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Genetic Resources

Plant Chitinase Consensus Sequences

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Abstract: Eighty-six plant chitinase sequences from 29 different species and one hybrid were obtained from the on-line GenBank nucleotide database. These sequences were grouped into five gene families based on previously published guidelines (Meins et al., 1994), and the amino-acid and nucleotide sequences of each gene family were aligned. Consensus amino-acid and nucleotide sequences were derived for each gene family based on the alignments. The consensus sequences were analyzed to determine their amino-acid composition, hydropathy profiles, and codon usage.

Chitinases catalyze the hydrolysis of chitin, a biopolymer of N-acetyl-D-glucosamine. The patterns of chitinase expression in plants (Meins and Ahl, 1989), *in-vitro* studies of fungal growth inhibition by chitinases (Schlumbaum et al., 1986) and enhanced resistance of transgenic plants to fungal pathogens (Broglie et al., 1991) are consistent with the hypothesis that chitinases are an important component of plant defense systems. Consequently, plant chitinases are the subject of intensive research that may ultimately lead to disease resistant crops and decreased use of ecologically harmful pesticides.

The classification of plant chitinases is based on the presence or absence of an N-terminal hevein domain and on sequence similarity to an archetypal catalytic domain. *Chia1* chitinases have an N-terminal hevein domain and a catalytic domain that is at least 50 percent identical to tobacco *Chia1* chitinase (Meins et al., 1994; Neuhaus et al., 1996). The *Chia2* chitinases lack an N-terminal hevein domain, but contain catalytic

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domains that are at least 50 percent identical to the catalytic domain of tobacco *Chia1* chitinase (Meins et al., 1994; Neuhaus et al., 1996). The *Chia4* chitinases are at least 50 percent identical to the *Phaseolus vulgaris* PR-4 chitinase (Meins et al., 1994; Neuhaus et al., 1996). *Chia4* chitinases have an N-terminal hevein domain and weak homology to *Chia1* chitinases, but have several distinct deletions (Meins et al., 1994; Neuhaus et al., 1996).

Chib1 chitinase sequences are not similar to *Chia1*, *Chia2*, or *Chia4* chitinases, but are at least 30 percent identical to tobacco *Chib1* chitinases, which also have lysozyme activity (Meins et al., 1994; Neuhaus et al., 1996). *Chic1* chitinases share no similarity with *Chia1*, *Chia2*, *Chia4*, or *Chib1* chitinases, but their amino-acid sequences are at least 50 percent identical to a group of tobacco chitinases that are similar to bacterial exochitinases (Meins et al., 1994; Neuhaus et al., 1996).

A full-length amino-acid consensus sequence of five *Chia1* and three *Chia2* chitinase sequences has been published (Meins et al., 1994). Because of the small number of sequences involved, variable residues or regions may appear conserved in the consensus sequence. In addition, aligning *Chia1* and *Chia2* sequences for a common consensus sequence may prevent the identification of subtle differences between the two chitinase classes.

We obtained 86 plant chitinase sequences from the on-line GenBank nucleotide database, and used these sequences to construct amino-acid and nucleotide consensus sequences for five plant chitinase gene families. We have identified highly conserved residues and regions located within the consensus sequences. These conserved regions may be useful in the design of primers for amplification with the polymerase chain reaction (PCR) or to identify residues that would be appropriate for mutational analyses.

Materials and Methods

Plant chitinase sequences were obtained from the on-line Genbank nucleotide database. The gene family, organism, accession number, and member number (from the *Mendel* database) for each of the sequences is shown in Table I. The sequences were grouped into five gene families: *Chia1*, *Chia2*, *Chia4*, *Chib1*, and *Chic1*. If the sequence report identified the group classification, then the chitinase was classified accordingly. If the chitinase sequence had not been classified in the sequence report, the sequence was placed in a group based on amino-acid homology with

Table I. Sources of chitinases used in this study.

