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Biochemical and Molecular Characterization of Three Barley Seed Proteins with Antifungal Properties*

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We have purified three proteins from barley (*Hordeum vulgare* L.) seeds which synergistically inhibit the growth of fungi measured in a microtiter well assay. The proteins are a 26-kDa chitinase, a 30-kDa ribosome-inactivating protein, and a 32-kDa (1–3)-β-glucanase. Full-length cDNAs encoding them were isolated and sequenced to determine the complete primary structures of the proteins. Northern hybridizations with the cDNAs as probes showed that the corresponding mRNAs accumulate differentially during seed development and germination. Chitinase mRNA accumulates to high levels in aleurone cells during late seed development and early germination, while high levels of mRNA encoding the ribosome-inactivating protein accumulate only in the starchy endosperm during late seed development. The glucanase mRNA accumulates to low levels during seed development and to higher levels in aleurone and seedling tissues during germination. Southern hybridizations showed that the three proteins are encoded by small families of three to eight genes. Their biological roles and potential use in genetic engineering studies are discussed.

Plants use various defense mechanisms for protecting themselves against infection by pathogens (Boller, 1985). These mechanisms include inducible modifications of plant cell walls, the synthesis of toxic phytoalexins, and the accumulation of pathogenesis related proteins, often protease inhibitors or pathogen targeted hydrolytic enzymes (Hahlbrock and Grisebach, 1979; Darvill and Albersheim, 1984; van Loon, 1985). Examples of the latter group are chitinase and (1–3)-β-glucanase, which are synthesized in the vegetative tissues of many plants in response to fungal invasion (Legrand *et al.*, 1987; Kauffmann *et al.*, 1987). These enzymes limit fungal growth by degrading chitin and (1–3)-β-glucan, major structural polysaccharides of fungal cell walls (Boller, 1988; Bartnicki-Garcia, 1968). Furthermore, the glycosidic fragments released by this degradation act as elicitors of host stress metabolite biosynthesis (Ryan, 1987). Chitinase and (1–3)-β-glucanase are thus involved in host signaling during pathogen attack. The extent to which these two enzymes and other pathogen-related proteins contribute to pathogen resistance is being studied using genetic and molecular techniques. For example, chitinase and (1–3)-β-glucanase appear to be synthesized in response to infection by *Cladosporium fulvum* and *Fusarium oxysporum* earlier and to higher levels in resistant than in susceptible tomato cultivars (Joosten and DeWit, 1989; Benhamou *et al.*, 1990).

Nonvegetative plant parts, such as flowers and fruits, must also defend themselves against pathogen attack. Because the growth of these organs is under developmental control, the expression of defensive mechanisms in them may be strictly programmed (Lotan *et al.*, 1989). For example, mature cereal seeds are often surrounded by a lignified husk (Stoskopf, 1985), and a testa-pericarp layer rich in phenolic compounds (Aastrup *et al.*, 1984). Furthermore, the storage tissues of the cereal seed, like the white of an avian egg, are rich in inhibitors of mammalian and microbial hydrolases (Shewry and Miflin, 1985).

We are interested in determining how cereal plants and their seeds defend themselves against infection by fungal pathogens. To begin this work, we isolated three proteins exhibiting antifungal properties from barley seeds. They are: 1) a 26-kDa chitinase (CHI 26),[1] 2) a 30-kDa ribosome-inactivating protein (RIP 30), and 3) a 32-kDa (1–3)-β-glucanase (BGL 32). As noted above, chitinase and (1–3)-β-glucanase are thought to retard fungal growth by interfering with fungal cell wall synthesis. RIP 30, like its wheat homologue tritin (Coleman and Roberts, 1982) and the related cytotoxin lectin ricin (Lamb *et al.*, 1985), inhibits protein synthesis in target cells by specifically modifying 28 S rRNA such that elongation factor 2 binds inefficiently (Endo *et al.*, 1988). Such ribosome-inactivating proteins do not inactivate "self" ribosomes, but show varying specificities toward ribosomes of distantly related species, including fungi (Roberts and Selitrennikoff, 1986; Stirpe and Hughes, 1989). This specificity may be due to interactions both with the 28 S rRNA substrate and with associated ribosomal proteins. These properties suggest that ribosome-inactivating proteins may be involved in the inhibition of pathogen infection in a less direct manner than chitinase and (1–3)-β-glucanase.

Here, we report the purification of the three proteins from barley seeds and measurements of their synergistic inhibitory effects on the growth of fungi in a microtiter plate assay. Furthermore, we report the isolation of full-length cDNAs encoding these proteins and an analysis of the genomic organization and patterns of expression of their genes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M36989, M36990, M36991, and M36992.

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[1] The abbreviations used are: CHI 26, 26-kDa chitinase protein; RIP 30, 30-kDa ribosome-inactivating protein; BGL 32, 32-kDa (1–3)-β-glucanase protein; DPA, day post anthesis; ABA, abscisic acid; GA_3, gibberellic acid; bp, base pair; kb, kilobase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

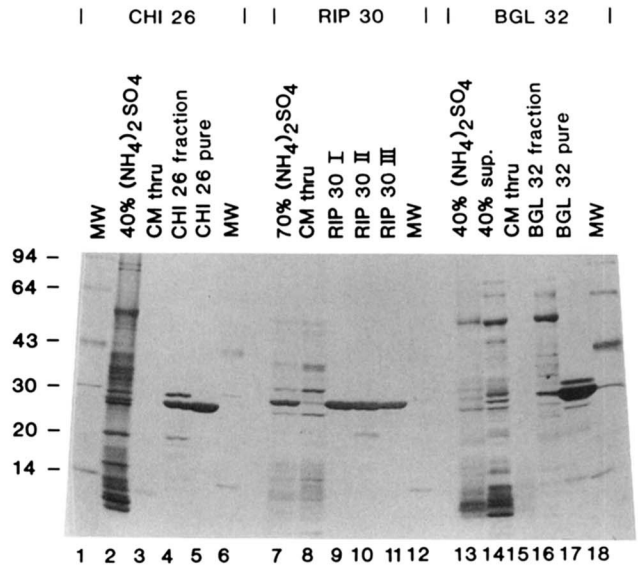

FIG. 1. **Purification of chitinase, ribosome-inactivating protein, and (1–3)-β-glucanase.** SDS-PAGE of protein fractions obtained at various steps in the purification of chitinase (*lanes 2–5*), ribosome-inactivating protein (*lanes 7–11*), and (1–3)-β-glucanase (*lanes 13–17*). CHI 26 and RIP 30 were purified from mature barley seed flour, while BGL 32 was purified from 12-day germinated seedlings (see "Experimental Procedures"). % $(NH_4)_2SO_4$ is protein precipitated by the salt and % *sup.* is supernatant proteins not precipitated by salt. *CM thru* is CM column flow-through fraction (unbound proteins). *RIP 30 I, II,* and *III* are chromatographic isoforms described by Asano *et al.* (1986). Molecular weight markers (*lanes 1, 6, 12, 18*) are indicated (× 1000).

EXPERIMENTAL PROCEDURES[2]

RESULTS

Purification of Chitinase, Ribosome-inactivating Protein, and (1–3)-β-Glucanase—CHI 26 and RIP 30 were purified from mature barley seed flour as described previously (Leah *et al.*, 1987; Asano *et al.*, 1986, see "Experimental Procedures"). Proteins fractionated in the purification steps were resolved by silver-stained SDS-PAGE (Fig. 1). The 40 and 70% $(NH_4)_2SO_4$ precipitates (Fig. 1, *lanes 2, 7,* and legend), shown to contain CHI 26 and RIP 30 by Western blotting, were chromatographed on Whatman CM52 (Fig. 1, *lanes 3, 4, 8*), followed by final purification on Mono S columns (Fig. 1, *lanes 5, 9, 10, 11*). The three similar RIP 30 isoforms I, II, and III, previously described by Asano *et al.* (1986), eluted separately from the CM52 column (Fig. 1, *lanes 9, 10, 11* and legend). The specific activity of the purified CHI 26 fraction was 22 mg diacetylchitobiose/min/mg protein, within the expected range for the purified enzyme (Molano *et al.*, 1977). The RIP 30 isozyme fractions showed 50% inhibition of RNA translation (reticulocyte lysate) at concentrations of 3–30 nM, essentially the same inhibitory activities as described by Asano *et al.* (1986).

