert WF, Peumans WJ** (1988) Pectic polysaccharides elicit chitinase accumulation in tobacco. Physiol Plant **74:** 740–744
4. **Broglie KE, Biddle P, Cressman R, Broglie R** (1989) Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell **1:** 599–607
5. **Broglie KE, Gaynor JJ, Broglie RM** (1986) Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc Natl Acad Sci USA **83:** 6820–6824
6. **Corbin DR, Sauer N, Lamb CJ** (1987) Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. Mol Cell Biol **7:** 4337–4344
7. **Gaynor JJ** (1988) Primary structure of an endochitinase mRNA from *Solanum tuberosum*. Nucleic Acids Res **16:** 5210
8. **Hedrick SA, Bell JN, Boller T, Lamb CJ** (1988) Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. Plant Physiol **86:** 182–186
9. **Henikoff S** (1987) Unidirectional digestion with exonuclease III in DNA sequence analysis. Meth Enzymol **155:** 156–165
10. **Hooft van Huijsduijnen RAM, Kauffmann S, Brederode FT, Cornelissen BJC, Legrand M, Fritig B, Bol JF** (1987) Homology between chitinases that are induced by TMV infection of tobacco. Plant Mol Biol **9:** 411–420
11. **Klee HJ, Muskopf YM, Gasser CS** (1987) Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol Gen Genet **210:** 437–442
12. **Klein TM, Gradziel T, Fromm ME, Sanford JC** (1988) Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Biotechnology **6:** 559–563
13. **Kombrink E, Schroder M, Hahlbrock K** (1988) Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc Natl Acad Sci USA **85:** 782–786
14. **Kunkel TA, Roberts JD, Zakour DA** (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol **154:** 367–382
15. **Legrand M, Kauffmann S, Geoffroy P, Fritig B** (1987) Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754
16. **Mauch F, Hadwiger LE, Boller T** (1984) Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol **76:** 607–611
17. **Mauch F, Hadwiger LA, Boller T** (1988) Antifungal hydrolases in pea tissue I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol **87:** 325–333
18. **Mauch F, Mauch-Mani B, Boller T** (1988) Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936–942

19. **Mauch F, Staehelin LA** (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1:** 447–457
20. **Metraux JP, Burkhart W, Moyer M, Dincher S, Middlesteadt W, Williams S, Payne G, Carnes M, Ryals J** (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc Natl Acad Sci USA **86:** 896–900
21. **Meyerowitz EM, Pruitt RE** (1985) *Arabidopsis thaliana* and plant molecular genetics. Science **229:** 1214–1218
22. **Mount SM** (1982) A catalogue of splice junction sequences. Nucleic Acids Res **10:** 459–472
23. **Roberts WK, Selitrennikoff CP** (1988) Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol **134:** 169–176
24. **Rochester DE, Winer JA, Shah DM** (1986) The structure and expression of maize genes encoding the major heat shock protein, hsp 70. EMBO J **5:** 451–458
25. **Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ** (1987) Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol Gen Genet **210:** 219–233
26. **Shah D, Horsch R, Klee H, Kishore G, Winter J, Tumer N, Hironaka C, Sanders P, Gasser C, Aykent S, Siegal N, Rogers S, Fraley R** (1986) Engineering herbicide tolerance in transgenic plants. Science **233:** 478–481
27. **Shinshi H, Mohnen D, Meins F** (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA **84:** 89–93
28. **Tuzun S, Nageswara R, Vogeli U, Schardl C, Kuc J** (1989) Induced systemic resistance to blue mold: Early induction and accumulation of β-1,3-glucanases, chitinases and other pathogenesis-related proteins (b-proteins) in immunized tobacco. Phytopathology **79:** 979–983
29. **van Loon LC** (1989) Stress proteins in infected plants. *In* T Kosuge, EW Nester, eds, Plant-Microbe Interactions, Vol 3. McGraw-Hill, New York, pp 198–237
30. **Vieira J, Messing J** (1987) Production of single-stranded plasmid DNA. Methods Enzymol **153:** 3–11