Gene Family	Member Number	Organism	Accession Number
Chia1	1	*Allium sativum*	M94105
Chia1	2	*Allium sativum*	M94106
Chia1	1	*Arabidopsis thaliana*	M38240
Chia1	2	*Hordeum vulgare*	L34211
Chia1	3	*Hordeum vulgare*	U02287
Chia1	1	*Lycopersicon esculentum*	Z15140
Chia1	1	*Nicotiana tabacum*	M15173
Chia1	2	*Nicotiana tabacum*	A21091
Chia1	2	*Nicotiana tabacum*	S44869
Chia1	2	*Nicotiana tabacum*	X16939
Chia1	3	*Nicotiana tabacum*	X64518
Chia1	4	*Nicotiana tabacum*	A16119
Chia1	2	*Oryza sativa*	D16222
Chia1	3	*Oryza sativa*	D16221
Chia1	4	*Oryza sativa*	X56787
Chia1	5	*Oryza sativa*	Z29961
Chia1	6	*Oryza sativa*	Z29962
Chia1	7	*Oryza sativa*	U02286
Chia1	8	*Oryza sativa*	L37289
Chia1	9	*Oryza sativa*	D16223
Chia1	10	*Oryza sativa*	X87109
Chia1	1	*Phaseolus vulgaris*	M13968
Chia1	3	*Phaseolus vulgaris*	S43926
Chia1	1	*Pisum sativum*	X63899
Chia1	2	*Pisum sativum*	L37876
Chia1	1	*Populus trichocarpa*	X59995
Chia1	1	*Populus trichocarpa x Populus deltoides*	U01660
Chia1	1	*Solanum tuberosum*	X15494
Chia1	2	*Solanum tuberosum*	X14133
Chia1	3	*Solanum tuberosum*	U02605
Chia1	4	*Solanum tuberosum*	U02606
Chia1	5	*Solanum tuberosum*	U02607
Chia1	6	*Solanum tuberosum*	U02608
Chia1	1	*Theobroma cacao*	U30324
Chia1	1	*Ulmus americana*	L22032
Chia1	1	*Vigna unguiculata*	X88800
Chia1	1	*Vitis vinifera*	Z54234
Chia1	1	*Zea mays*	L00973
Chia1	2	*Zea mays*	L16798
Chia2	1	*Arachis hypogaea*	X82329
Chia2	2	*Arachis hypogaea*	X82330
Chia2	1	*Citrus sinensis*	Z70032
Chia2	1	*Gossypium hirsutum*	Z68152
Chia2	1	*Hordeum vulgare*	L34210

Table I, continued.

Gene Family	Member Number	Organism	Accession Number
Chia2	2	*Hordeum vulgare*	X78671
Chia2	2	*Hordeum vulgare*	X78672
Chia2	1	*Lycopersicon chilense*	M97210
Chia2	2	*Lycopersicon chilense*	L19342
Chia2	2	*Lycopersicon esculentum*	Z15139
Chia2	3	*Lycopersicon esculentum*	Z15141
Chia2	4	*Lycopersicon esculentum*	U30465
Chia2	1	*Nicotiana tabacum*	M29869
Chia2	2	*Nicotiana tabacum*	X51425
Chia2	1	*Petunia hybrida*	X51427
Chia2	1	*Populus trichocarpa x Populus deltoides*	M25336
Chia2	1	*Solanum tuberosum*	X67693
Chia2	2	*Solanum tuberosum*	U49969
Chia4	1	*Beta vulgaris*	A23392
Chia4	3	*Beta vulgaris*	L25826
Chia4	1	*Brassica napus*	X61488
Chia4	2	*Brassica napus*	U21848
Chia4	1	*Daucus carota*	U52846
Chia4	2	*Daucus carota*	U52847
Chia4	3	*Daucus carota*	U52848
Chia4	1	*Phaseolus vulgaris*	X57187
Chia4	1	*Phaseolus vulgaris*	X74919
Chia4	1	*Picea glauca*	L42467
Chia4	1	*Sambuscus nigra*	Z46948
Chia4	2	*Sambuscus nigra*	Z46950
Chia4	1	*Vigna unguiculata*	X88803
Chia4	1	*Zea mays*	M84164
Chia4	2	*Zea mays*	M84165
Chia5	1	*Urtica dioica*	M87302
Chia6	1	*Beta vulgaris*	X79301
Chib1	1	*Arabidopsis thaliana*	M34107
Chib1	1	*Beta vulgaris*	S66038; J03714
Chib1	1	*Cicer arietinum*	X70660
Chib1	1	*Cucumis sativus*	M84214
Chib1	2	*Cucumis sativus*	M84214
Chib1	3	*Cucumis sativus*	M84214
Chib1	1	*Nicotiana tabacum*	Z11563; S41954
Chib1	2	*Nicotiana tabacum*	Z11564; S41955
Chib1	1	*Psophocarpus tetragonolobus*	D49953
Chib1	1	*Vigna angularis*	D11335
Chib1	1	*Vigna unguiculata*	X88802
Chib1	2	*Vigna unguiculata*	X88801
Chic1	1	*Nicotiana tabacum*	X77110
Chic1	1	*Nicotiana tabacum*	X77111