BGL 32 was purified from 12-day-old barley seedlings by $(NH_4)_2SO_4$ precipitation and chromatography on CM52 and Mono S columns (see "Experimental Procedures"). Purified proteins were analyzed by Western blotting with an antibody provided by Dr. M. Ballance (Department of Plant Science, Faculty of Agriculture, Winnipeg, Manitoba, Canada) and by SDS-PAGE (Fig. 1, *lanes 13–17*). The final BGL 32 fraction was shown by NH_2-terminal amino acid sequencing to contain only the high pI isoform II (Ballance and Svendsen, 1988) without detectable contaminants. The specific activity of the purified BGL 32 fraction was 25 mg glucose equivalents/min/mg enzyme, within the expected range for the purified enzyme (Høj *et al.*, 1989).

Synergistic Inhibition of Fungal Growth by the Purified Proteins—The microtiter well plate assay used in this study measures the protein concentrations required for 50% growth inhibition (IC_{50}) of fungal growth at 0.05–1.5 μg of protein/135 μl of growth media (Fig. 2). This corresponds to protein concentrations from 1.25×10^{-8} to 3.75×10^{-7} M, 10^2–10^3 times less protein than reported in other studies on the inhibition of fungal growth by a chitin-binding lectin (Broekaert *et al.*, 1989) and thionins (Bohlmann *et al.*, 1988). This suggests that the microtiter plate assay is more sensitive than the generally used disc plate diffusion assay. It also suggests that our purified protein fractions are at least as inhibitory to fungal growth *in vitro* as those reported elsewhere.

Growth of *Trichoderma reesei* was inhibited 50, 20, and 90% by 1.5 μg/well of CHI 26, RIP 30, or BGL 32, respectively (Fig. 2*A*). In contrast, growth was inhibited greater than 95% by a mixture of 0.25 μg each of CHI 26, BGL 32, and RIP 30 (Fig. 2*A*). The same level of inhibition was obtained with mixtures of 1.0 μg/well each of RIP 30 and BGL 32 or 1.5 μg/well each of CHI 26 and RIP 30.

Growth of *Fusarium sporotrichioides*, a barley seed rot, was inhibited more than 95% by a mixture of 0.5 μg/well of each of the three proteins. A mixture of 1.0 μg/well each of CHI 26 and RIP 30 inhibited growth to approximately the same extent (Fig. 2*B*). 1.5 μg/well of the three proteins alone, or of RIP 30 and BGL 32 in combination, inhibited growth to a lesser extent. As a control, heat-denatured CHI 26, RIP 30, and BGL 32 did not inhibit growth of the test fungi at 1.5 μg/well protein (data not shown). The same concentrations of bovine serum albumin and cytochrome *c* (pI values = 4.9 and 10.6, respectively) also showed no inhibitory effect.

These results show that combinations of the purified proteins exhibit synergistic inhibitory effects on the growth *in vitro* of both *Trichoderma* and *Fusarium*. Inhibition by RIP 30 alone is slight, but is greatly enhanced in the presence of CHI 26 (Fig. 2, *A* and *B*) or BGL 32 (Fig. 2*A*).

Isolation and Sequencing of cDNAs Encoding the Three Proteins—Antiserum raised against purified CHI 26 was used to identify a corresponding, full-length cDNA clone (cCHI26) from a lambda gt11 library made from mRNA of seeds harvested 30 days post anthesis (Leah and Mundy, 1989). The nucleotide sequence of cCHI26 and the deduced amino acid sequence of CHI 26 are presented in Fig. 3. The NH_2-terminal amino acid residue (S + 1) of the mature CHI 26 protein has previously been determined by amino acid sequencing (Leah *et al.*, 1987). Upstream of this residue, cCHI26 encodes a 23 amino acid polypeptide with the characteristics of a eukaryotic signal sequence (von Heijne, 1983). The open reading frame of cCHI26 predicts a polypeptide of 28,159 Da. The 3′-untranslated region contains a putative polyadenylation signal (AATAAG) similar to that reported for other plant mRNAs (Lamb *et al.*, 1985; Leah and Mundy, 1989). The open reading frame of cCHI26 is high in G + C (68%), as has been noted for other cereal genes (Rogers, 1985).

The mature CHI 26 has a total molecular weight of 25,933. Its sequence is almost identical to that determined by earlier, partial sequencing of the CHI 26 protein (Fig. 3, Leah *et al.*, 1987). This indicates that at least two CHI 26 isoforms are expressed in barley seeds. The sequence of CHI 26 is compared in Fig. 4 with that encoded by another barley cDNA isolated

[2] Portions of this paper (including "Experimental Procedures" and Figs. 3–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

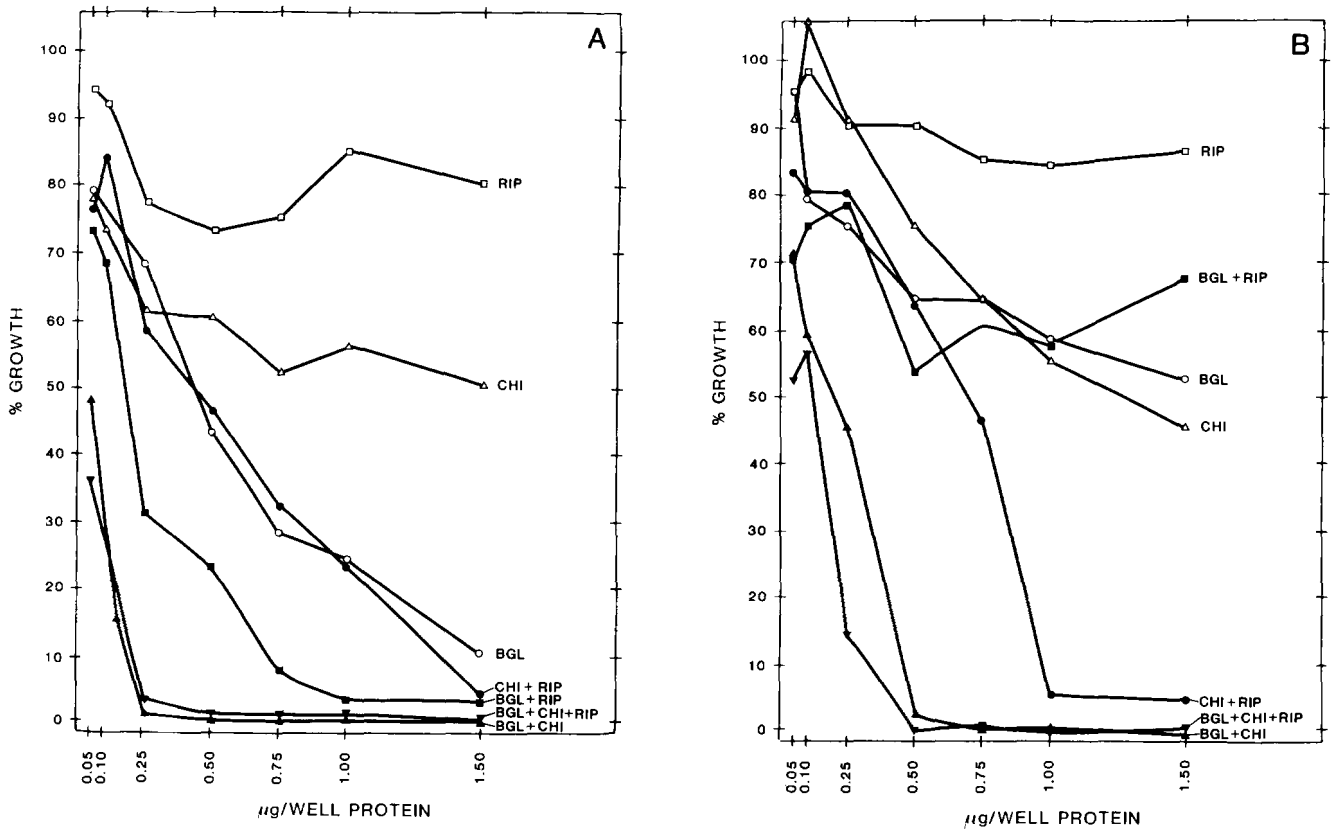

FIG. 2. **Effect of the purified proteins on fungal growth.** Spores of *T. reesei* (*A*) and *F. sporotrichioides* (*B*) were grown in a final volume of 135 µl/microplate well for 48 h with 0.05–1.5 µg of each of the indicated purified proteins (see "Experimental Procedures"). Each point represents the average of five independent measurements with relative standard deviations of 3.6% (*A*) and 7.3% (*B*). 100% growth represents an $OD_{540\ nm}$ of 0.40 (*A*) and 0.41 (*B*).

from Himalaya barley (*clone 10*, Swegle *et al.*, 1989). While *clone 10* encodes a homologous chitinase, differences in the nucleic acid sequence clearly show that it is derived from a different transcript than cCHI26 (data not shown). Furthermore, *clone 10* was shown by hybrid selection to encode a 36-kDa protein (Swegle *et al.*, 1989). *Clone 10* and cCHI26 thus appear to encode two different chitinase enzymes expressed in barley seeds.

