

# Regulation of Cucumber Class III Chitinase Gene Expression

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The chromosomal region encoding the acidic class III chitinase from cucumber has been isolated and characterized. As a result of an apparent gene triplication, the pathogen-induced gene (*CHI2*) is flanked by two closely related genes with complete open reading frames (ORF). The high level of conservation within the three ORFs suggests an essential role for each encoded protein in plant growth and development. The developmental and tissue-specific expression of RNA from each gene was analyzed using both gene-specific probes and RNA-PCR. The expression of each gene in response to various inducing treatments was also characterized. Only transcripts corresponding to *CHI2* were detected. Chitinase mRNA abundance increased slightly following cycloheximide application; however, its potent induction by salicylic acid was inhibited by cycloheximide treatment.

*Additional keywords:* systemic acquired resistance.

When infected with necrotizing pathogens many plant species react by inducing both a local and systemic resistance against subsequent infection by unrelated fungi, viruses, and bacteria (Chester 1933). In tobacco, this protection is observed in both the previously infected leaves (local acquired resistance, LAR) as well as in the uninfected leaves (systemic acquired resistance, SAR) (Ross 1961a, 1961b). Cucumber plants also become resistant to pathogen infection following an initial infection with either the necrotizing fungus *Colletotrichum lagenarium* or tobacco necrosis virus (TNV) (Kúc 1982). The protection is effective against a broad range of pathogens and can last for weeks to months following initial infection.

Acquired resistance can also be induced by compounds such as 2,6-dichloroisonicotinic acid (INA), salicylic acid (SA), or certain other benzoic acid derivatives (Métraux *et al.* 1991; Uknes *et al.* 1992; Ward *et al.* 1991). These chemical "activators" are thought to act by stimulating the mechanisms of resistance in the host plant (Métraux *et al.* 1991; Ward *et al.* 1991). Pretreatment of crop plants with such activators to improve resistance against a broad range of pathogens could be an attractive disease control practice.

In cucumber and tobacco an increase in SA levels precedes the onset of SAR (Malamy *et al.* 1990; Métraux *et al.* 1990).

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At least nine tobacco SAR gene families are coordinately induced at the RNA level by TMV, SA, and INA (Ward *et al.* 1991). Included in these are the pathogenesis-related (PR) protein gene families (Gianinazzi *et al.* 1970; Van Loon and Van Kammen 1970). A unique form of chitinase accumulates to high levels in cucumber following pathogen infection and is correlated with SAR (Métraux *et al.* 1989). This class III chitinase protein is extracellularly located, acidic in charge, and structurally unrelated to either the class I or class II chitinases of tobacco (Shinshi *et al.* 1990). The mRNA encoding this protein accumulates after SA and TNV treatment (Métraux *et al.* 1989). As such, chitinase induction provides a useful molecular and biochemical marker for the induction of systemic acquired resistance in cucumber.

Cucumber provides a good model system for dissection of the SAR response. In particular, cucumber has a relatively small genome size (Arumuganathan and Earle 1991) which may be useful for protein-DNA studies *in vivo*, and a well-established SAR response with inducible pathosystems (Kúc 1982). Moreover, cucurbits exude phloem sap when petioles are cut, which has facilitated the isolation of putative signal molecules (Métraux *et al.* 1990). As a first step toward analyzing the signal transduction cascade responsible for SAR in cucumber, we isolated and characterized the class III chitinase gene. Sequence analysis of a 12,124-bp genomic clone revealed the presence of three complete open reading frames encoding the chitinase gene product. These genes share greater than 90% identity throughout the coding regions, but the homology drops significantly in the 5' and 3' flanking regions. The flanking genes, *CHI1* and *CHI3*, although containing features of functional genes, are not expressed under our experimental conditions, or are expressed at levels below the limits of detection. In contrast, *CHI2* is induced by pathogens, SA, INA and in response to tissue-specific and developmental cues.

## RESULTS

### Cucumber class III chitinase gene structure.

The cucumber chitinase gene was isolated from a genomic library constructed in  $\lambda$ EMBL4. Sequence analysis of a 12,124-bp genomic clone revealed the presence of three genes closely related or identical to the cucumber class III chitinase cDNA (Fig. 1). Each gene contains a complete, uninterrupted open reading frame oriented in the same direction. The 5' flanking sequences contain potentially functional promoters, based on the presence of TATAAA and CAAT sequences. Further-

