Regulation of Cucumber Class III Chitinase Gene Expression

Kay A. Lawton, James Beck, Sharon Potter, Eric Ward, and John Ryals

Department of Molecular Genetics, Ciba Agricultural Biotechnology, P.O. Box 12257, Research Triangle Park, NC 27709 U.S.A.

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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).

Corresponding author: John Ryals.

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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 λEMBL4. 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-

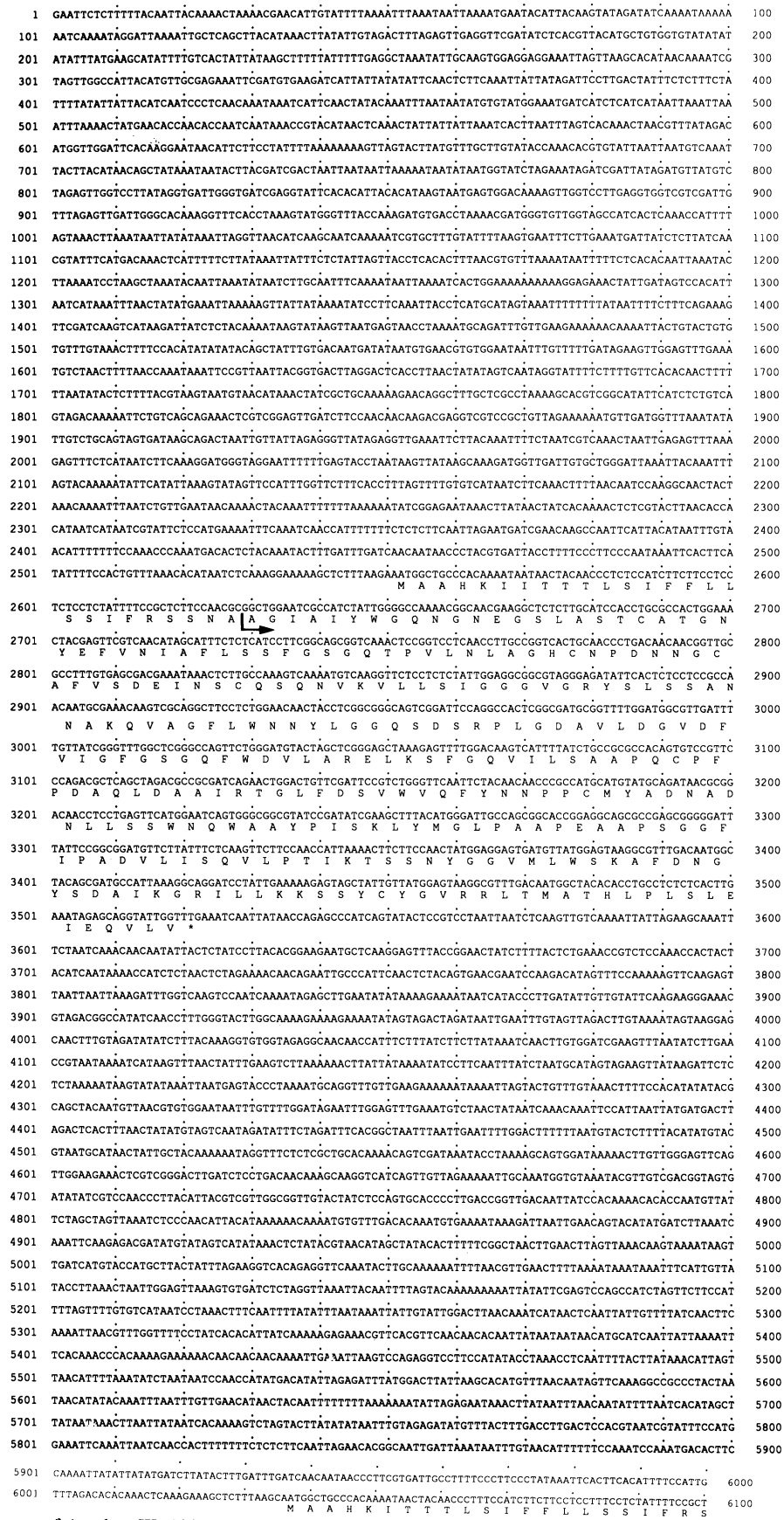


Fig. 1. Nucleotide sequence of the class III chitinase genomic clone and predicted amino acid sequence of each of the ORFs. Arrows indicate start of mature protein. *(Continued on next page)*