The amino acid sequence of CHI 26 is also compared in Fig. 4 with that of the 35-kDa bean chitinase reported by Broglie *et al.* (1986). The major difference between the two proteins is that the bean chitinase is extended at both the NH_2 and COOH termini. The NH_2-terminal extension of the bean chitinase is related to a domain of the wheat germ agglutinin isolectin which binds aminoacetylated amino sugars (Lucas *et al.*, 1985).

Antiserum raised against purified 30-kDa ribosome-inactivating protein was used to identify a corresponding full-length cDNA clone, cRIP30, from the lambda gt11 expression library. The nucleotide sequence of cRIP30 and the deduced amino acid sequence of the encoded protein are presented in Fig. 5*A*. The GC-rich open reading frame of cRIP30 predicts a polypeptide of 29,976 Da. The cRIP30 clone does not encode a signal peptide extension. The open reading frame starts with an ATG (methionine, M − 1) just preceding the first amino acid (A + 1) of the mature RIP 30 protein sequence. This indicates that the protein encoded by cRIP30 is the mature form, suggesting that RIP 30 is a cytosolic protein.

The RIP 30 protein encoded by the cDNA differs in 6 residues from that of the barley 30-kDa ribosome-inactivating protein isoform II sequenced by Asano *et al.* (1986) (Fig. 5, legend). A partial cDNA clone (cRIP30A), isolated in the same screenings which identified the cRIP30 clone, encodes a RIP 30 isoform that is identical to the isoform II protein (Fig. 5*B*). These results suggests that cRIP30A encodes RIP 30 isoform II, while the full-length cRIP30 encodes either isoform I or III.

The full-length (1–3)-β-glucanase cDNA clone, cBGL32, was isolated from a cDNA library made from GA_3-treated aleurone layer mRNA using a 70-mer synthetic oligodeoxynucleotide as probe (Fig. 6, legend). This probe, derived from a recently published sequence (Høj *et al.*, 1989), was selected from the 5′-most region of the (1–3)-β-glucanase open reading frame with least homology to that of (1–3,1–4)-β-glucanase, a closely related protein (Fincher *et al.*, 1986). The nucleotide sequence of cBGL32 and the deduced amino acid sequence of the encoded protein are presented in Fig. 6. cBLG32 differs from the clone described by Høj *et al.* (1989) at 35 nucleotides, only 2 of which cause amino acid substitutions in the open reading frame (Fig. 6, legend).

The GC-rich open reading frame of cBGL32 predicts a polypeptide of 35,212 Da. The 306 COOH-terminal amino acids differ at a single residue (V43:L) from the protein sequence determined for the endo-(1–3)-β-glucanase isoform II (Ballance and Svendsen, 1988). In addition, cBGL32 encodes a 28-amino acid polypeptide with the characteristics of an eucaryotic signal sequence. The mature BGL 32 protein therefore has a predicted molecular weight of 32,343.

Organization of Barley Chitinase, Ribosome-inactivating Protein, and (1–3)-β-Glucanase Structural Genes—Southern

hybridization was used to investigate the organization and copy number of the genes encoding the three proteins. DNA isolated from embryos was digested with *Eco*RI, *Hin*dIII, or *Bam*HI and probed with the cDNAs. The full-length cCHI26 probe hybridizes to six *Eco*RI, eight *Hin*dIII, and six *Bam*HI restricted DNA fragments (Fig. 7*A*). In contrast, a probe from the 3′-untranslated region of cCHI26 (112-bp *Mnl*I-*Eco*RI fragment, Fig. 3) hybridizes to a single fragment in each of the restricted genomic DNAs (indicated by * in Fig. 7*A*). These results indicate that the barley chitinases are encoded by a small family of six to eight genes.

The RIP 30 cDNA hybridizes to three *Eco*RI, three *Hin*dIII, and three *Bam*HI restricted DNA fragments (Fig. 7*B*). This indicates that barley contains three RIP 30 genes per haploid genome, consistent with the isolation of three ribosome-inactivating protein isoforms by Asano *et al.* (1986).

The cBGL32 cDNA hybridizes strongly to two *Eco*RI DNA fragments and three *Hin*dIII and two *Bam*HI restricted genomic fragments (Fig. 7*C*). The strongly hybridizing bands suggest that barley contains two highly homologous BGL 32 structural genes per haploid genome. This is consistent with the recent characterization of the two highly similar 32-kDa (1–3)-β-glucanase isoenzymes I and II by Høj *et al.* (1989). The weakly hybridizing DNA fragments may be due to hybridization of the cBGL32 probe to sequences encoding the related (1–3,1–4)-β-glucanase isoenzymes.

Differential Accumulation of mRNAs Encoding the Three Proteins—Northern hybridization was used to monitor the accumulation of mRNAs encoding CHI 26, RIP 30, and BGL 32 in seed and vegetative tissues (Fig. 8). In whole developing seeds, accumulation of a single 1.2-kb CHI 26 mRNA is detectable at 23 days post anthesis (DPA), and increases to a maximum about 30 DPA (Fig. 8*A*, *lanes 1–5, 10, 11*). Accumulation of this mRNA appears to be aleurone-specific (Fig. 8*A*, *lanes 12, 13 versus 14, 15*). The low level of CHI 26 transcript detected in mRNA from 30 DPA starchy endosperms is probably due to contamination of the starchy endosperm tissue fraction with aleurone cells (Fig. 8*A*, *lane 13*). This was confirmed by performing *in situ* RNA hybridization on whole 30 DPA barley seeds with the CHI 26 cDNA as probe after Raihkel *et al.* (1989). This experiment showed that hybridizable CHI 26 mRNA only accumulates in aleurone cells (data not shown). The deposition of CHI 26 protein in mature seeds was also shown to be aleurone-specific by tissue-print blots (Cassab and Varner, 1987) probed with the monospecific anti-chitinase antibody (data not shown).

The time course of RIP 30 mRNA accumulation in seeds is similar to that of CHI 26 mRNA (Fig. 8*B*, *lanes 1–5, 10, 11*). In contrast to that of CHI 26 mRNA, accumulation of RIP 30 mRNA is confined to the starchy endosperm (Fig. 8*B*, *lane 13*). In keeping with these results, the RIP 30 protein was shown to be localized to the starchy endosperm of mature seeds when analyzed by tissue-print immunoblots (data not shown).

Low levels of BGL 32 mRNA accumulate early during seed development (Fig. 8*C*, *lanes 1–5*). Lower levels of BGL 32 mRNA are also observed in other RNA preparations from 20 and 30 DPA developing seeds (Fig. 8*C*, *lanes 10, 11*) and from 20- and 30-DPA starchy endosperm tissue (Fig. 8*C*, *lanes 12, 13*). Higher levels of BGL 32 mRNA accumulate during germination where it is detectable in growing shoots (coleoptile and primary leaf) and in young roots (Fig. 8*C*, *lanes 6, 7*). Levels of BGL 32 mRNA in these tissues are much lower than those of CHI 26 and RIP 30 mRNAs in developing seeds (see Fig. 8, legend).