1 GAATTCCTTTTACAATTAACAAATAAACGAACATTGTATTTAAAATTTAAAATAATAAAATGAATACATTACAGTATAGATATCAAAAATAAAA 100

101 AATCAAAATAGGATTAATAATGTCTCAGCTTACATAAACTATATTTAGAGCTTAGAGTTGAGGTTTCGATATCTCAGCTTACATGCTGGTGTATATAT 200

201 ATATTTATGAGCATATTTTGTCACTATTATAAGCTTTTATTTTGGGCTAAATATGCAAGTGGAGGAGAAATAGTTAAGCACATAACAAAATCG 300

301 TAGTTGGCCATTACATGTGGGAGAAATTCGATGTGAAGATCAATATATATATCAACTCTCAAAATATTATAGATCTCTGACTATTTCTCTTTCTA 400

401 TTTTATATATTACATCAATCCCTCAACAAATAAATCATTCACTATACAAAATTTAAATAATATGTGATGGAATGATCATCTCATATAAATAA 500

501 ATTTAAACATATGACACCAACCAATCAATAAACCGTACATAAATCAACTATTATTATAAATCACTTAATTTAGTCACAAACTAACGTTTATAGAC 600

601 ATGGTTGGATTCACAAAGGAATAACACTTCTCTATTTTAAAAAAGTTAGTACTTATGTTTGGTTGTATACCAAACAGCTGATTAATTAATGTCAAAT 700

701 TACTTACATAACAGCATATAAATAACTTACAGTCACTAATAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 800

801 TAGATTTGGCTTATAGGTGATTGGGTATCGAGGTATTACACATTACACATAAGTAATGAGTGGACAAAAGTTGGTCTTGAGGTGGCTCGGATG 900

901 TTTAGAGTTGATTGGGCACAAAGTTTCCACTAAAGTATGGGTTTACCAAAAGTGTGACCTAAAAGATGGGTGGTGGTGGCCATCACTCAACCAATTT 1000

1001 AGTAAACTTAATAATATATAAATAGGTTAACAATCAAGCAATCAAAAATCGTGCTTGTATTTAAGTGAATTTCTTGAATGATTAATCTCTTATCAA 1100

1101 CGTATTCATGACAAACTATTTTCTTATAAATATTCTCTATAGTACCTCACACTTTAACGTTTAAAAATAATTTTCTCACACAATAAATAATC 1200

1201 TTAATACTCTAAGCTAAATCAATTAATAATACTTGCATTTCAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 1300

1301 AATCATAAAATTTAACTATATGAATTAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 1400

1401 TTCGATCAAGTCATAAGTTATCTCTACAAAATAAGTATAAGTTAATGAGTAACTAAAATGCAGATTGTTGAAGAAAACAAAATACTGTACTGTG 1500

1501 TGTTTGAATTTTCCACATATATACAGCTATTGTGACAAATGATAAATGTGAACGTGGAAATAATTTGTTTGTAGAAAGTTGGAAGTTGAAA 1600

1601 TGCTCAACTTTTAAACAAATAAATCCGTTAATACGGTACTTAGGACTCACCTTAACATATAGTCAATAGTATTCTTCTTGGTCCACAACTTTT 1700

1701 TTAATATACTTTTACGTAAGTAATGAACTAACTATCGCTCAAAAAGAACAGGCTTTGCTCGCTTAAAGCACGTCGGCATATTCATCTCTGTCA 1800

1801 GTACAAAATACTGTCTGACGAGAATCGTGGAGTTGATCTTCAACAACAAGACGAGTCCGCTGGTGTAGAAAATAATGTTGATGGTTTCAATAATA 1900

1901 TTGCTGAGTATGATAGCAGACTAATTTGTTATAGAGGTTATAGAGGTTGAAATCTTACAAAATTTCTAATCGTCAAACTAATGAGAGTTAAA 2000

2001 GAGTTTCTCATATCTCAAAGGATGGTAGGAATTTTGTAGTACCTAATAAGTTATAAGCAAGATGGTGTGATGTGCTGGGATTAATACAAATTT 2100

2101 AGTACAAAATATTCATATTAAGATAGTTCATTTGGTCTTCCACTTTAGTTTGTGTCATAATCTCAAACTTTAACAAATCAAAGGCACTACT 2200

2201 AAACAAAATTAATCTGTGAATAACAAACTACAAATTTTTTAAAAAATAATCGGAGAATAAATTAATCACTACAAAACCTCGTACTTAACACCA 2300

2301 CATAAATAAATTTAACTATATGAATTAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 2400

2401 ACATTTTTTCCAAACCCAAATGACACTTACAAATACTTTGATTTGATCAACAATAACCTACGTGATTACCTTTTCCCTTCCCAATAAATCACTTCA 2500

2501 TATTTCCACTGTTTAAACACATAATCTCAAGAAAAGCTCTTAAAGAAATGGCTGGCCAAAATAAATACTACAACTTCCATCTTCTCTCC 2600

2601 TCTCTCTATTTTCCGCTTCCACACGGCTGGAATCGCCATCTATTGGGCCAAAACGGCAACGAAGCTCTCTTGCATCCACTGGCCACTGGAAA 2700

2701 CTACGAGTTGGTCAACATAGCATTCTCTCATCTTCCGACGGGTCAAACCTCGGCTTCAACCTTCCGCGTCACTGCAACCTGACAAACGCTTTGC 2800

2801 GCCTTTGTGAGGAGCAAAATAAATCTTCCAAAGTCAAATGTCAAGTTCTCTCTCTATTGGAGGCGGGTAGGAGATATTCACCTTCTCCCGCC 2900

2901 ACAATGGAAACAAGTGGCAGGCTTCTCTGGAACAACCTCTCGGCGGCGAGTCCGATCCAGGCCACTGGCGATCGGTTTGGATGGCTTGATTT 3000

3001 TGTATCGGTTTGGCTGGGCGACTTCTGGATGACTAGCTCGGAGCTAAAGAGTTTGGACAAGTCAATTTATCTGCCGCCCAAGTGTCCGTTCT 3100

3101 CCAGACGCTCAGCTAGACCGCCGCGATCAGAAGTGGACTGTTCGATTCGCTGGGTTCAATTTCAACAACCCCGCATGATGAGGATAACCGGG 3200

3201 ACAACCTCTGAGTATGGAATCAGTGGGCGGCTATCCGATATCGAAGCTTTACATGGGATGGCAGCGCCAGGAGCGCCGAGCGGGGGTT 3300

3301 TATTCGGGGGATGTTCTTATTTCTCAAGTTCTTCAACCAATTAATAAATCTTCCAACTATGGAGGAGTGTGTTATGGATTAAGCGGTTGCAATATGGC 3400

3401 TACAGCATGCCATTAAAGCCAGGATCCATTGAAAAGAGTAGCTATTGTTAGGACTAAGCGGTTGCAATGGCTAGACACCTGCCCTCTCACTG 3500

3501 AAATAGACAGGATTTGGTTGAAATCAATATAACAGAGCCCATCAGTATACCTCGTCTAATTAATCTCAAGTTGTCAAAATATTATGAGCAAAAT 3600

3601 TCTAATCAAACAATAATTACTCTATCTTACACGGAAGAATGCTCAAGGAGTTACCAGCACTATCTTTTACTCTGAACCGCTCCCAACCACTACT 3700

3701 ACATCAATAAAACCATCTCACTCTAGAAAACAACAGAAATGGCCATCAACTCTACAGTGAACGAATCCAAGACATAGTTTCCAAAAGTTCAAGAGT 3800

3801 TAATTAATTAAGATTTGGTCAAGTCCAATCAAATAGAGCTTGAATATATAAAGAAAATAATACACCTTGATATTTGTTGATTAAGAAGGAAAC 3900

3901 GTAGACGGCCATATCACTTTGGTACTTGGCAAAAAGAAAAGAAAATAATAGTACATAGATAATGAATTTGTAGTTAGACTGTAAAATAGTAAGAG 4000

4001 CAACCTTGTAGATATATCTTACAAAGGTGTGTAGAGGCAACCAACTTTCTTATCTCTTATAAATCAACTTGGGATCGAAGTTAATATCTGAA 4100

4101 CGTAATAAATCATAAGTTAACTATTGAAGCTTAAAAAATCTTATATAAATACTCTCAATTTATCTAATGCATAGTAGAAGTTATAAGATCTC 4200

4201 TCTAAAATAAGTATATAAATAAATGAGTACCTAAAATGCAAGTTTGTGAAGAAAATAAATAATAGTACTGTTGTGAACCTTTTCCACATATACG 4300

4301 CAGTACAATGTAACGTGGGAATAATTTGTTTGGATAGAATTTGGAGTTTGAATGTCAACTATAATAAACAATAAATCCATTAATTTATGATGACT 4400

4401 AGACTCATTAACTATATGATGATCAATAGATTTCTAGATTTCACGGCTAATTAATGAATTTGGACTTTTTAATGTACTCTTTACATATGTAC 4500

4501 GTAATGCATAACTTGTCTCAAAAATAAGTTTCTCTCGCTGCACAAAACAGTCGATAAATACCTAAAAGCAGTGGATAAAAATCTGTTGGGAGTTCAG 4600

4601 TTGAAGAAAATCGTGGGACTTGATCTCTGACAAACAAGCAAGTCACTAGTTGTAGAAAAATGCAATGGTGAATAACGTTGTGACGAGGTAGT 4700

4701 ATATATCTGCCAACCCCTTAACTACGTCTGGGCGTTGTACTATCTCCAGTGCACCCCTTGACCGGTTGACAATATCCGACAAAACAACCAACTATG 4800

4801 TCTAGTATGTAATCTCCCAACATTACATAAAAACAATAATGTTTGGACAAAATGTGAAAATAAAGATTAATGAACAGTACATATGATCTTAAATC 4900

4901 AAAATCAAGAGACGATATGATAGTCAATAAATCTATAGTACATAGCTATACACTTTTCGGCTAATCTGAACTTGTAAACAAGTAAAATAAGT 5000

5001 TGATCATATACCATCTCTACTATTAGAAAGTCCACAGGTTCAAATCTTGCAAAATAATTAACGTTGAACCTTTAAAATAAATAAATTTCACTGTTA 5100

5101 TACCTTAACTAATGGAGTTAAGGTGATCTCTAGGTTAAATTAACAATTTAGTACAAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 5200

5201 TTAGTTTTGTGCTAATCTAACTTAACTTTCAATTTTATATTTAATAAATTTAGTATGGACTTAAACAATAAATAAATAAATAAATAAATAAATAA 5300

5301 AAAATTAAGCTTTGGTTTCTCATCACATATATCAAAAAGAGAAAGTCCAGTCAACAACAATAAATAAATAAATAAATAAATAAATAAATAAATAA 5400

5401 TCACAAACCCAAAAGAAAACAACAACAACAATAAATGPAATTAAGTCCAGAGGCTTCCATATACCTAAACCTCAATTTTACTTATAAACAATAGT 5500

5501 TAACATTTTAAATATCAATAATCCAACCATATGACATATAGAGATTTATGGACTTATTAAGCAGATGTTAACAATAGTTCAAAGGCGCCCTACTAA 5600

5601 TAACATATAAATAAATAAATTTGTTGAACATAACTACAATTTTTTTTAAAAAATAAATAGAGAATAAATAAATAAATAAATAAATAAATAAATAA 5700

5701 TATAA 5800

5801 GAAATCAAAATTAATCAACCACTTTTTTCTCTCTCAATTAGAACACGGCAATGATTAATAAATTTGTAACATTTTTTCCAAAATCAACAACTG 5900

5901 CAAAATATATATATAGTCTTATACTTTGATTGATCAACAATAACCCCTCGTGATTGCGTTTTCCCTTCCCTATAAATCACTTACAGATTTTCCATG 6000

6001 TTGACACACAACTCAAAGAGCTCTTAAGCAATGGCTGCCACAAAATAACTACAGCCCTTCCATCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 6100