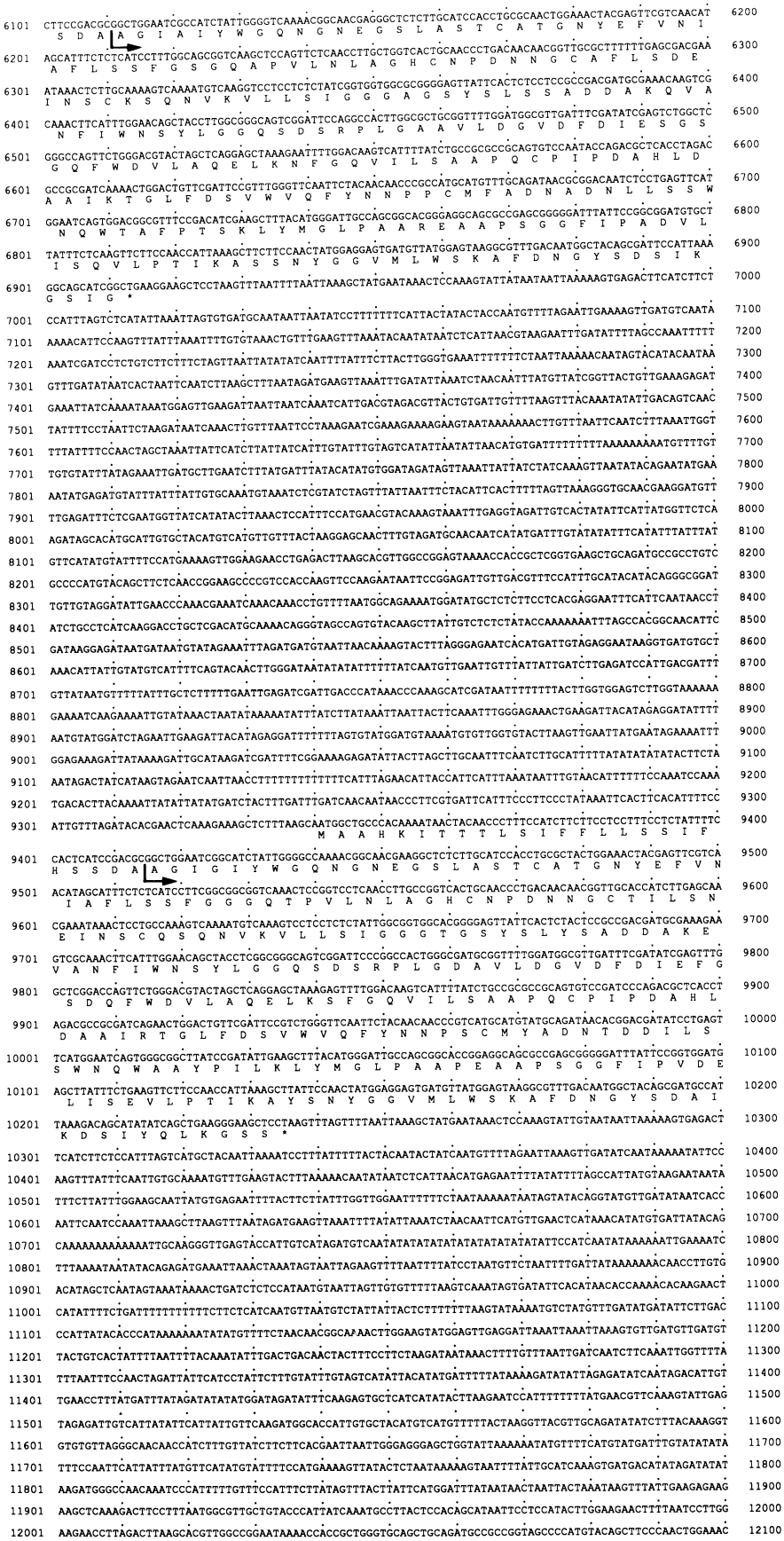


Fig. 1. *Continued from preceding page.*

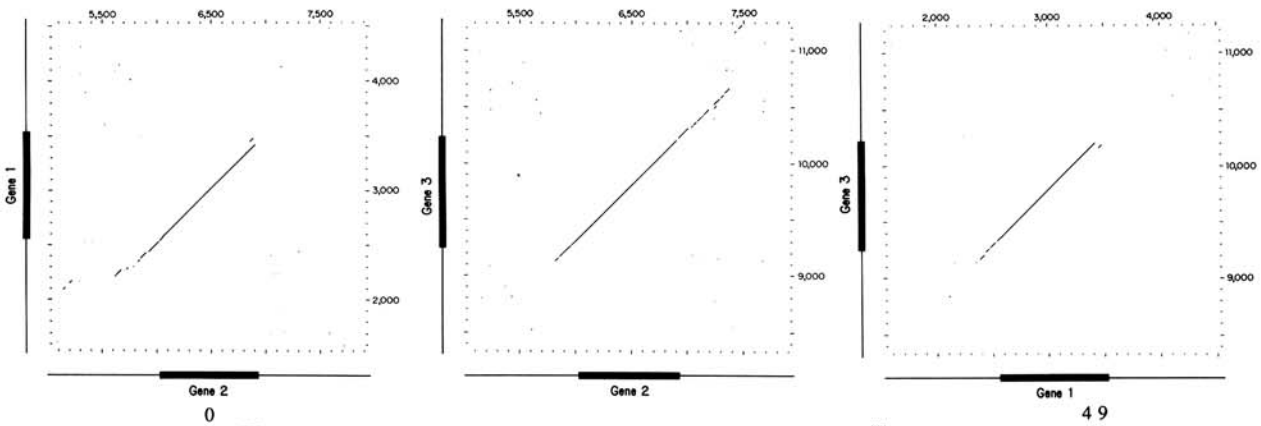
more, the 3′ regions of each gene contain polyadenylation sites. The high degree of similarity among the coding regions and the divergence at the 5′ and 3′ flanking regions are illustrated in the pairwise dot matrix comparisons shown in Figure 2A. The mature protein coding sequences of the three genes 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 various 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.

A



B

	0				49
Gene 3	AGIGIYWGQN	GNEGSLASTC	ATGNYEFVNI	AFLSSFGGGQ	TPVLNLAGHC
Gene 2	AGIAIYWGQN	GNEGSLASTC	ATGNYEFVNI	AFLSSFGSGQ	APVLNLAGHC
Gene 1	AGIAIYWGQN	GNEGSLASTC	ATGNYEFVNI	AFLSSFGSGQ	TPVLNLAGHC
	50				99
Gene 3	NPDNNGCTIL	SNEINSCQSQ	NVKVLLSIGG	GTGSYSLYSA	DDAKEVANFI
Gene 2	NPDNNGCAFL	SDEINSCKSQ	NVKVLLSIGG	GAGSYSLSSA	DDAKQVANFI