Accumulation Patterns of the mRNAs in Germinating Seed Tissues—Northern hybridization was used to monitor the presence of CHI 26, RIP 30, and BGL 32 mRNAs in normally germinating seedlings (Fig. 9). The cCHI26 probe hybridizes to two different chitinase mRNAs (Fig. 9*A*, *lanes 1 versus 4*). The cCHI26 gene-specific probe (Fig. 3) hybridizes solely to the 1.2-kb mRNA present between 4 and 72 h (Fig. 9*A*, *lanes 1–4*) (data not shown). The 1.4-kb mRNA transcript, which presumably encodes another chitinase form, is accumulated later, from 24 to 96 h of germination (Fig. 9*A*, *lanes 2–5*). In contrast, RIP 30 mRNA is not detectable in germinating seedlings (Fig. 9*B*), while the BGL 32 mRNA transcript accumulates to detectable levels first after 72 h (Fig. 9*C*, *lanes 4, 5*).

The accumulation of the mRNAs was also monitored in aleurone layers cultured in the presence of the phytohormones gibberellic and abscisic acids (Fig. 10). This organ system has been used to study the effect of these hormones on the expression of genes involved in seed dormancy and germination (Mundy *et al.*, 1986). Control hybridizations with an ABA-responsive cDNA probe and a GA_3-responsive cDNA probe has previously confirmed that the filter used here contains ABA and GA_3-responsive mRNAs (Leah and Mundy, 1989).

The cCHI26 probe recognizes both the 1.2- and 1.4-kb mRNAs in germinating seedlings and in hormone-treated aleurone layers (Fig. 10*A*). Short exposure of the same blot as well as hybridization with the cCHI26 gene specific probe reveals that levels of the less abundant 1.2-kb mRNA are not affected by the hormone treatments. The longer exposure (Fig. 10*A*) shows that levels of the 1.4-kb mRNA are unaf-

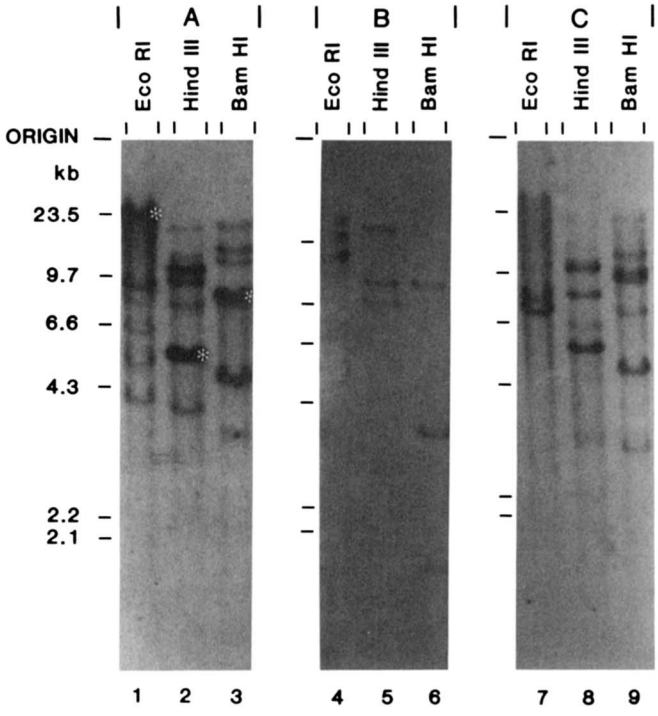

FIG. 7. **Organization of genes encoding the three proteins.** Southern blot analysis of the organization of genes encoding chitinase (*A*), ribosome-inactivating protein (*B*), and (1–3)-β-glucanase structural genes (*C*). Barley embryonic DNA was digested with the restriction enzymes *Eco*RI (*lanes 1, 4, 7*), *Hin*dIII (*lanes 2, 5, 8*), and *Bam*HI (*lanes 3, 6, 9*), fractionated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled cCHI26 (*A*), cRIP30 (*B*), and cBGL32 (*C*). The asterisks in *A* indicate the single DNA fragments which hybridize to a gene-specific 26-kDa chitinase probe (see "Results"). Molecular weight markers are indicated (× 1000) at the *left*.

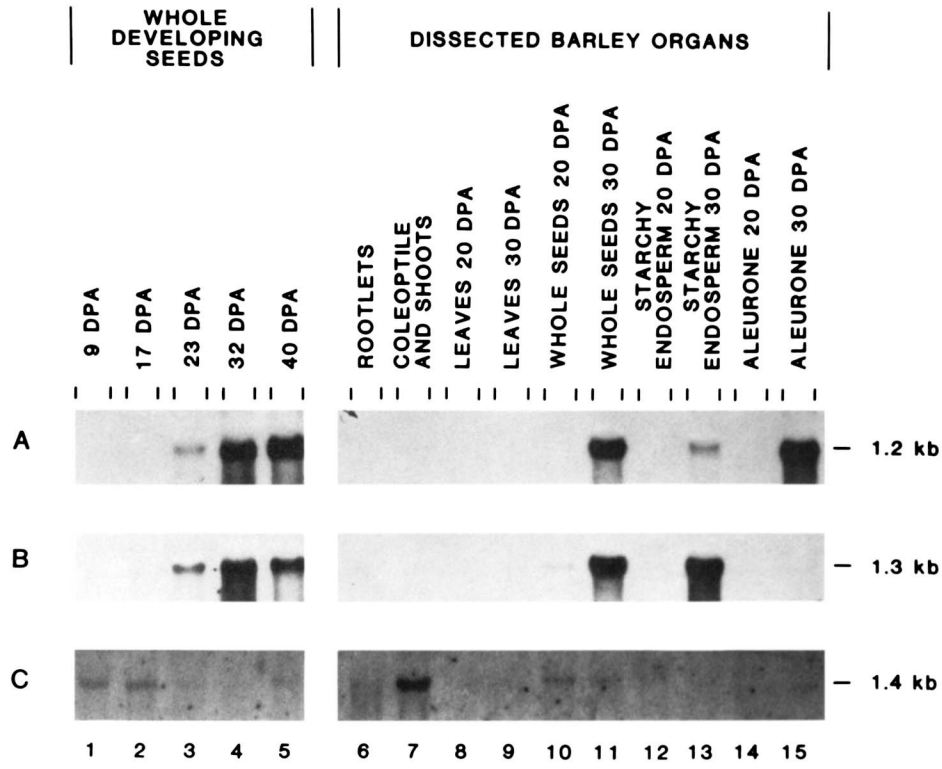

FIG. 8. **Tissue-specific and differential accumulation of mRNAs encoding the three proteins.** Northern blot analysis of chitinase (*A*), ribosome-inactivating protein (*B*), and (1–3)-β-glucanase mRNA (*C*) from whole developing barley seeds 9–40 DPA (*lanes 1–5*), from rootlets (*lane 6*), and coleoptile/shoots) (*lane 7*) of 5-day germinated seedlings, and from leaves (*lanes 8, 9*), whole developing seeds (*lanes 10, 11*), developing starchy endosperm (*lanes 12, 13*) and aleurone cells (*lanes 14, 15*) from plants 20 days (*lanes 8, 10, 12, 14*) and 30 days (*lanes 9, 11, 13, 15*) post anthesis (see "Experimental Procedures"). The size of the transcripts are 1.2 kb for the chitinase mRNA, 1.3 kb for the ribosome-inactivating protein mRNA, and 1.4 kb for the (1–3)-β-glucanase mRNA. The exposure time of the Northern blots in *C* was three times that of *A* and *B* (see "Results").

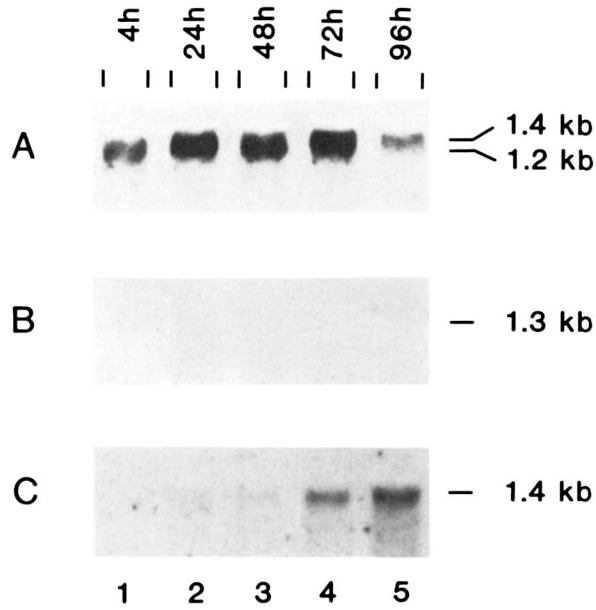

FIG. 9. **Differential accumulation during germination of mRNAs encoding the three proteins.** Northern blot analysis of chitinase (*A*), ribosome-inactivating protein (*B*), and (1–3)-β-glucanase mRNA (*C*) at indicated time points during germination. The same Northern blot filter was sequentially hybridized with cCHI26, cRIP30, and cBGL32 inserts as probes. Hybridization of the Northern blot filter with the 26-kDa chitinase gene specific probe showed that the 1.2-kb mRNA transcript is present throughout the germination, but is not detectable after 96 h of germination (data not shown).