Fig. 1. Nucleotide sequence of the class III chitinase genomic clone and predicted amino acid sequence of each of the ORFs. Arrows indicate start of mature protein. (Continued on next page)

6101 CTTCCGACGGCGTGAATCGCCATCTTTGGGGTCAAACGGCAACGAGGGCTCTCTTGCATCCACCTGCGCACTGGAACTAGCAGTTCGTCAACAT 6200  
S D A A G I A I Y W G Q P V L N L A G H C N P D N N G C A F L S D E

6201 AGCATTCTCTCATCTTTGGCGGGTCAAGCTCAAGTCTCAACCTTGGTGGTCAACCTGACAAACAGGTTGGCGTTTTTTGAGCGACGAA 6300  
A F L S S F G S G Q P V L N L A G H C N P D N N G C A F L S D E

6301 ATAACTCTGCAAAAGTCAAATGCAAGTCCCTCTCTATCGGTGGTGGCGGGAGTATTCACTCTCCGCGGACGATGGAAACAGTGG 6400  
I N S C K S Q N V K V L L S I G G G A G S Y S L S S A D D A K Q V A

6401 CAACTCTAATTTGGACAGCTACCTTGGCGGCGAGTCCGATTCCAGGCCACTTGGCGCTGCGGTTTTGGATGGCGTATTTCGATATCGAGTTCGGCT 6500  
N F I W N S Y L G G Q S D S R P L G A A V L D G V D F D I E S G S

6501 GGCGCAGTCTGGACGCTAGCTCAGGAGCTAAGAATTTGGACAGTCAATTTATCGCGCGCGGAGTCCCAATACCGACGCTCACCTAGAC 6600  
G Q F W D V L A Q E L K N F G Q V I L S A A P Q C P I P D A H L D

6601 GCCCGCATCAAACCTGGACTGTTCCGATTCGGTTGGGTTCAATCTACAACAACCGCCATGCAATGTTTCGAGATACCGCGCAATCTCCTGAGTTCAT 6700  
A A I K T G L F D S V W V Q F Y N N P P C M F A D N A D N L L S S W

6701 GGAATCAGTGGACGGCTTCCGACATCGAAGTTTACATGGGATGCGCGGACCGGAGGAGCGCGCGGAGCGGGGATTTATCCGCGGAGTGTCT 6800  
N Q W T A F P T S K L Y M G L P A A R E A A P S G G F I P A D V L

6801 TATTTCCTAAGTCTTCCAACCTTAAAGCTTCTCAACTATGGAGGAGTGTATGATGAGTAAAGGTTTGCACATGGCTACAGGATTCATTA 6900  
I S Q V L P T I K A S S N Y G G V M L W S K A F D N G Y S D S I

6901 GGACGCATCGCTGAAGGAGCTCCTAAGTTTAAATTTAAATTAAGCTAAGAATCTCAAAGTATTATAATAATTAAGAGTGGAGCTTCATCTCT 7000  
G S I G \*

7001 CCATTTAGTCTCATATAATTAATAGTGTGATGCAATAAATAATCTTTTTTCTTACTACTACCAATGTTTTAGAATGAAAAGTTGATGCAATA 7100  
7101 AAAACATCCCAAGTTTAAATTTAAATTTGTAACCTGTTGAAGTTTAAATACAATAATACTCTAATAAGTAAAGATTTGATATTTAGCCAAATTTT 7200  
7201 AAATCGATCCCTGTCTCTTCTAGTAAATATATATCAATTTTATTTCTACTGGGTAAATTTTTTCTAATTAAGAAACATAGTACATACAATA 7300  
7301 GTTTGATATACTCAATCAATCTTAAGCTTTAATAGATGAAGTAAATTTGATATTAATCTAACAATTTATGTTATCGGTTACTGTTGAAGAGAT 7400  
7401 GAAATTAACAATAAAGGAGTGAAGTAAATTAATCAATCATTGACGTAGACGTTACTGTGATTTTTAAGTTTCAAAATATATGACAGTCAAC 7500  
7501 TATTTCCTAATCTAAGATAAATAACTTGTTTAATCTTAAGAATCGAAAGAAAGAAATAAAAACCTGTTTAACTCAATCTTAAATGGT 7600  
7601 TTTATTTCCAACCTAGTCAATTTATTCATCTTATATCATTTGTTATTTGATGATTAATTAACATGATGTTTTTTTTTAAAAAAAATGTTTTGT 7700  
7701 TGTGATTTATGAAATGATGCTTGAATCTTATGATTTATACATATGTTGATAGATGTTAAATTTATCTATCAAAAGTAAATACAGAATAGAA 7800  
7801 AATAGAGATGATTTATTTATGTCGAAATGAAATCTGCTATCTAGTTTATTAATTTCTACATCTACTTTTTAGTTAAAGGTGCAACGAAGGAT 7900  
7901 TGGAGATTTGCAAGTGGTATCATATACTTAACTCCATTTCCATGAAGTCAAAAGTAAATTTGAGGTAGATGTCACATATTCATATGTTCTCA 8000  
8001 AGATGACATGTCATGTTGCTACATGCTGTTTACTAAGGACCACTTTGATAGTCAACAATCATATGATTTGATATATATTCATATTTATTTAT 8100  
8101 GTTCATATGATTTTTCCATGAAAAGTTGGAAAGCTGAGACTTAAGCAGCTTGGCGGGAGTAAACCCAGCGCTCGGTGAAGTGCAGATGCGCCCTGTC 8200  
8201 GCCCATGACAGCTTCTCAACCGGAAGCCCGCTCCACCAAGTCCAAGAAATAATCCGGAGATGTTGACGTTTCCATTTGCATACATACGCGCGGAT 8300  
8301 TGTGTAGGATTTGAACCCAAACGAAATCAAACCACTGTTTAAAGGCAAAATGATGATGCTCTCTCTCCACAGGAAATTTCAATCAATCACT 8400  
8401 ATCTGCTCATCAAGGACTGCTGCGACATGCAAAAAGGATGACCGTGTACAGCTTATGCTCTCTTACAAAAAATTTAGCCACGCAACATCTC 8500  
8501 GATAAGGAGATAATGATAATGATAGAAATTTAGATGATTAATAACAAGTACTTTAGGAGAAATACATGTTGAGGAAATAAGGATGATGCT 8600  
8601 AAACATTTATGATGCTATTTTCAGTCAACTTTGGGATAATATATTTTTTCAATGTTGAATGTTTTATTTGATCTTGAGATCCATTTGACGATTT 8700  
8701 GTTATAATGTTTTTTTGGCTTTTTGAAATGAGATCGATGACCCATAACCCAAAGCATCGATAATTTTTTACTTGGTGGAGTCTTGTAAAAAA 8800  
8801 GATAAACAAGAAAATGATAAACTAATAAAAAATTTATCTTATAAATTAATTACTTCAAATTTGGGAAACCTGAAAGTACATAGAGGATATTT 8900  
8901 AATGATGGATCAGAAATGAAAGTACATAGAGGATTTTTAGTGTATGGATGAAAATGCTTGGTGTACTTAAATGAAATGATAAGAAAATTT 9000  
9001 GGAGAAAGATTAATAAGATTTGCAATGATGATTTCCGAAAGAGATTTACTTACTTGAATTTCAATCTGCAATTTTATATATATATCTCTA 9100  
9101 AATGACATACAAAGTAAATTAATTAACCTTTTTTTTTTTTCAATTTAGAACATTAACCATTTAAATTTGTAACATTTTTTCCAATCCAAA 9200  
9201 TGACACTACAAAATTAATATATGATCTACTTTGATTTGATCAACAATAACCCCTTCGTGATTCATTTCCCTCCCTAATAAATCACTTCAATTTCC 9300  
9301 ATTTGATGATACAGAACTCAAAGAAAGCTCTTAAGCAATGGCTGCCACAAAATACTACAACCTTTCCATCTCTCTCCCTTTCTCTATTTTC 9400  
M A A H K I T T T L S I F F L L S S I F