Gene 1	NPDNNGCAFV	SDEINSCQSQ	NVKVLLSIGG	GVGRYSLSSA	NNAKQVAGFL
	100				149
Gene 3	WNSYLGGQSD	SRPLGDAVLD	GVDFDIEFGS	DQFWDVLAQE	LKSFGQVILS
Gene 2	WNSYLGGQSD	SRPLGAAVLD	GVDFDIESGS	GQFWDVLAQE	LKNFGQVILS
Gene 1	WNNYLGGQSD	SRPLGDAVLD	GVDFVIGFGS	GQFWDVLARE	LKSFGQVILS
	150				199
Gene 3	AAPQCPIPDA	HLDAAIRTGL	FDSVWVQFYN	NPSCMYADNT	DDILSSWNQW
Gene 2	AAPQCPIPDA	HLDAAIKTGL	FDSVWVQFYN	NPPCMEADNA	DNLLSSWNQW
Gene 1	AAPQCPFPDA	QLDAAIRTGL	FDSVWVQFYN	NPPCMYADNA	DNLLSSWNQW
	200				249
Gene 3	AAYPILKLYM	GLPAAPEAAP	SGGFIPVDEL	ISEVLPTIKA	YSNYGGVMLW
Gene 2	TAFPTSKLYM	GLPAAREAAP	SGGFIPADVL	ISQVLPTIKA	SSNYGGVMLW
Gene 1	AAYPISKLYM	GLPAAPEAAP	SGGFIPADVL	ISQVLPTIKT	SSNYGGVMLW
	250				297
Gene 3	SKAFDNGYSD	AIKDSIYQLK	GSS*		
Gene 2	SKAFDNGYSD	SIKGSIG*			
Gene 1	SKAFDNGYSD	AIKGRILLKK	SSYCYGVRRL	TMATHLPLSL	EIEQVLV*

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 tissue 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