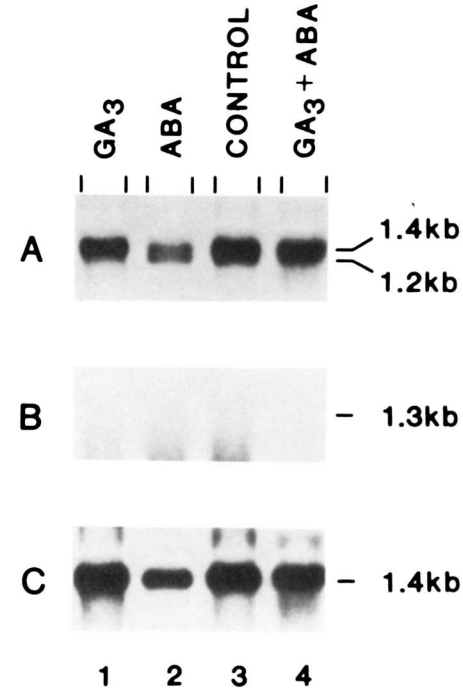

FIG. 10. **Differential accumulation of mRNAs encoding the three proteins in hormone-treated aleurone layers.** Northern blot analysis of chitinase (*A*), ribosome-inactivating protein (*B*), and (1–3)-β-glucanase mRNA (*C*) levels in *lane 1*, GA_3 (10 μM); *lane 2*, ABA (50 μM); *lane 3*, − hormones; *lane 4*, GA_3 + ABA. 2 μg poly(A)-rich mRNA were loaded in each lane. The same Northern blot filter was sequentially hybridized with cCHI26, cRIP30, and cBGL32 inserts as probes. Hybridization with the 26-kDa chitinase gene-specific probe (the 112-bp *Mnl*I-*Eco*RI, Fig. 3) showed that the 1.2-kb mRNA corresponds to cCHI26 (data not shown).

fected by GA_3 but reduced by ABA (Fig. 10*A*, *lane 2 versus 1*, *3*, and *4*). The larger size of this mRNA and the inhibition of its accumulation by ABA suggest that it corresponds to the chitinase encoded by the *clone 10* recently reported by Swegle *et al.* (1989).

As expected from the foregoing results (Fig. 9*B*), RIP 30

mRNA does not accumulate to detectable levels in cultured aleurone layers (Fig. 10*B*, *lanes 1–4*). RIP 30 mRNA is therefore only accumulated in developing seeds (Fig. 8). In contrast, BGL 32 mRNA does accumulate in aleurone layers. Its accumulation is not markedly affected by the hormone treatments (Fig. 10*C*, *lanes 1–4*). This indicates that expression of (1–3)-β-glucanase, in contrast to the related (1–3,1–4)-β-glucanase, is not responsive to GA_3 (Mundy and Fincher, 1986).

DISCUSSION

We are interested in studying the molecular basis of the resistance of cereal plants and seeds to fungal pathogens and seed rots. As part of these studies, we isolated three proteins from barley seeds which exhibit antifungal properties *in vitro*. Two of them, a 26-kDa chitinase and a 30-kDa ribosome-inactivating protein, accumulate to high levels (approximately 80 μg/g seed) late during seed development. The third protein, a 32-kDa (1–3)-β-glucanase, accumulates at lower levels (approximately 3 μg/g seed) during seed formation and germination.

Here we present evidence that these purified proteins inhibit the growth of *T. reesei* and *F. sporotrichioides*, a barley seed rot, as measured in a microtiter plate assay. Similar patterns of inhibition were also measured for *Rhizoctonia solani* and *Botrytis cinerea*, pathogens of potato and pea, respectively (data not shown). The microtiter plate assay used here is more sensitive and precise than the disc-plate diffusion assay used in other studies (Roberts and Seletrennikoff, 1986). Mycelial growth was inhibited by the individual purified proteins at concentrations as low as 1×10^{-8} M, indicating that the protein fractions are as active as any reported elsewhere (Mauch *et al.*, 1988). Inhibition was far more pronounced when mixtures of the proteins were used, suggesting that they synergistically retard fungal growth via different mechanisms.

Previous work has indicated that chitinase and (1–3)-β-glucanase inhibit fungal growth by degrading chitin and (1–3)-β-glucan, major structural cell-wall polysaccharides in growing hyphae (Bartnicki-Garcia, 1968). The results presented here and elsewhere (Roberts and Selitrennikoff, 1986) indicate that single chain ribosomal inactivating proteins, like the barley RIP 30, are cytotoxic to fungal cells (Endo *et al.*, 1988). Furthermore, the synergistic inhibition of fungal growth by mixtures of RIP 30 with CHI 26 and BGL 32 suggests that inhibition by RIP 30 is enhanced when hyphal cell walls are permeabilized by the action of these hydrolases.

Full-length cDNAs encoding CHI 26, RIP 30, and BGL 32 were isolated and sequenced to determine the primary structures of these proteins. The cDNAs were also used as probes in Southern and Northern hybridization experiments to determine the structure and patterns of expression of the corresponding genes. The Southern experiments show that the three proteins are encoded by small families of two to eight genes. At present, the specific functions *in vivo* of the different protein isoforms encoded by these genes is not known. Northern hybridizations show that the patterns of expression of these genes in seeds are very different. High levels of CHI 26 mRNA accumulate late during seed development and are present during germination specifically in aleurone cells. Corresponding high levels of RIP 30 mRNA accumulate only in the starchy endosperm. In contrast, BGL 32 mRNA does not show such tissue-specific accumulation. It accumulates to lower levels first during seed development, and later in the aleurone layer and seedling tissues during germination. Furthermore, the accumulation of BGL 32 and CHI 26 mRNAs during germination appears to be unaffected by the phytohormone gibberellic acids, which controls the expression on several germination-specific enzymes (Mundy and Fincher, 1986).

The different patterns of expression of the three proteins presumably reflect their different biological functions. For example, CHI 26 may be specifically deposited in the outer aleurone layer to retard attack by fungi and insects which contain chitin in their cell walls and exoskeletons. As chitin is not a component of barley seeds, the accumulation in them of high levels of the 26-kDa chitinase suggests that the enzyme plays a protective role. In contrast, low levels of BGL 32 are synthesized at various developmental stages where it may function in the metabolism of endogenous barley cell wall (1–3)-β-glucan. At present, it is not known whether the enzyme plays a defensive role in seeds against attack by pathogens and seed rots.

The starchy endosperm-specific deposition of RIP 30 suggests that it may function as an albumin storage polypeptide. However, its inhibitory activities, measured by *in vitro* translation and fungal growth assays, suggest that it may play a protective role as well. Starchy endosperm cells differentiate terminally during development and are metabolically senesced at maturity. It is possible that RIP 30, despite its inhibitory specificity toward "foreign" ribosomes (Stirpe and Hughes, 1989), is mildly cytotoxic to barley cells. In this case, starchy endosperm cells would form one of the tissues where high levels of ribosome inactivating proteins could accumulate in cereal plants. These proteins might even be determinants of the terminally differentiated fate of this cell type.

Clearly, much work remains to determine the specific functions of these proteins in seeds. One approach to this question would entail phenotypic analysis of transgenic plants which overexpress the proteins. To this end, expression vectors are being constructed to express these proteins singly and in pairs under the control of selected plant promoters. These experiments may increase our understanding of the functions of these proteins in seeds, and may result in the production of transgenic plants with heightened resistance to fungal pathogens.