9401 CACTCATCCGACGGCGTGAATCGGCATCTATTGGGGTCAAACGGCAACGAGGGCTCTCTTGCATCCACCTGCGCACTGGAACTAGCAGTTCGTCA 9500  
H S S D A A G I G I Y W G Q N G N E G S L A S T C A T G N Y E F V N

9501 ACATAGCATTTCTCTATCTTCCGCGGGTCAAACCTGCGGCTCAACCTTGGCGGCTACTGCAACCTGCAACAACGATGGAAACAGTGG 9600  
I A F L S S F G G Q P V L N L A G H C N P D N N G C T I L S N

9601 CGAAATAAATCTCCGCAAGTCAAATGCAAGTCCCTCTCTATTGGCGGTGGCAGGGGAGTATTCACTCTCCGCGGACGATCGAAAGAA 9700  
E I N S C Q S Q N V K V L L S I G G G T G S Y S L S A D D A K E

9701 GTCGCAACTCATTGGAACGCTACTCTCCGCGGAGTCCGATTCCCGCCACTGGCGGCTGCGGTTTTGGATGGCGTATTTCGATATCGAGTGG 9800  
V A N F I W N S Y L G G Q S D S R P L G D A V L D G V D F D I E F G

9801 GCTCGGACAGTCTGGGACTACTGCTCAGGAGTAAAGAGTTTGGACAGTCAATTTATCGCGCGGAGTTCGCGATCCGACGCTCAACCT 9900  
S D Q F W D V L A Q E L K S F G Q V I L S A A P Q C P I P D A H L

9901 AGACGCGGATCAGAACTGGACTGTTGATTCGCTGGGTTCAATCTACAACAACCGCTCATGATGATGAGATTAACCGGACATATCTCGAGT 10000  
D A A I R T G L F D S V W V Q F Y N N P S C M Y A D N T D D I L S

10001 TCAATGATCAAGTGGGCTTCCGATTTGAAGCTTTACATGGGATGCGCGGACCGGAGGACGCGCGAGCGGGGATTTATCCGCGGATG 10100  
S W N Q W A A Y P I L K L Y M G L P A A P E A A P S G G F I P V D E

10101 AGCTTATTTGAACTTTCCAACCTTAAAGCTTATCCAACTATGGAGGAGTGTATGATGAGTAAAGGTTTGAACATGGCTACAGGATGGCAT 10200  
L I S E V L P T I K A Y S N Y G G V M L W S K A F D N G Y S D A I

10201 TAAAGCAGCATATATCAAGTGAAGGAGCTCCTAAGTTTAAATTTAAAGCTAAGAATCAACCTCAAAGTATTGTAATAATTAAGAGTGGAGCT 10300  
K D S I Y Q L K G S S \*

10301 TCATCTCTCAATAGTCTGCTACAATTAATCTCTTTTACTACTACTACTAATGTTTTAGAATTAAGTTGATATCAATAAAAAATTTCC 10400  
10401 AAGTTTATTTCAATTTGCAAAAATGTTGAAAGTCTTTAAACCAATATAATCTCATTAAACATGAGAAATTTATATTTAGCCATATGTAAGAATA 10500  
10501 TTTCTTATTTGGAAGCAATATGAGAAATTTACTCTTATTTGGTGGAAATTTTTCTAATAAAAAATAGTATACAGGATGATGATATAATCAC 10600  
10601 AATTCATCCAAATTAAGCTTAAAGTTAATAGATGAAGTTAAATTTATATAAATCAACAATTCATGTTGAACCTATAAAGATATGATTATACAG 10700  
10701 CAAAAAATAAATGCAAGGTTGAGTACCATGTCATAGATGCAAT 10800  
10801 TTTAAATAATATACAGAGTAAATTAACCTAAATAGTAAATGAAAGTTTAAATTTTCTAATGTTCTAATTTGATTTAATAAAAAACCACTTGT 10900  
10901 ACATAGCTCAATGATAAATAAAGCTGCTCCCAATGTAATAGTGTGTTTTAAGTCAAAATGATGATTTACATAACCAAAAACAAGAACT 11000  
11001 CATATTTCTGATTTTTTTTTTCTCTCAATGATTAAGTCTATTAATCTTTTTTAAAGTATAAATGCTATGTTGATATGATATCTTGAC 11100  
11101 CCATTATACCCCAATAAAAAATATATGTTTTCTAACAAACGCAAACTTGAAGTATGGAGTGGAGATTAATTAATTAAGGTTGATGTTGATG 11200  
11201 TACTGCTCAATTTTAAATTTTCAAAATATTTGACTGACACTACTTCTCTAAGATAAATAACTTTTGTTTAATGATCAACTCTCAATTTGTTTT 11300  
11301 TTTAATTTCCAACTAGATTTATCTCCTATTTCTGTTATTTGATGATATATACATATGATTTTTTAAAGATATATATAGAGATATCAATAGACATTT 11400  
11401 TGAAGCTTTGATTTATAGATATATATGATAGATTTCAAGAGTGCATCATATCTTAAAGATCCATTTTTTTTTATGAAGCTCAAAGATTTGAG 11500  
11501 TAGAAGTGTCTATATCTAATTTATTTGTCGAAGTGGACCATTTGCTACATGCTCATGTTTTTACTAAGGTTACGTTGAGATATATCTTTACAAAGT 11600  
11601 GTGTGTTAGGGCAACAACCTCTTTGTTATCTTCTCAGCAATAATTTGGGAGGAGCTGTTATTAATAATAGTTTTCTGATGATGTTGATATATA 11700  
11701 TTTCCAAATCTATTTATGTTATGATGATGATTTTCCATGAAAAGTTACTCTAATAAAAAGTAAATTTTATGATCAAGTGTACATATAGATATA 11800  
11801 AAGTGGGCAACAATAACCTTTTTTGTTCCTTCTTATGTTTACTTATTCTGATGTTTTAATAAATACTAATAAATAAGTTTATGGAAGAG 11900  
11901 AAGCTCAAAGACTTCTTTAAAGGCTGCTGACCAATTAATCAATGCTTACTCCACAGCAATAATCCCTCATCTGGAAGAACTTTAATCCCTGG 12000  
12001 AAGAACCTTAGACTTAAGCAGTTGCGCGAATAAAACCCGCTGGTGCAGTGCAGATGCCCGGTAGCCCATGACAGCTTCCCACTGAAAC 12100  
12101 CCGCTCCACCGTTCCAAAGATTC 12124

Fig. 1. Continued from preceding page.

more, the 3' regions of each gene contain polyadenylation sites. The high degree of similarity among the coding regions and the divergence at the 5' and 3' flanking regions are illustrated in the pairwise dot matrix comparisons shown in Figure 2A. The mature protein coding sequences of the three genes are >93% identical at the nucleotide level. This similarity drops dramatically outside the coding region. Comparison of cloned cDNAs to the genomic clone revealed that all 10 previously isolated cDNA clones (Métraux *et al.* 1989) matched the middle genomic ORF (*CHI2*). Figure 2B shows comparisons of the amino acid sequences of the predicted mature proteins. A high degree of homology is apparent at the amino acid level. The predicted protein encoded by *CHI1* contains a carboxy-terminal extension relative to the others, suggesting it may be localized in the vacuole (Neuhaus *et al.* 1991).

### Chemical regulation.

To determine the pattern of class III chitinase expression in cucumber and which of the three genes were active under var-

ious conditions, we analyzed RNA extracted from chemically treated leaf tissue for chitinase message. We also analyzed vegetative tissues at various developmental stages and reproductive tissues during flower development for chitinase mRNA expression. These are tissues where chitinase activity or protein have been reported to be present in tobacco (Lotan *et al.* 1989; Neale *et al.* 1990; Trudel *et al.* 1989). Initial experiments were conducted with a cDNA probe that did not distinguish among the genes. When expression was detected, a variety of approaches were used to determine which gene was expressed in a given tissue.