previously classified chitinase sequences. If an N-terminal hevein domain was present, then that chitinase was grouped with the *Chia1* chitinases. Chitinases that lacked a hevein domain or hinge region that were homologous to the catalytic region of *Chia1* and *Chia2* chitinases were grouped with *Chia2* chitinases. The *Chib1* chitinases were grouped based on the presence of the WNQW amino-acid motif.

The amino-acid sequences of each class were aligned using the Eyeball Sequence Editor v.1.04 (Cabot, 1987). Gaps were manually introduced to maximize homology. The amino-acid sequence alignments were used to align the nucleotide sequences. Amino-acid and nucleotide consensus sequences were manually derived from the alignments.

The designated consensus residue occurs most frequently at a given position. Residues identified as invariant are present in all full-length sequences. Positions that exhibit no clear consensus are represented as an "X" in the consensus sequences, while positions that were not present in at least 50 percent of the sequences were not included in the consensus sequence. Because the hinge region in the *Chia1* chitinases varies in length and amino-acid composition, the hinge region was not used to construct the *Chia1* consensus sequence. In the *Chia1* consensus sequence described here, the catalytic domain immediately follows the N-terminal hevein domain.

The consensus sequences were compared with the Eyeball Sequence Editor (Cabot, 1987). The amino-acid consensus sequences were aligned, and the percent identity determined. In addition, the nucleotide consensus sequences were translated and compared to the relevant amino-acid consensus sequence.

The predicted molecular weights and amino-acid compositions of the amino-acid consensus sequences were manually determined. The amino-acid consensus sequences were also used as the subjects in BLAST searches (Basic Local Alignment Search Tool) (Altschul et al., 1990) to identify those sequences that shared the highest percent identity with the consensus sequences.

Finally, the consensus sequences were imported into DSAS v2.2 (Neigel, The University of Southwestern Louisiana) to identify homologies between the nucleotide consensus sequences of each chitinase gene family. Matches of $\geq$15 nucleotides in a 20-nucleotide sliding window were scored. The DSAS program also allowed the codon usage and amino-acid composition of the nucleotide consensus sequences to be determined.

Results and Discussion

Nucleotide and amino-acid consensus sequences were derived from the alignments. Because the amino-acid and nucleotide consensus sequences were determined independently, there are differences between the amino-acid consensus sequences and the amino-acid sequences encoded by the consensus nucleotide sequences. For example, if serine occupied a given position in five sequences, proline occupied that same position in three sequences, and threonine occupied that position in two other sequences, the consensus amino acid would be serine, but the consensus codon could be CCN, which encodes proline. The amino-acid consensus sequences were compared to the corresponding translated nucleotide consensus sequence. On average, the sequences exhibited 91.8 percent identity. Because there are only two members of the *Chic1* gene family, the amino-acid consensus sequence and the translated nucleotide consensus sequence are 98 percent identical. Once residues of weak consensus (less than 50 percent) become a factor, the percent identity decreases. Consequently, the *Chia1* sequences exhibit 94 percent identity, the *Chia2* sequences are 90 percent identical, the *Chia4* sequences are 87 percent identical, and the *Chib1* sequences are 90 percent identical.

When the hevein domain is discounted, the *Chia1* amino-acid consensus sequence exhibits 73 percent identity to the *Chia2* amino-acid sequence. The homology between consensus sequences for these two chitinase classes degenerates immediately after the SHETTGG motif and does not resume until the YYGRGPIQ motif. The length of this variable region differs between the two classes; the region is 39 amino acids long in the *Chia1* chitinase consensus sequence and 22 amino acids long in the *Chia2* consensus sequence. The beginning of this variable region coincides with the start of exon 2 in several *Chia1* and *Chia2* chitinases.