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SUPPLEMENTAL MATERIAL TO

Biochemical and Molecular Characterization of Three Barley Seed Proteins with Anti-Fungal Properties

Robert Leah, Henrik Tommerup, Ib Svendsen, and John Mundy

EXPERIMENTAL PROCEDURES

Plant Material

Developing barley (*Hordeum vulgare* L. cv. Piggy) seeds were harvested various days after anthesis, frozen in liquid N_2 and stored at -80°C. The preparation of whole germinating seedlings, roots and shoots thereof, and hormone-treated aleurone layers were prepared according to Mundy et al. (1986).

Purification of the Three Proteins

CHI 26 and RIP 30 were purified from flour of whole mature barley seeds as previously described (Leah etal., 1987; Asano et al., 1986). Monospecific rabbit antibodies raised against the proteins purified in these earlier studies were used to follow the course of the large-scale purifications described here. Briefly, after extraction (10kg flour/10 liter buffer) CHI 26 and RIP 30 were recovered in the 40% and 70% $(NH_4)_2SO_4$ precipitates, respectively. After dialysis, the proteins were initially purified by ion-exchange chromatography on CM 52 (Whatman), followed by chromatography on Mono S columns (Pharmacia). BGL 32 was extracted (1,6 kg lyophilized seedlings/25 liter buffer) from whole seedlings germinated 12 days. Following precipitation with 40% $(NH_4)_2SO_4$, the supernatant was dialyzed and chromatographed on CM 52 and Mono S columns. Purified protein fractions were tested for purity by silver-stained SDS-PAGE, Western blotting and N-terminal sequence analysis by automated Edman degradation. The activity of CHI 26 and RIP 30 was measured according to Molano et al. (1977) and Asano et al. (1984), respectively. Activity of BGL 32 was calculated as release of glucose equivalents/min/mg enzyme (Høj et al., 1989).

Fungal Growth Inhibition Assay

Trichoderma reesei and *Fusarium sporotrichioides* were obtained from ATCC, Rockville. Spores were collected from 8 day old cultures grown at 25°C on potato dextrose agar (Difco) plates. The spores were harvested according to Broekaert et al. (1990) and stored in 20% glycerol at -20°C. In the growth inhibition assay, fungal spore suspensions (10,000 spores/ml) were grown at 25°C in 100ul potato dextrose broth per microtiter well and incubated with protein fractions (total volume 135ul) as outlined in the Results. Measurements of culture absorbances at 540nm correlate linearly with fungal biomass within a broad OD range. Plots of the OD_{540} *versus* concentration of added protein permitted the calculation of the inhibitory activity of the purified proteins.

Isolation of cDNA Clones Encoding the Three Proteins

A cDNA expression library was constructed in lambda gt11 from poly(A)-rich RNA isolated from 30 DPA developing *Hordeum vulgare* L. cv. Piggy seeds (Leah and Mundy, 1989). The library was immuno-screened according to standard protocols (Young and Davis, 1983) with monospecific rabbit antisera raised against purified 26kD chitinase (Leah et al., 1987) and 30kD ribosome-inactivating protein (Mundy et al., 1986). Another cDNA library was constructed in lambda ZAP II (Stratagene) from poly(A)-rich RNA isolated from GA_3-treated aleurone layers of cv. Himalaya. This library was screened according to standard protocols (Maniatis et al., 1983) with a synthetic 70-mer oligodeoxynucleotide probe to identify a full-length clone encoding (1-3)-ß-glucanase. This probe was selected from the 5' region of a barley (1-3)-ß-glucanase cDNA (Høj et al., 1989) with least homology to that of a cDNA encoding the homologous (1-3,1-4)-ß-glucanase (Fincher et al., 1986).

DNA Nucleotide Sequence Analysis

Phage were plaque-purified and the inserts identified by sequencing the phage DNA. Selected cDNAs were then subcloned and completely sequenced in both directions by the dideoxynucleotide chain termination method (Sanger et al., 1977). Suitable sequencing primers were synthesized on an Applied Biosystem Model 380A synthesizer. Sequences were analyzed with Beckman Micro Genie Sequence software.

RNA and DNA Blot Analysis

Total RNA was prepared from barley tissues using aurintricarboxylic acid as RNase inhibitor (Leah and Mundy,1989). RNAs (20ug/lane) were separated by formaldehyd agarose gel electrophoresis (Maniatis et al., 1982) and blotted onto Biodyne A nylon membranes (Pall Corporation) without denaturation or neutralization prior to transfer. Poly(A)-rich RNA was purified according to Leah and Mundy (1989). Genomic DNA was extracted from developing embryos collected from barley spikes harvested 20 DPA. The embryos were ground to a powder under liquid N_2, thawed in 10 volumes of 10mM Tris-HCl (pH8), 100mM NaCl and 1mM EDTA , treated with Proteinase K and pancreatic RNase, followed by phenol extraction and ethanol precipitation. Restricted DNA was separated in gels and blotted to nylon membranes with 20 x SSC as transfer buffer (Maniatis et al., 1982).
Hybridizations were performed at 42°C in 50% Formamide, 5 x SSC, 5 x Denhardt's solution, 0.2% SDS, 10ug/ml poly(A), 100ug/ml denatured salmon sperm DNA and denatured ^{32}P-random-primer-labeled DNA probes (spc. act. 1 - 5 x 10^8cpm/ug DNA). The hybridized membranes were washed under stringent conditions (3 changes of 2 x SSC, 0.2% SDS at room temperature for 15 min followed by 3 changes of 0.2 x SSC, 0.1% SDS at 65°C for 15 min).

RESULTS

Figure 3.

Sequence of cCHI26 and of the Encoded 26kD Chitinase.

```
CCTACGACAGTAGCGTAACGGTAAACACCGAGTACGGTACTCTGTGCTTTGTTGGCTCGC    60
                *   *
ACAATGAGATCGCTCGCGGTGGTGGTGGCCGTGGTAGCCACGGTGGCCATGGCCATCGGC   120
  M  R  S  L  A  V  V  V  A  V  V  A  T  V  A  M  A  I  G
          -20                                  -10
ACGGCGCGCGGCAGCGTGTCCTCCATCGTCTCGCGCGCACAGTTTGACCGCATGCTTCTC   180
  T  A  R  G  S  V  S  S  I  V  S  R  A  Q  F  D  R  M  L  L
       -1  1                                 10
CACCGCAACGACGGCGCCTGCCAGGCCAAGGGCTTCTACACCTACGACGCCTTCGTCGCC   240
  H  R  N  D  G  A  C  Q  A  K  G  F  Y  T  Y  D  A  F  V  A
             20                                  30
GCCGCAGCCGCCTTCCCGGGCTTCGGCACCACCGGCAGCGCCGACGCCCAGAAGCGCGAG   300
  A  A  A  A  F  P  G  F  G  T  T  G  S  A  D  A  Q  K  R  E
             40                                  50
GTGGCCGCCTTCCTAGCACAGACCTCCCACGAGACCACCGGCGGGTGGGCGACTGCACCG   360
  V  A  A  F  L  A  Q  T  S  H  E  T  T  G  G  W  A  T  A  P
             60                                  70
GACGGGGCCTTCGCCTGGGGCTACTGCTTCAAGCAGGAACGTGGCGCCTCCTCCGACTAC   420
  D  G  A  F  A  W  G  Y  C  F  K  Q  E  R  G  A  S  S  D  Y
             80                                  90
TGCACCCCGAGCGCACAATGGCCGTGCGCCCCCGGGAAGCGCTACTACGGCCGCGGGCCA   480
  C  T  P  S  A  Q  W  P  C  A  P  G  K  R  Y  Y  G  R  G  P
            100                                 110
ATCCAGCTCTCCCACAACTACAACTATGGACCTGCCGGCCGGGCCATCGGGGTCGATCTG   540
  I  Q  L  S  H  N  Y  N  Y  G  P  A  G  R  A  I  G  V  D  L
            120                                 130
CTGGCCAACCCGGACCTGGTGGCCACGGACGCCACTGTGGGCTTTAAGACGGCCATCTGG   600
  L  A  N  P  D  L  V  A  T  D  A  T  V  G  F  K  T  A  I  W
            140                                 150
TTCTGGATGACGGCGCAGCCGCCCAAGCCATCGAGCCATGCTGTGATCGCCGGCCAGTGG   660
  F  W  M  T  A  Q  P  P  K  P  S  S  H  A  V  I  A  G  Q  W
            160                                 170
AGCCCGTCAGGGGCTGACCGGGCCGCAGGCCGGGTGCCCGGGTTTGGTGTGATCACCAAC   720
  S  P  S  G  A  D  R  A  A  G  R  V  P  G  F  G  V  I  T  N
            180                                 190
ATCATCAACGGCGGGATCGAGTGCGGTCACGGGCAGGACAGCCGCGTCGCCGATCGAATC   780
  I  I  N  G  G  I  E  C  G  H  G  Q  D  S  R  V  A  D  R  I
            200                                 210
GGGTTTTACAAGCGCTACTGTGACATCCTCGGCGTTGGCTACGGCAACAACCTCGATTGC   840
  G  F  Y  K  R  Y  C  D  I  L  G  V  G  Y  G  N  N  L  D  C
            220                                 230
TACAGCCAGAGACCCTTCGCCTAATTAATTAGTCATGTATTAATCTTGGCCCTCCATAAA   900
  Y  S  Q  R  P  F  A  *
            240
ATACAATAAGAGCATCGTCTCCTATCTACATGCTGTAAGATGTAACTATGGTAACCTTTT   960

ATGGGGAACATAACAAAGGCATCTCGTATAGATGCTTTGCTA12                   1013
```