Spray application of SA resulted in a 10-fold increase in chitinase mRNA within 1 day (Fig. 3). Transcripts continued to accumulate for the next several days, reaching peak abundance of 62-fold induction over controls on day four, then decreasing on day five. Application of INA also strongly induced expression of the class III chitinase mRNA (Fig. 3). While the initial induction parallels that of SA, the transcript accumulated to much higher levels (486-fold induced over controls) and remained elevated.

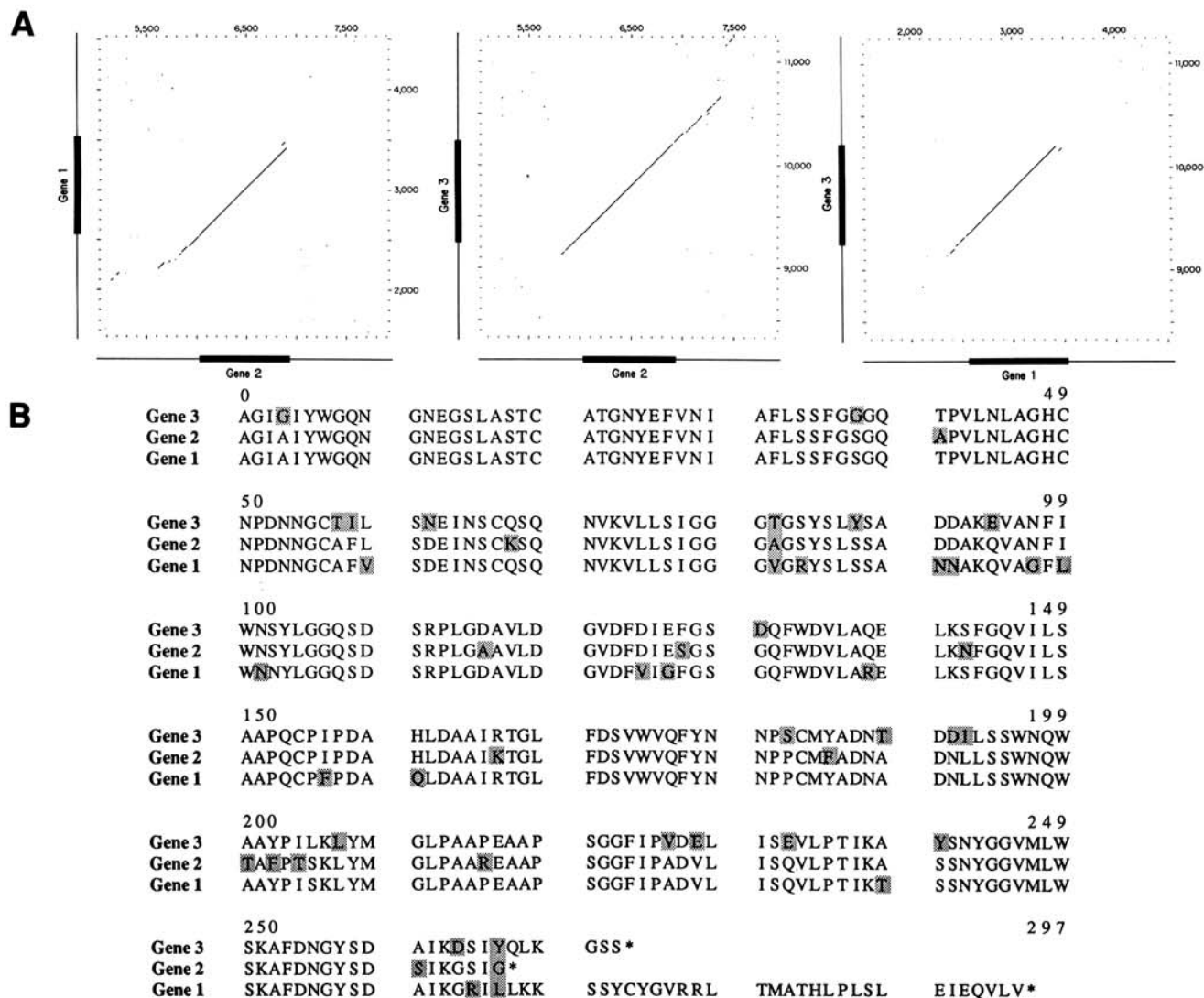


Fig. 2. Comparisons of cucumber class III chitinase genes and amino acid sequences. **A**, Pairwise dot matrix comparisons of the coding sequence (thick line) and 5' and 3' flanking regions (thin line) of the 3 chitinase genes. **B**, Predicted amino acid sequence comparisons for the mature proteins encoded by the three chitinase genes. Shaded residues highlight the differences.

### Developmental regulation in vegetative tissues.

The accumulation of class III chitinase transcripts in vegetative tissue of uninfected plants increased with age (Fig. 4). In the seedling (7–10 days postgermination), chitinase mRNA was detected at low levels in roots and cotyledons, while in plants that have begun to exhibit the vining growth habit (3–5 wk), chitinase message was present in older and mature, fully expanded leaves but not in young, expanding leaves. A gradient of expression was observed in stems of flowering plants (6–8 wk), with the maximum mRNA accumulation in basal (older) stems and decreasing levels in middle and top (younger) stems of the plant. In leaves of flowering plants chitinase transcripts were most abundant in mature leaves harvested from the middle of the plant as compared to older, basipetal leaves and younger, expanding leaves from the acropetal portion of the plant. The pattern of expression in petioles, although reduced in amount, reflected that observed in leaves. Tendrils also accumulated high levels of chitinase mRNA. In fact, the middle leaves, middle stems, and tendrils of flowering plants contained chitinase mRNA at levels approaching those found in INA-treated leaves. Furthermore, basal stems from flowering plants contained more chitinase message than the leaves induced with INA. These results indicate that cucumber chitinase mRNA is expressed in response to developmental cues in vegetative tissues of healthy plants.

### Developmental regulation in reproductive tissues.

Chitinase is developmentally regulated in a tissue-specific manner in flowers from healthy plants (Fig. 5). Chitinase mRNA was first detected during the expanding petal stage of flower development in corollas/sepals, styles, and stamens. In further experiments with RNA isolated from the corolla and sepals separately, chitinase mRNA was only detected in corolla tis-

sue and not in the sepals (data not shown). It was also present in these same tissues when the flowers were open, although at reduced levels. Open male flowers had variable chitinase mRNA levels relative to male flowers at other developmental stages as well as to female flowers. In this particular experiment transcript levels in open male flower parts were unusually low. In senescing flowers chitinase mRNA was more abundant in the female than in the male flowers. Chitinase mRNA was not detected in ovary tissue at any stage of development.

### Gene-specific expression analysis.

Several approaches were taken to determine whether the accumulation of chitinase mRNA in response to pathogen, chemical, developmental, and tissue-specific signals results from the differential regulation of the three genes or the activation of a single gene. Oligonucleotides specific to divergent regions of the 3' untranslated sequences of each gene were synthesized and used to probe RNA blots. Figure 6 shows autoradiograms of RNA blots hybridized with the gene-specific oligonucleotide probes. Only *CHI2* expression was detected in response to chemical inducers. Expression of genes 1 and 3 was not detected under these conditions. In similar experiments with RNA isolated from vegetative and reproductive tissues during development, *CHI2* appeared to be the only gene expressed (data not shown).