The *Chia1* and *Chia2* amino-acid consensus sequences were compared to a previously published plant chitinase consensus sequence (Meins et al., 1994). Our *Chia1* amino-acid consensus sequence is 87 percent identical to the previously published amino-acid sequence and 92 percent identical within the catalytic region. Our *Chia2* consensus sequence is identical to the composite chitinase sequence in 71 percent of the positions.

The *Chia1* and *Chia4* amino-acid consensus sequences are 40 percent identical overall and 43 percent identical within the catalytic region. The catalytic region of the *Chia2* consensus sequence is identical to the *Chia4*

consensus sequence in 40 percent of the positions. Again, the region that follows the SHETTGG motif is highly variable in length and composition. This region is 30 amino acids long in the *Chia4* consensus sequence.

The *Chia1* and *Chia4* nucleotide sequences were also examined for the presence of imperfect direct repeats flanking the chitin-binding domains. These 9- to 10-bp imperfect direct repeats suggest that the chitin-binding domains may have been added to the catalytic domains by a transposition event (Shinshi et al., 1990). The imperfect direct repeats in the *Chia1* consensus sequence are identical at seven of nine positions while the imperfect direct repeats in the *Chia4* consensus sequence are identical at six of nine positions. The imperfect direct repeats in the *Chia1* consensus sequence are most similar to the imperfect direct repeats found in tobacco chitinase 48 (Shinshi et al., 1990) while the *Chia4* imperfect direct repeats share little homology with the *Chia1* imperfect direct repeats.

The *Chia1*, *Chia2* and *Chia4* amino-acid consensus sequences share 75 common residues; this represents approximately 38 percent of the *Chia2* catalytic region. Fourteen residues are invariant when 72 representatives of these three gene families are considered: E89, A91, E100, T101, C118, Q151, N157, P173, G223, E236, C237, R248, G264 and C269 (numbers refer to the position in the *Chia1* consensus sequence). The positions of these residues relative to each other are largely conserved. Only the distances between C118 and Q151, which includes an intron splice site, and the distances between P173 and G223 vary among the classes.

Chia5 chitinase sequences contain a unique duplicated N-terminal hevein domain, while *Chia6* chitinase sequences have a truncated N-terminal hevein domain and an extremely long hinge region. We were only able to find a single member of each of these gene families: the *Chia5* lectin of *Urtica dioica* (*URTdi;Chia5;1.*) and the *Chia6* proline-rich chitinase from *Beta vulgaris* (*BETvu;Chia6;1*). The amino-acid sequence of the *Chia5* sequence is identical to the *Chia1* amino-acid consensus sequence at 51

Fig. 1. Alignment of the *Chia1*, *Chia2* and *Chia4* amino-acid consensus sequences with the *Chia5* and *Chia6* sequences (opposite). Capitol letters in the consensus sequences identify invariant residues within a class while lower-case letters identify residues that occur in at least 50 percent of the sequences within a class. Dashes represent residues of weak consensus that occur in less than 50 percent of the sequences within a class. Gaps introduced to maximize homology are identified by dots. Residues that are invariant in all five classes are in **bold** print. Lower-case letters beneath the alignment identify residues that are present in all of the aligned sequences. Regions where at least 50 percent of the residues are present in all of the consensus sequences are identified by gray boxes.

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Chia1   //FdqMl-hRNd-ac-akgfYtydafi-aa--fpgfgttgdtttrKrEiAAFlaQTshET
Chia2   //f-emlk-rnd--CpakgFyTYdaFiaAansFpgFGttGddtqrkkEiAAFfgQtsHET
Chia4   //Ffngii-qa-s-C-GknFYtr-aFlsAl--yp-fg--gs-dd-kREiAafFAh-tHET
Chia5   //FDQMFSHRKD..CPSQGFYSYHSFLVAAESFPAFGTIGDVATRKREVAAFLAHISQAT
Chia6   //FNEFLLNRIQPRCPGRWFYTYQAFITAAETFPEFGNTGNDEIRKREIAAFFGQTSHET
          f          c     fy     f   a    p fg   g     k  e aaf         t