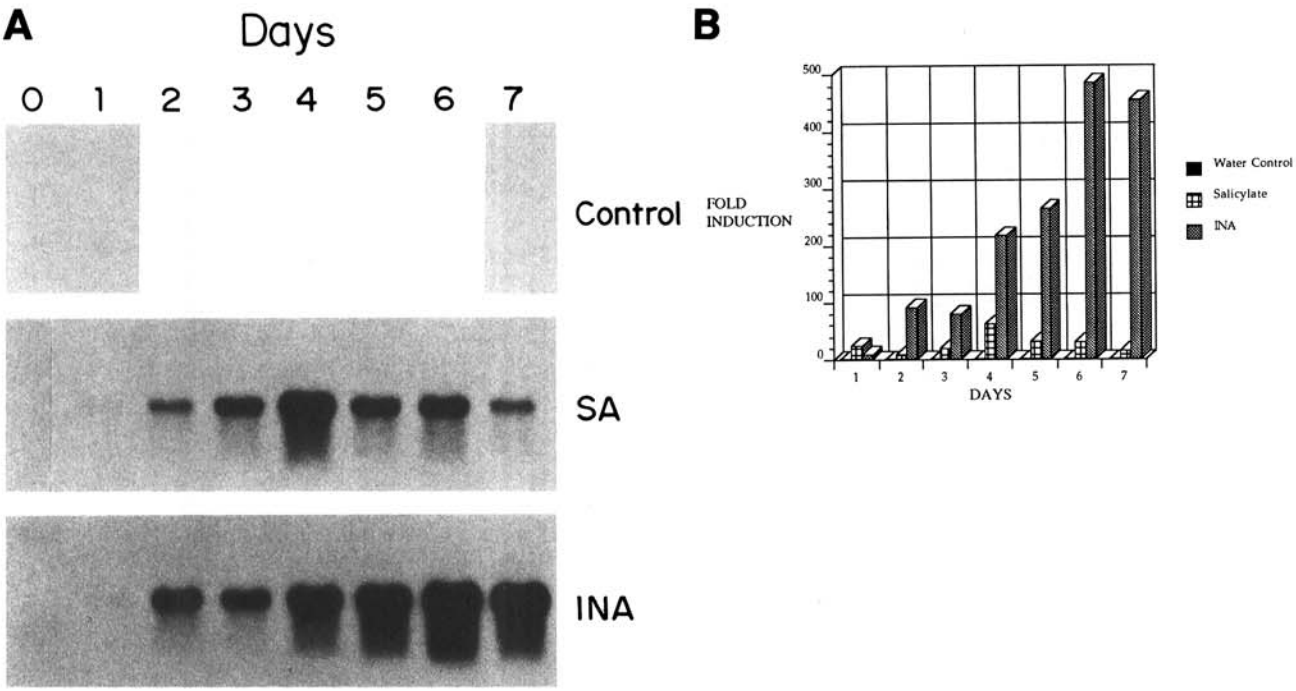


Fig. 3. Chemical induction of class III chitinase mRNA. Leaf tissue was harvested for total RNA extraction at various times after chemical application. **A,** RNA gel blots (10 µg/lane) hybridized with ^{32}P-labeled cucumber chitinase cDNA clone. **B,** Quantitation of induction of chitinase transcripts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