An alternative approach to determine mRNA levels is RNA-PCR. First-strand cDNA was synthesized from mRNA isolated from vegetative and reproductive tissue at the developmental stages when chitinase transcripts were detected with a chitinase cDNA probe (see Figs. 4 and 5). This first-strand cDNA was subsequently used as template in PCR reactions with either nonspecific 5' and 3' oligonucleotide primers or with nonspecific 5' and gene-specific 3' primers. Each approach

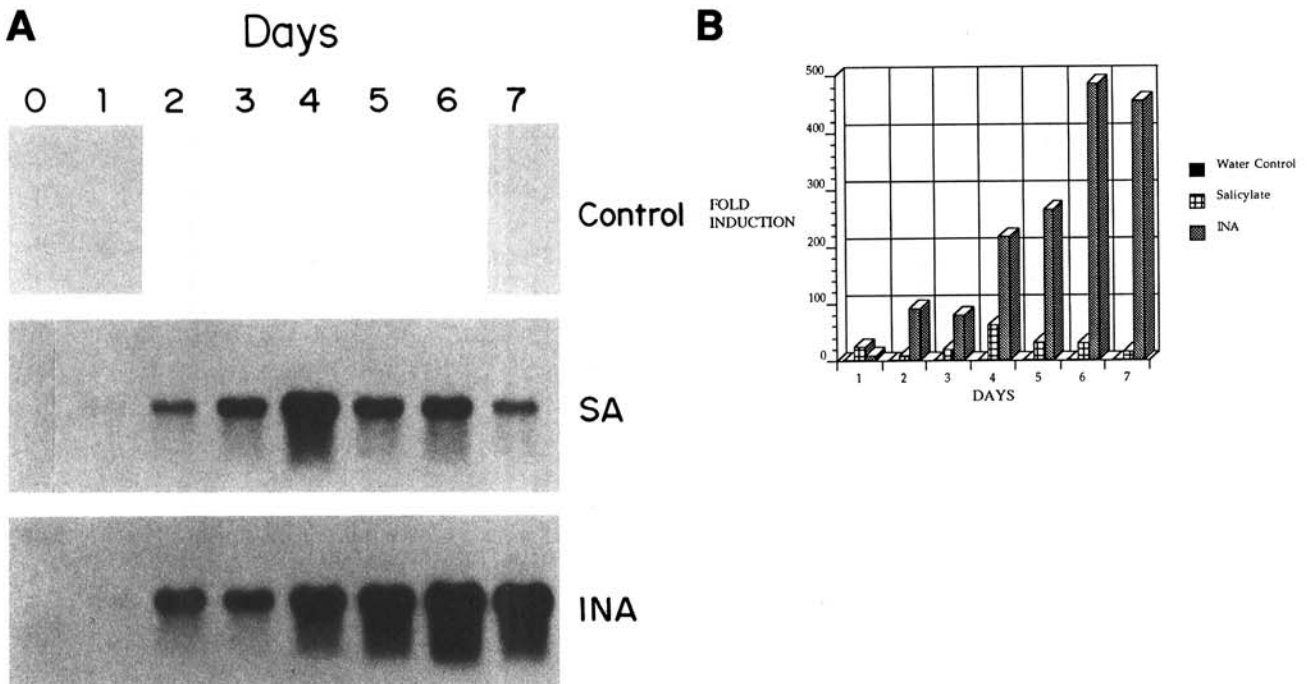


Fig. 3. Chemical induction of class III chitinase mRNA. Leaf tissue was harvested for total RNA extraction at various times after chemical application. A, RNA gel blots (10  $\mu$ g/lane) hybridized with  $^{32}$ P-labeled cucumber chitinase cDNA clone. B, Quantitation of induction of chitinase transcripts relative to the untreated control.



was repeated several times. Plasmids containing cDNA corresponding to all three genes or to each individual gene were used as control templates to test primer specificity and as controls for PCR. Nonspecific primer pairs resulted in PCR products from each of the tissue types in which chitinase mRNA had been detected by gel blot hybridization (Figs. 4 and 5). Each of these products was sequenced in both directions; all sequences analyzed corresponded to *CHI2* (data not shown). In experiments using the first-strand cDNAs as templates with gene-specific 3' primers, a PCR product was reproducibly obtained with the *CHI2* specific primer, but no product was detected using the primers specific for *CHI1* or *CHI3* (data not shown). Thus, both experimental approaches detected only *CHI2* transcript, indicating that this is the only active gene under the conditions described.

**Requirement of protein synthesis for SA induction.**

Treatments with cycloheximide (CHX) were performed to investigate the requirement for *de novo* protein synthesis for the induction of chitinase gene expression by SA. Figure 7 shows that chitinase mRNA increased in response to CHX alone. However, this increase was less than that observed with SA alone. Furthermore, when SA was applied following CHX application, chitinase mRNA did not accumulate to the level observed in response to SA treatment alone. These results suggest that protein synthesis is required for chitinase mRNA induction by SA.

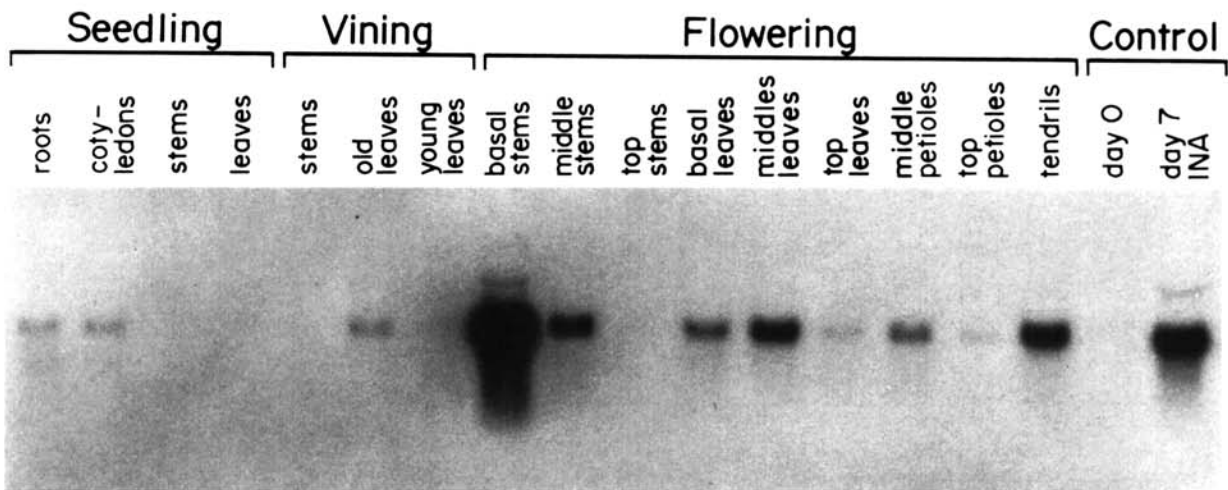
**DISCUSSION**

Expression of the class III chitinase from cucumber is regulated by chemical, developmental, and tissue-specific cues that activate a single gene. Previous work showed that chitinase activity is associated with pathogen infection and induced resistance in cucumber (Métraux and Boller 1986). We show here that chitinase mRNA accumulates in response to chemical activators of this resistance response, SA and INA, and that the time course of expression is consistent with that observed for the chemical induction of the SAR gene families in tobacco (Ward *et al.* 1991). The accumulation of cucumber