Chia1   tGgwatapdgpyawGYCf-rEq----..dYC-ps-.qwpCapg-kYyGrGpiQis-NyNy
Chia2   tGG---apdgpf-wGYCf--e--------...............yyGRGPIQlt--sNY
Chia4   .............ghfCyieEing----.YCd----qyPC--gk-Y-grgplQlsWNyNy
Chia5   SGE.RSDVENPHAWGLCHI.NTTTVTENDFCTSS..DWPCAAGKKYSPRGPIQLTHNFNY
Chia6   SGE.PTAQHGPFTWGYCFIEEIGAGPLSQYCAPSV.EWPCIRGRFYYGRGPVQLTWNFNY
                        c                              y  rgp q       ny

Chia1   GpcgraigvdllnnPdlvatddvisfkta-wfwMtpqspkPSchdVitgrW-Ps-aD-aa
Chia2   --aG-aig-dlvnnPdlvatda-isFktaiWFWMtaq-nKPScHdVi-g-wtPsaaD-aa
Chia4   gpaG-sigfdgL--Pe-Va-d-vvsfktalWfWm-...................nv-s
Chia5   GLAGQAIGEDLIQNPDLVEKDPIISFKTALWFWMSQHDNKPSCHDIV......LNANSAA
Chia6   GKQVKHLGLDLLFNPDIVAHDPVISFETAIWFWMTPEGNKPSSHEVITGQWTPTPADIAR
              g d    p  v  d   sf  ta  wfwm

Chia1   gr-pGyGviTNIiNgGlECg-g-d.srv-dRigfy-rYC-ilgv--G-NldC-nQr-f//
Chia2   gRvpGyGviTNIINGgiECg-g-n.-avedRIGfyrrycg-lnv--G-nLDCynQrnf//
Chia4   v--qGfgatiraing-lECnggnp.--vnaRv-yy--yC-qlgV-pG-Nl-C......//
Chia5   NRIPNKGVIGNI...ISRAFGHDDFAVRSSSIGFYKRYCDMLGVSYGHDLKYWFDNTP//
Chia6   NRLPGYGLITNIFNGALECGTHGPDNRGENRIQFYQRYCDLLDVSYGDNLDCY.RQTP//
            g                              y  yc  l v  g   l
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percent of catalytic region positions. The catalytic regions of the *Chia6* sequence and the *Chia1* amino-acid consensus sequence are 60 percent identical. In contrast, the catalytic regions of the *Chia5* and *Chia6* sequences are only 34 percent identical. This suggests that the *Chia5* and *Chia6* sequences may be more closely related to *Chia1* sequences than to each other.

The catalytic domains of the *Chia1*, *Chia2*, and *Chia4* consensus sequences were aligned with the *Chia5* and *Chia6* sequences (Fig. 1). Forty-five residues are common to all five sequences. While all of the 14 invariant residues are present in the *Chia6* sequence, only 8 of the invariant residues are present in the *Chia5* sequence: E89, A91, T101, C118, Q151, N157, P173 and G264.

The *Chib1* and *Chic1* amino-acid consensus sequences share no appreciable homology with the consensus sequences of the other classes. These two consensus sequences do share two motifs that are common to bacterial and fungal chitinases: the SXXG motif and the GXDXDXE motif. When these motifs are used to align the *Chib1* and *Chic1* amino-acid consensus sequences with each other, only 17 percent of the positions are identical. Mutational studies with *Altermonas* chitinase (Tsujibo et al., 1993) and *Bacillus circulans* chitinase (Watanabe et al., 1993) have shown that, when the glutamate within the GXDXDXE motif is changed to either aspartate or glutamine, activity is lost. X-ray structural analysis of a hevamine-inhibitor complex shows that the glutamate of the GXDXDXE motif is properly positioned to serve as a proton donor (van Scheltinga et al., 1995). This glutamate is variable in the plant *Chib1* sequences. The first open reading frame (ORF) of a genomic clone (AC M84214) that encodes three members of the *Chib1* chitinase family of cucumber contains a glycine at this position, instead of a glutamate. This cucumber chitinase gene was not expressed after treatment with salicylic acid or 2,6-dichloroisonicotinic acid (Lawton et al., 1994). Thus, it appears that expression of this chitinase gene is not regulated in the same manner as other plant defense genes.