Nucleotide sequence of cCHI26 (GenBank accession # M36989). The cDNA contains 1013 nucleotides with a 63 bp 5′-untranslated leader, a 798bp open reading frame and a 152 bp 3′-untranslated tail. (*) indicates in frame stop codons, the underlined AT-rich sequence at position 905 a putative polyadenylation signal. The deduced amino acid sequence of the barley 26kD chitinase preprotein of 266 amino acids is shown below the nucleotide sequence. The putative signal peptide sequence (M-23 to G-1) is heavy underlined. The NH_2- and COOH- terminal residues of the mature 26kD chitinase are S+1 and A243, respectively. More than 75% of the amino acid sequence was determined in parallel by sequencing the purified protein (underlined). Two discrepancies (G150:A and D182:D or S) were observed between this protein sequence and the open reading frame of cCHI 26. The 3′-cCHI26 gene specific probe is the 112bp MnlI - EcoRI fragment at position 902 -1013 (overlined).

Figure 4.

Sequence Similarities between Barley and Bean Chitinases

```
C)  MKKNRMMMMIWSVGVVWMLLLVGGSYGEQCGRQAGGALCPGGNCCSQFGWC

A)  M R S L A V V V A V V A T V A M A I G T A R G S V   25
B)
C)  G S T T D Y C G P G C Q S Q C G G P S P A P T D L

A)  S S I V S R A Q F D R M L L H R N D G A C Q A K G   50
B)
C)  S A L I S R S T F D Q M L K H R N D G A C P A K G

A)  F Y T Y D A F V A A A A A F P G F G T T G S A D A   75
B)
C)  F Y T Y D A F I A A A K A Y P S F G N T G D T A T

A)  Q K R E V A A F L A Q T S H E T T G G W A T A P D  100
B)                      H E T T G G W A T A P D
C)  R K R E I A A F L G Q T S H E T T G G W A T A P D

A)  G A F A W G Y C F K Q E R G A S S D Y C T P S A Q  125
B)  G A F A W G Y C F K Q E R G A T S N Y C T P S A Q
C)  G P Y A W G Y C F V R E R N P S T - Y C S A T P Q

A)  W P C A P G K R Y Y G R G P I Q L S H N Y N Y G P  150
B)  W P C A P G K S Y Y G R G P I Q L S H N Y N Y G P
C)  F P C A P G Q Q Y Y G R G P I Q I S W N Y N Y G Q

A)  A G R A I G V D L L A N P D L V A T D A T V G F K  175
B)  A G R A I G V D L L R N P D L V A T D P T V S F K
C)  C G R A I G V D L L N K P D L V A T D S V I S F K

A)  T A I W F W M T A Q P P K P S S H A V I A G Q W S  200
B)  T A M W F W M T A Q A P K P S S H A V I T G Q W S
C)  S A L W F W M T A Q S P K P S S H D V I T S R W T

A)  P S G A D R A A G R V P G F G V I T N I I N G G I  225
B)  P S G T D R A A G R V P G F G V I T N I V N G G I
C)  P S S A D V A A R R L P G Y G T V T N I I N G G L

A)  E C G H G Q D S R V A D R I G F Y K R Y C D I L G  250
B)  E C G H G Q D M R V A D R I G F Y K R Y C D I L G
C)  E C G R G Q D S R V Q D R I G F F K R Y C D L L G

A)  V G Y G N N L D C Y S Q R P F A *                  266
B)  V G Y G N N L D C Y S Q R P F A *
C)  V G Y G N N L D C Y S Q T P F G (NSLLLSDLVTSQ*)
```

The amino acid sequence of A) CHI 26, compared with that of B) the partial amino acid sequence of the 36kD barley chitinase (Swegle et al., 1989), and of C) the 35kD ethylene-induced bean chitinase (Broglie et al., 1986). Identical amino acids are boxed and putative signal sequences are underlined. The amino acid sequence of CHI 26 and the 35kD ethylene-induced bean chitinase show 84% identity when conserved amino acid changes are included. Alignment of the barley CHI 26 signal sequence (M-23 to G-1) with the bean chitinase preprotein exhibits no homology.

Figure 5.

Sequence of cRIP30 and cRIP30A and of the Encoded 30kD Ribosome-Inactivating Proteins

A

```
CTTAATAGCACATCTTGTCCGTCTTAGCTTTGCATTACATCCATGGCGGCAAAGATGGCG   60
                          *              M  A  A  K  M  A
                                        -1  1

AAGAACGTGGACAAGCCGCTCTTCACCGCGACGTTCAACGTCCAGGCCAGCTCCGCCGAC  120
 K  N  V  D  K  P  L  F  T  A  T  F  N  V  Q  A  S  S  A  D
          10                            20

TACGCCACCTTCATCGCCGGCATCCGCAACAAGCTCCGCAACCCGGCGCACTTCTCCCAC  180
 Y  A  T  F  I  A  G  I  R  N  K  L  R  N  P  A  H  F  S  H
          30                            40

AACCGCCCCGTGCTGCCGCCGGTCGAGCCCAACGTCCCGCCGAGCAGGTGGTTCCACGTC  240
 N  R  P  V  L  P  P  V  E  P  N  V  P  P  S  R  W  F  H  V
          50                            60

GTGCTCAAGGCCTCGCCGACCAGCGCCGGGCTCACGCTGGCCATTCGGGCGGACAACATC  300
 V  L  K  A  S  P  T  S  A  G  L  T  L  A  I  R  A  D  N  I
          70                            80

TACCTGGAGGGCTTCAAGAGCAGCGACGGCACCTGGTGGGAGCTCACCCCGGGCCTCATC  360
 Y  L  E  G  F  K  S  S  D  G  T  W  W  E  L  T  P  G  L  I
          90                           100

CCCGGCGCCACCTACGTCGGGTTCGGCGGCACCTACCGCGACCTCCTCGGCGACACCGAC  420
 P  G  A  T  Y  V  G  F  G  G  T  Y  R  D  L  L  G  D  T  D
         110                           120

AAGCTGACCAACGTCGCTCTCGGCCGGCAGCAGCTGGCGGACGCGGTGACCGCCCTCCAC  480
 K  L  T  N  V  A  L  G  R  Q  Q  L  A  D  A  V  T  A  L  H
         130                           140

GGGCGCACCAAGGCCGACAAGCCGTCCGGCCCGAAGCAGCAGCAGGCGAGGGAGGCGGTG  540
 G  R  T  K  A  D  K  P  S  G  P  K  Q  Q  Q  A  R  E  A  V
         150                           160

ACGACGCTGCTCCTCATGGTGAACGAGGCCACGCGGTTCCAGACGGTGTCTGGGTTCGTG  600
 T  T  L  L  L  M  V  N  E  A  T  R  F  Q  T  V  S  G  F  V
         170                           180

GCCGGGTTGCTGCACCCCAAGGCGGTGGAGAAGAAGAGCGGGAAGATCGGCAATGAGATG  660
 A  G  L  L  H  P  K  A  V  E  K  K  S  G  K  I  G  N  E  M
         190                           200

AAGGCCCAGGTGAACGGGTGGCAGGACCTGTCCGCGGCGCTGCTGAAGACGGACGTGAAG  720
 K  A  Q  V  N  G  W  Q  D  L  S  A  A  L  L  K  T  D  V  K
         210                           220

CCTCCGCCGGGAAAGTCGCCAGCGAAGTTCGCGCCGATCGAGAAGATGGGCGTGAGGACG  780
 P  P  P  G  K  S  P  A  K  F  A  P  I  E  K  M  G  V  R  T
         230                           240

GCTGTACAGGCCGCCAACACGCTGGGGATCCTGCTGTTCGTGGAGGTGCCGGGTGGGTTG  840
 A  V  Q  A  A  N  T  L  G  I  L  L  F  V  E  V  P  G  G  L
         250                           260

ACGGTGGCCAAGGCGCTGGAGCTGTTCCATGCGAGTGGTGGGAAATAGGTAGTTTTCCAG  900
 T  V  A  K  A  L  E  L  F  H  A  S  G  G  K  *

GTATACCTGCATGGGTAGTGTAAAAGTCGAATAAACATGTCACAGAGTGACGGACTGATA  960

TAAATAAATAAATAAACGTGTCACAGAGTTACATATAAACAAATAAATAAATAATTAAAA 1020

ATGTCCAGTTTA47                                                1078
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B