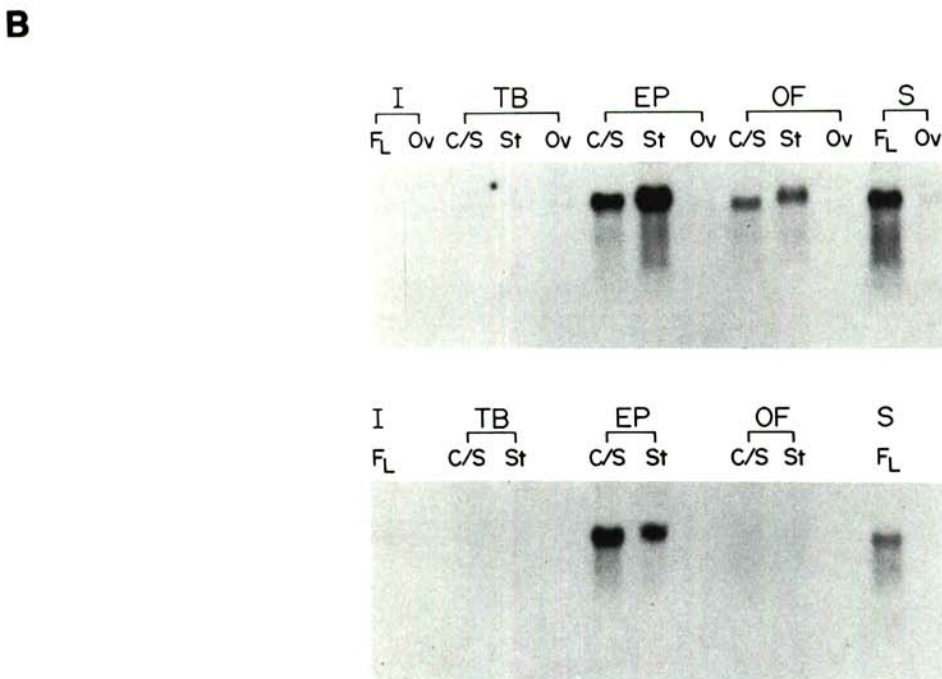
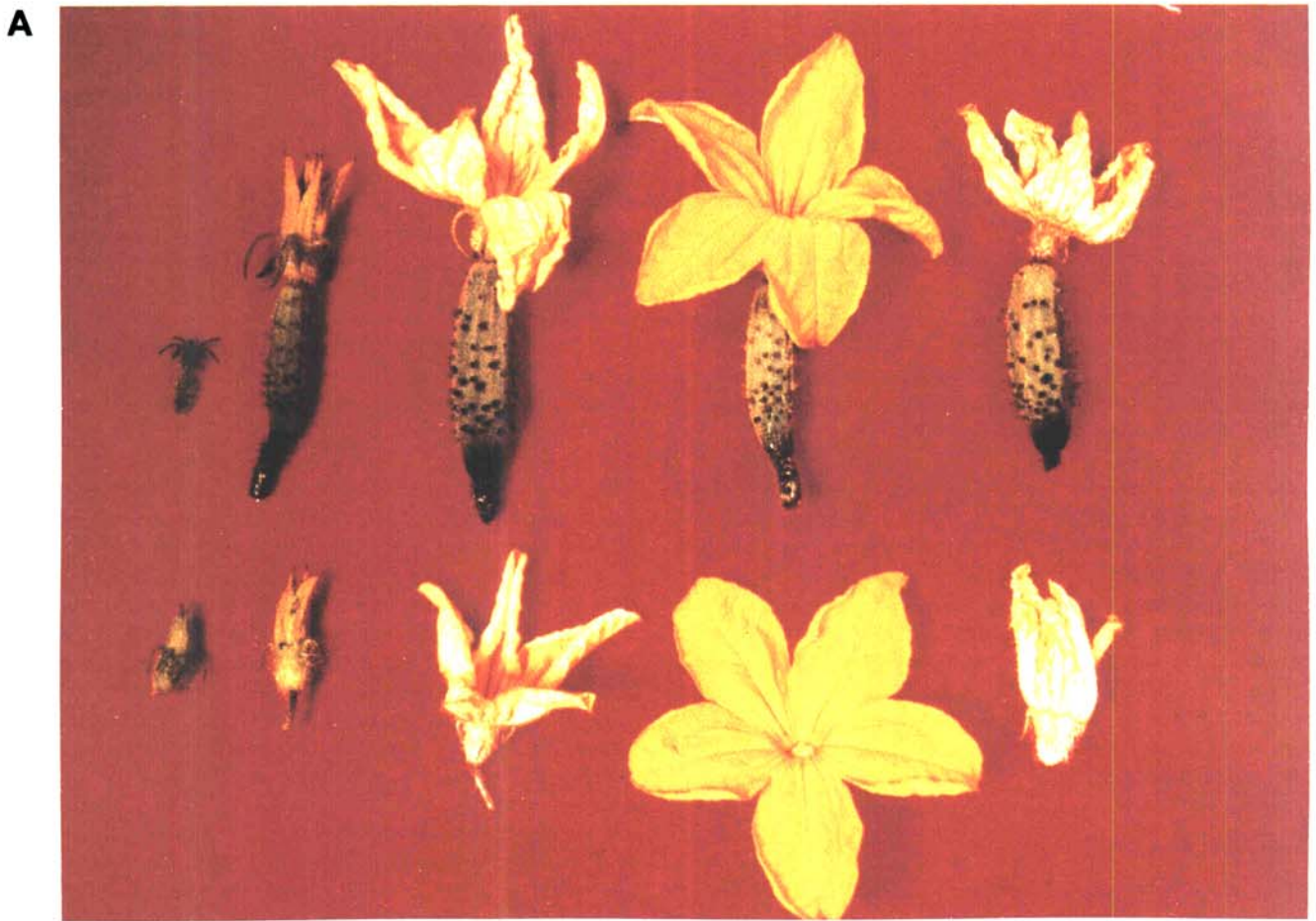
class III chitinase mRNA in response to SA and other resistance-inducing compounds further supports a role for chitinase in defense against pathogens. Furthermore, the pattern of mRNA accumulation is consistent with the increase in chitinase activity previously reported (Métraux and Boller 1986), indicating that the increased enzyme activity is at least partially due to increased message abundance.

In cucumber, chitinase mRNA expression is regulated in response to developmental signals in specific tissues. Although the class III chitinase is not structurally related to the class I and class II chitinases, the developmental expression in vegetative organs is consistent with the reported patterns of expression of these unrelated chitinases in tobacco. Increased chitinase activity in leaf tissue, cotyledons, stems, and seed homogenates from uninfected tobacco plants has been reported (Trudel *et al.* 1989). Using antibody probes, basic chitinase protein has been detected in older leaves, seedlings, and roots of uninfected tobacco plants (Neale *et al.* 1990). In tobacco flowers, low levels of chitinase activity were found in all flower parts except stamens (Trudel *et al.* 1989). In addition, PR-P and PR-Q proteins have been detected in pedicels, sepals, anthers, and ovaries using antibody probes. The results reported here support and extend these findings. We show that not only is chitinase expression confined to specific floral organs, but that this expression is also developmentally regulated. Furthermore, expression in both vegetative and reproductive organs is at least partially regulated at the level of mRNA abundance.

Chitinase transcript accumulation in young leaf tissue is sensitive to inhibition of cytoplasmic protein synthesis. Protein synthesis inhibition results in mRNA accumulation but at reduced levels compared to SA alone. Furthermore, inhibition of protein synthesis followed by SA application prevents transcript accumulation to the level induced by SA alone. Therefore, for full induction by SA, protein synthesis must not be compromised. This observation is consistent with studies in tobacco, in that small amounts of CHX that only partially inhibit protein synthesis induce PR-1a mRNA expression but high levels of CHX block mRNA accumulation in inhibited tissue (Uknes *et al.* 1993). The reason for gene induction in



**Fig. 4.** Cucumber class III chitinase mRNA levels in vegetative tissue during development. Gel blot RNA extracted from vegetative tissues at the seedling (7–10 days postgermination), vining (when tendrils appear), and flowering stages of development hybridized with a <sup>32</sup>P-labeled chitinase cDNA clone. Each lane contained 10 ug total RNA. RNA from INA-treated tissue was used as a control for hybridization.



**Fig. 5.** Cucumber class III chitinase mRNA expression in reproductive tissue during development. **A**, Developmental stages of cucumber flowers. Female (top) and male (bottom) flowers during development. Left to right: Immature (I), Tight Bud (TB), Expanding Petals (EP), Open Flower (OF) and Senescent Flower (S). **B**, Gel blot analysis of mRNA accumulation in flower tissues during development. RNA was extracted from female (top) and male (bottom) cucumber flower tissues collected at various developmental stages. (FL) Whole flower, (Ov) Ovary, (C/S) Corolla and sepals, (St) Style or stamen.

response to partial protein synthesis inhibition is not clear. Conceivably, the plant cell could monitor potential pathogen attack by sensing perturbation of protein synthesis and trigger expression of certain defense genes.

A particularly intriguing finding reported here is the presence of three closely linked genes that encode chitinase, only one of which appears active. Interpretation of genomic Southern blot data led us to the conclusion originally that the class III chitinase in cucumber was the product of a single gene (Métraux *et al.* 1989). After isolating and sequencing the genomic clone we now know that three closely linked genes encode isoforms of the class III chitinase in cucumber. However, only *CHI2* is expressed to a detectable level under the conditions presented here, even though all three genes appear structurally competent for expression. In contrast, other proteins that are encoded by multigene families (e.g., ACC synthase,

PAL) exhibit differential gene regulation in response to various environmental, tissue-specific, and developmental signals (Cramer *et al.* 1989; Rottmann *et al.* 1991). Furthermore, these genes do not show the strong homology in the coding region that is observed with the cucumber class III chitinase genes. The high degree of similarity among the class III chitinase coding sequences suggests that there is strong evolutionary pressure to maintain these open reading frames. Alternatively, the triplication could be a relatively recent event; we consider this unlikely given the divergence of the regions flanking the open reading frames. The availability of the genomic region encoding the class III chitinase will facilitate future studies aimed at understanding the molecular basis for expression of these and other SAR-related genes.