To identify sequences with high percentages of identity to the consensus sequences, BLAST searches were performed with the *Chia1*, *Chia2*, *Chia4*, and *Chib1* amino-acid consensus sequences. As expected, the sequences that were most similar to the consensus sequences had been used to construct the alignments. The *Chia1* consensus sequence was found to be most identical (82 percent) to a potato *Chia1* sequence (U02605). The *Chia2* consensus sequence was found to be 82 percent identical to a tobacco *Chia2* sequence (M29869), and the *Chia4* consen-

sus sequence was 76 percent identical to a *Phaseolus Chia4* sequence (X57187). Finally, the *Chib1* consensus sequence was found to be 81 percent identical to an azuki bean *Chib1* sequence (D11335).

Table II. Distances between Invariant Residues in Chia1, Chia2 and Chia4 Gene Families.

	E-A	A-ET	ET-C	C-Q	Q-N	N-P	P-G	G-EC	EC-R	R-G	G-C
Chia1	1	8	16	32	5	15	49	12	10	15	4
Chia2	1	8	16	19	5	15	49	12	10	15	4
Chia4	1	8	16	21	5	15	28	12	10	15	4

The amino-acid composition and codon usage were determined for the consensus sequences. In *Chia1*, *Chia2* and *Chia4* chitinase consensus sequences, glycine is the most common amino acid, and accounts for an average of 13.3 percent of the amino-acid residues. In the *Chib1* and *Chic1* amino-acid consensus sequences, serine is the most common amino acid, accounting for an average of 11 percent of the amino acids.

Generally, codons exhibited fairly uniform usage patterns; there were, however, some codon preferences. The *Chia1*, *Chia2*, *Chia4*, and *Chib1* sequences exhibited a preference for AAC over AAT (asparagine). In the *Chic1* sequence, this pattern was reversed. Similarly, all of the sequences except *Chia1* exhibited a preference for GAT over GAC (aspartate). The codon CGG, encoding arginine, is only found in the *Chia1* consensus sequence. In addition, the codon GTA, encoding valine, is only found in the *Chia2* and *Chib1* sequences.

As expected, the majority of the invariant nucleotides in the nucleotide consensus sequences corresponded to first and second codon position pairs. An average of 48 percent of the invariant nucleotides were involved in such pairings. Unexpectedly, there were more invariant nucleotides in the second- than in the first-codon position. On average, there were twice as many invariant nucleotides in the second as in the first position. It may be that these invariant second-position nucleotides are involved in transcript stability.

Homology searches between the nucleotide consensus sequences were performed.

Regions where ≥ 15 of 20 nucleotides matched between *Chia1* and *Chia2*, *Chia1* and *Chia4*, *Chia1* and *Chib1*, *Chia2* and *Chia4*, *Chia2* and *Chib1*, *Chia2* and *Chic1*, *Chia4* and *Chib1*, *Chia4* and *Chic1* and *Chib1* and *Chic1* are presented in Table II. No 20-nucleotide spans of at least 75 percent identity were identified between *Chia1* and *Chic1*. Because many of the regions of homology among *Chia1*, *Chia2*, and *Chia4* overlap, it may be possible to design primers that would work with all three gene families. No 20-bp regions of at least 75 percent nucleotide identity were common, however, to all plant chitinase gene families, and it may not be possible to synthesize a universal primer that would anneal to all the known chitinase classes.

Conclusions

In this study, we have analyzed an expanded nucleotide database to generate consensus sequences for five different families of plant chitinases. These consensus sequences enhance and complement an earlier comparison of plant chitinases (Meins et al., 1994). We have identified 14 amino-acid residues that are invariant in 72 representatives of *Chia1*, *Chia2*, and *Chia4* chitinases. The relative positions of these residues with respect to each other are generally conserved. The identification of invariant or highly conserved residues, coupled with mutational analyses, may assist researchers in their efforts to identify specific amino acids involved in catalysis.

The homology among chitinase consensus nucleotide sequences varies among gene families. *Chia1*, *Chia2* and *Chia4* are very similar, but there do not appear to be nucleotide regions that are highly conserved between all five classes. Based on this study, it does not seem likely that enough homology exists at the nucleotide level among the classes to generate and utilize universal primers or probes that will target genes of all chitinase families.

The consensus sequences and the analyses of the consensus sequences described in the text are available from the electronic version of *PMBR* (http://www.uga.edu/~ispmb) or from the corresponding author with the submission of an IBM-formatted 3.5-inch diskette.

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