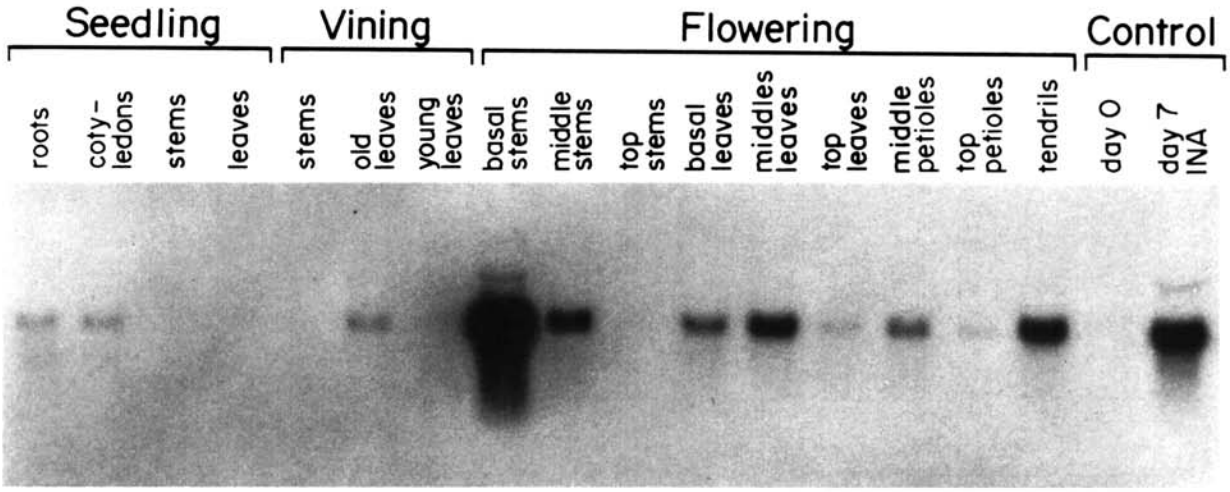


Fig. 4. Cucumber class III chitinase mRNA levels in vegetative tissue during development. Gel blot RNA extracted from vegetative tissues at the seedling (7–10 days postgermination), vining (when tendrils appear), and flowering stages of development hybridized with a ^{32}P-labeled chitinase cDNA clone.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