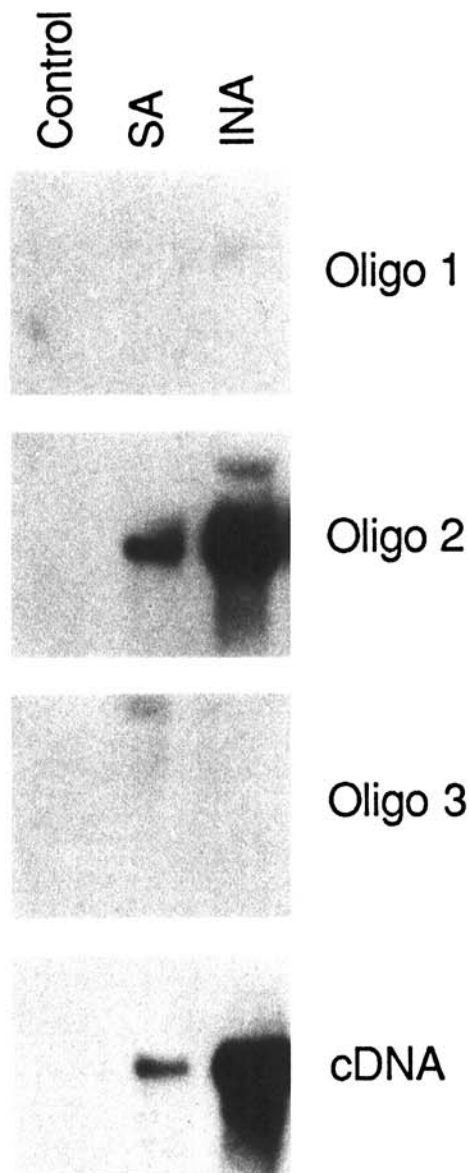
## MATERIALS AND METHODS

### Sequence analysis.

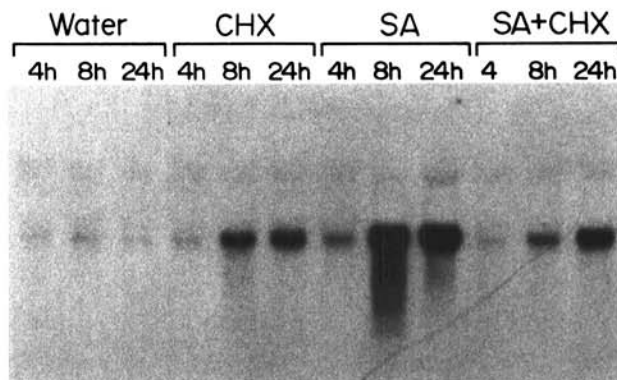
A  $\lambda$ EMBL4 genomic library was constructed from *EcoRI*-digested nuclear DNA and screened with the cucumber class III chitinase cDNA (Métraux *et al.* 1989). Purified positive plaques were restriction mapped and one 12.124-kbp clone was isolated and subcloned into pBluescript (Stratagene, La Jolla, CA). Restriction fragments of the parental clone, pBSucchrcht5, were subcloned into pBluescript and sequenced using the double-stranded dideoxy method (Hattori and Sakaki 1986). The nucleotide sequence has the GenBank accession number M84214. The genomic sequence was analyzed by dot matrix comparison using the GCG program Compare (Window: 20, Stringency: 16). Pairwise comparisons between individual genes were performed using the GCG program Gap (Deveraux *et al.* 1984).

### Plant material and treatment.

Cucumber seeds (Wisconsin SMR58) were sown in commercial growing media (Metro Mix 300, WR Grace & Co.) and grown in a glasshouse. Cucumber seedlings with two fully expanded leaves (2–3 wk postgermination) were sprayed with water, 50 mM SA or 1 mg/ml INA (as a wettable powder containing 25% active ingredient) for the chemical induction time course experiments. For experiments testing the effects of protein synthesis inhibition, 2-wk-old cucumber seedlings



**Fig. 6.** Analysis of gene-specific expression. Gel blots of total RNA (10  $\mu$ g) isolated from chemically induced leaf tissue 5 days after treatment were probed with 5' end-labeled oligos specific for the divergent 3' untranslated sequence for each gene or the chitinase cDNA clone.



**Fig. 7.** Effect of protein synthesis inhibition on salicylate induction of chitinase mRNA. Gel blot of RNA extracted from cucumber leaf tissue at the times indicated after spraying with water, SA, CHX, or CHX followed by SA 1 hr later. Each lane contained 10  $\mu$ g total RNA. The probe was a  $^{32}$ P-labeled chitinase cDNA clone.



were sprayed with water, 50 mM SA, 1 mg/ml CHX, or 1 mg/ml CHX followed by 50 mM SA after 1 hr. For developmental analysis, plants were grown in 4-L containers and trained onto trellises when tendrils appeared. Each experiment was performed at least three times with similar results.

#### Nucleic acid extraction and analysis.

Total RNA was extracted (Lagrimini *et al.* 1987), and analyzed by gel blot hybridization (Church and Gilbert 1984) with cucumber chitinase cDNA that had been <sup>32</sup>P-labeled by random priming (Feinberg and Vogelstein 1983) with a Prime Time C Kit (IBI) following manufacturer's instructions. Equal loading of lanes was confirmed by ethidium bromide staining followed by visualization under UV light. Following hybridization, washed blots were exposed to Kodak X-Omat film with intensifying screens for 18–24 hr at –80° C for autoradiography. Quantitation of hybridization signals was performed using a Betascope blot analyzer (Betagen Corp., Waltham, MA).

Gene-specific oligonucleotide probes were designed to 3' regions of each gene exhibiting little similarity to the other two genes. The following sequences were used for probes: 5'CTGGTTATAATTGATTTCAAACCAATA3' (*CHI1*); 5'CATCACACTAATTTAATATGAGACTAA3' (*CHI2*); 5'TAAAGGATTTAATTGTAGCATGACTA3' (*CHI3*). These oligonucleotides were radiolabeled with  $\gamma$ -<sup>32</sup>P-ATP in a kinase reaction (Maniatis *et al.* 1982). Specificity of hybridization was confirmed by specific hybridization of end-labeled oligos at low and high stringencies using plasmid DNA specific for each gene or *in vitro* transcribed RNA corresponding to *CHI2* as positive and negative controls (data not shown). RNA blots were hybridized with end-labeled oligos for 24 hr in hybridization buffer (1% bovine serum albumin/1 mM EDTA/0.5 M NaHPO<sub>4</sub>, pH 7.2/.24 M NaCl/7% NaDodSO<sub>4</sub>) at Tm –15° C (Tm = 16.6 [log [Na<sup>+</sup>]] + 0.41 [%G+C] + 81.5 – [820/L] where L is the length of the oligo), and washed at 37° C in 1× SSPE (0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA [pH 7.4]).

#### RNA-PCR analysis.

Total RNA was extracted as above, and the poly-A fraction was isolated using Poly A Quick Columns (Stratagene) following the manufacturer's protocol. mRNA was reverse transcribed to first-strand cDNA that was subsequently used in PCRs with primers specific for each gene. In addition, PCR was conducted with nonspecific primers and the resulting product was purified from low-melt agarose gels and sequenced to detect polymorphisms. Primer sequences: (Nonspecific primer pair) 5'CTGACAACAACGG3' (5' primer) and 5'CTTACTCCATAACATCACTC3' (3' primer). For gene-specific experiments this same 5' primer was paired with the gene specific oligos used in the hybridization experiments described above for use as 3' primers. PCR products were detected by ethidium bromide staining of 1% agarose gels followed by visualization under UV light.

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