```
GCGGTGACGACGCTGCTCCTCATGGTGAACGAGGCCACGCGGTTCCAGACGGTGTCGGGG   60
 A  V  T  T  L  L  L  M  V  N  E  A  T  R  F  Q  T  V  S  G
          170                           180

TTCGTGGCCGGGCTGCTGCACCCCAAGGCGGTGGAGAAGAAGAGCGGGAAGATCGGCAAT  120
 F  V  A  G  L  L  H  P  K  A  V  E  K  K  S  G  K  I  G  N
          190                           200

GAGATGAAGGCCCAGGTGAACGGGTGGCAGGACCTGTCCGCGGCGCTGCTGAAGACGGAC  180
 E  M  K  A  Q  V  N  G  W  Q  D  L  S  A  A  L  L  K  T  D
          210                           220

GTGAAGCCCCCGCCGGGAAAGTCGCCAGCGAAGTTCACGCCGATCGAGAAGATGGGCGTG  240
 V  K  P  P  P  G  K  S  P  A  K  F  T  P  I  E  K  M  G  V
          230                           240

AGGACTGCTGAGCAGGCTGCGGCTACTTTGGGGATCCTGCTGTTCGTTGAGGTGCCGGGT  300
 R  T  A  E  Q  A  A  A  T  L  G  I  L  L  F  V  E  V  P  G
          250                           260

GGGTTGACGGTGGCCAAGGCGCTGGAGCTGTTTCATGCGAGTGGTGGGAAATAGGTAGTT  360
 G  L  T  V  A  K  A  L  E  L  F  H  A  S  G  G  K  *
          270                           280

TTGCAGGTATACCTGCATGGGTAAATGTAAAAGTCGAATAAAAATGTCACAGAGTGACGG  420

ACTGATATAAATAAATTAATAAACATGTCATCATGAGTGACAGACTGATATAAATAAATA20499
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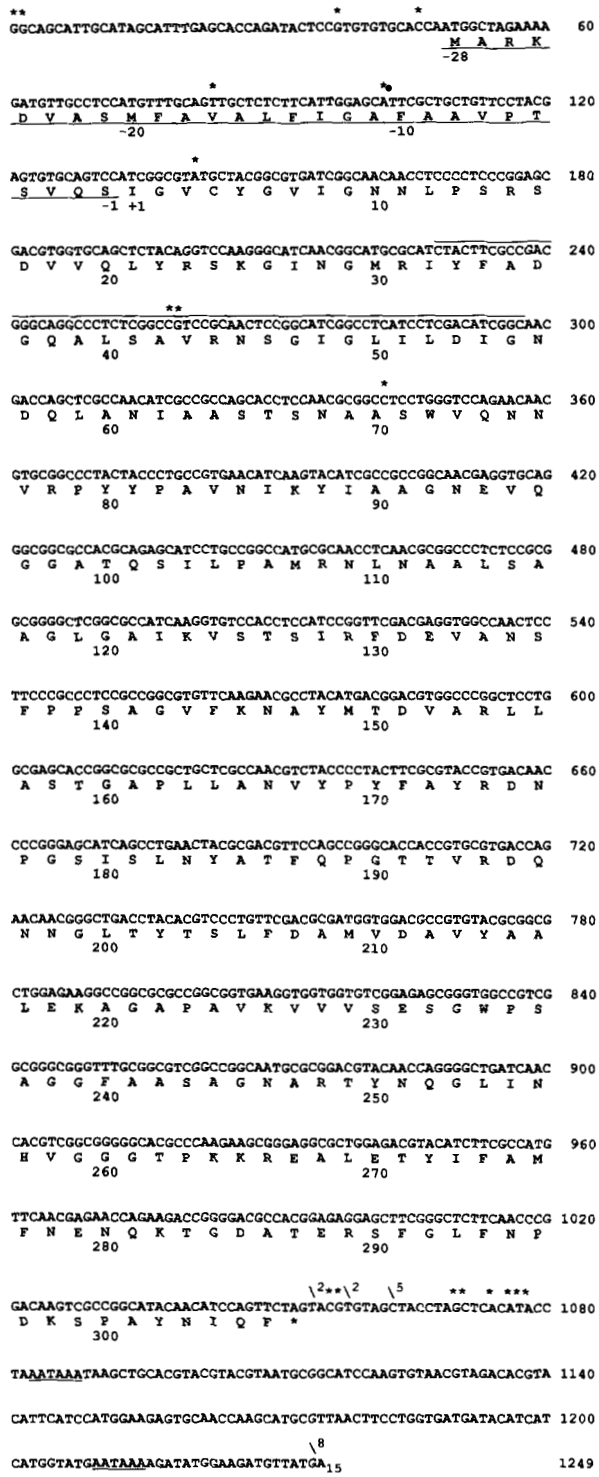
A) Nucleotide sequence of cRIP30 cDNA (GenBank accession # M36990). The cDNA contains 1078 nucleotides with a 42 bp 5′-untranslated leader, an 843 bp open reading frame, and a 193 bp 3′-untranslated tail. (*) indicates the in frame stop codon in the 5′ leader sequence. The underlined AT-rich sequences at positions 930, 963 and 1002 are putative polyadenylation signals. The deduced amino acid sequence of the preprotein of 281 amino acids is shown below the nucleotide sequence. The C-terminal 280 residues are 98% identical to that of the mature barley protein synthesis inhibitor isoform II determined by Asano et al. (1986). The differences are R47:E; A138:E; P153:A; A236:T; V247:E and N252:A. The first amino acid (M-1) of the preprotein is upstream of the first residue of the mature ribosome-inactivating protein (A+1).

B). Nucleotide sequence of the partial cRIP30A cDNA (GenBank accession # M36991). The open reading frame of 350 nucleotides encodes the C-terminal 117 residues of RIP 30 isoform II. The underlined AT-rich sequences at positions 397, 430 and 438 indicate putative polyadenylation signals.

Figure 6.

Sequence of cBGL32 and of the Encoded 32kD (1-3)-β-Glucanase.



Nucleotide sequence of cBGL32 (GenBank accession # M36992). The cDNA contains 1249 nucleotides with a 48 bp 5'-untranslated leader, a 1002 bp open reading frame, and a 199 bp 3'-untranslated tail. (*) and ($\backslash^{No.}$) indicate nucleotide substitutions and numbers of nucleotides deleted at position \, respectively, when the sequence is compared with that of the cDNA recently characterized by Høj et al. (1989). The underlined AT-rich sequences at positions 1083 and 1210 are putative polyadenylation signals. The deduced amino acid sequence of the encoded preprotein of 334 amino acids is shown below the nucleotide sequence. The open reading frame differs by two amino acid substitutions (V-17:A and V43:L) with that published by Høj et al. (1989). The putative signal peptide sequence (M-28 to S-1) is underlined. The NH_2- and COOH-terminal residues of the mature (1-3)-β-glucanase are I+1 and F306, respectively (Ballance et al., 1988). The 70-mer oligodeoxynucleotide used as probe to identify cBGL32 is overlined (bases 228 - 297).