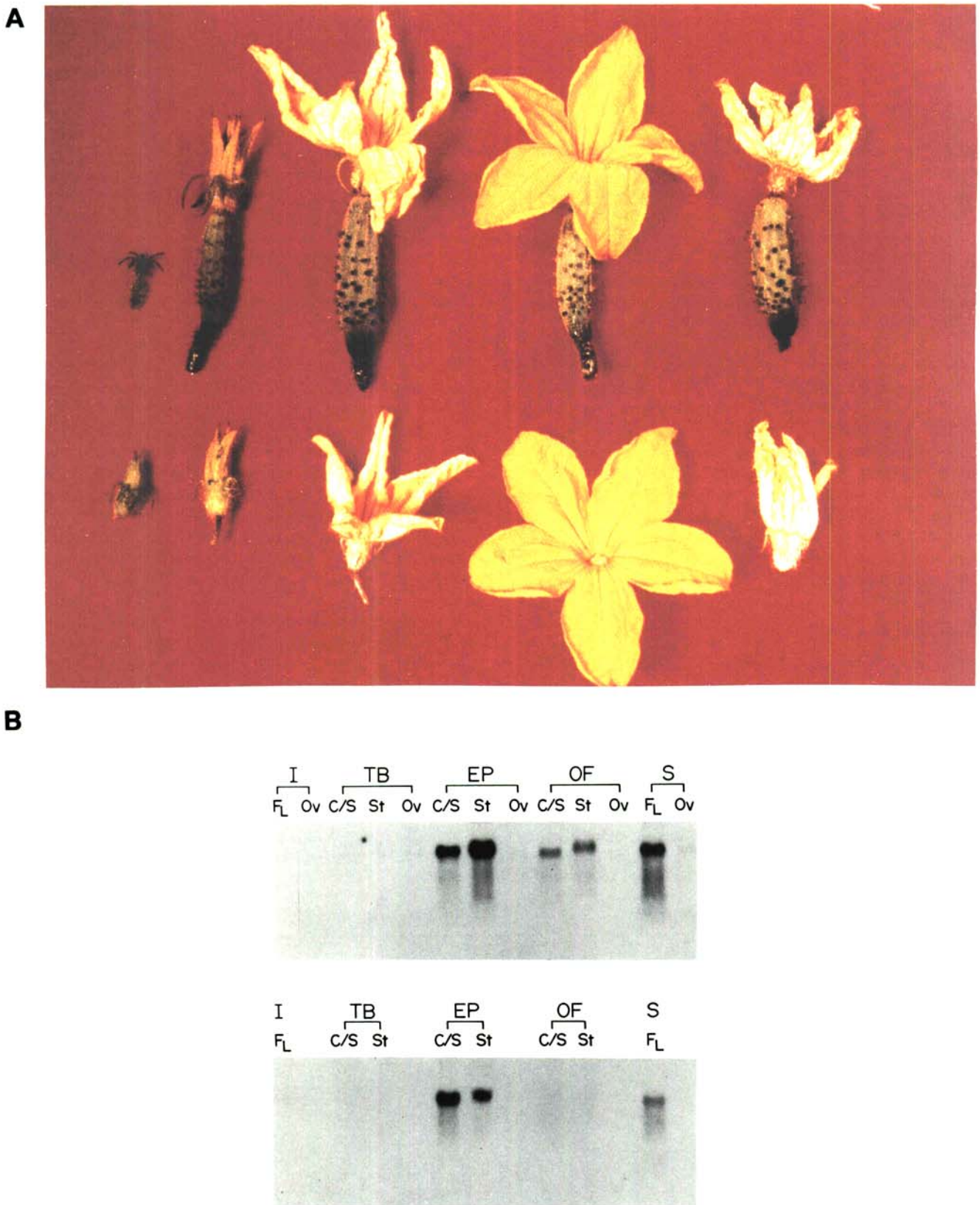


Fig. 5. Cucumber class III chitinase mRNA expression in reproductive tissue during development. **A,** Developmental stages of cucumber flowers. Female (top) and male (bottom) flowers during development. Left to right: Immature (I), Tight Bud (TB), Expanding Petals (EP), Open Flower (OF) and Senescent 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 flankng 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.

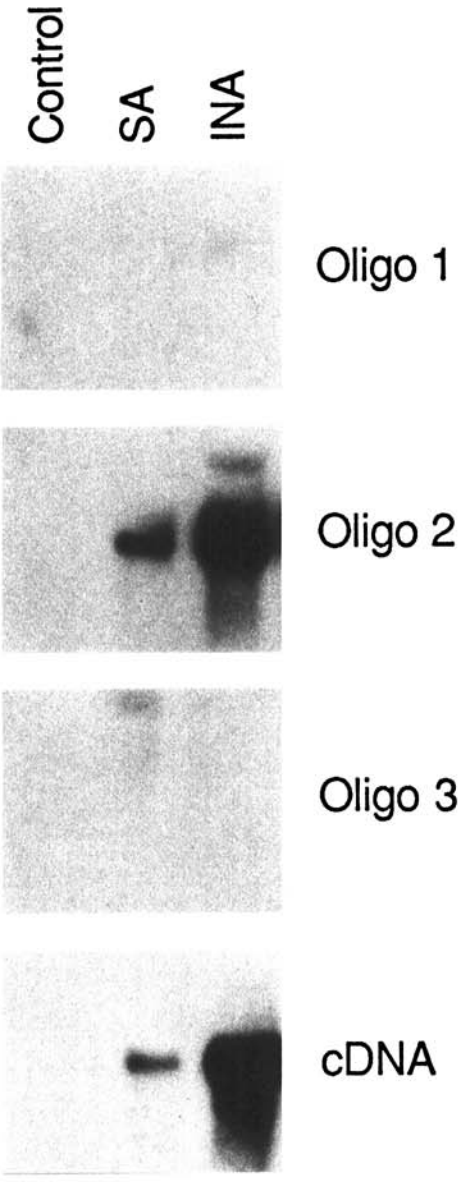


Fig. 6. Analysis of gene-specific expression. Gel blots of total RNA (10 μg) isolated from chemically induced leaf tissue 5 days after treatment were probed with 5′ end-labeled oligos specific for the divergent 3′ untranslated sequence for each gene or the chitinase cDNA clone.

MATERIALS AND METHODS

Sequence analysis.

A λEMBL4 genomic library was constructed from *Eco*RI-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, pBScucchrcht5, 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

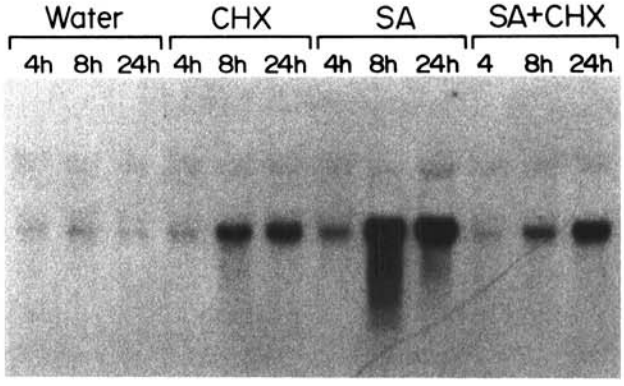


Fig. 7. Effect of protein synthesis inhibition on salicylate induction of chitinase mRNA. Gel blot of RNA extracted from cucumber leaf tissue at the times indicated after spraying with water, SA, CHX, or CHX followed by SA 1 hr later. Each lane contained 10 μg total RNA. The probe was a ^{32}P-labeled 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 ^{32}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′TAAAGGATTTTAATTGTAGCATGACTA3′ (*CHI3*). These oligonucleotides were radiolabeled with γ-^{32}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 $NaHPO_4$, pH 7.2/.24 M NaCl/7% $NaDodSO_4$) at Tm –15° C (Tm = 16.6 [log $[Na^+]$] + 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_2PO_4, 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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