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Two antifungal thaumatin-like proteins from barley grain

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Antifungal activity has been associated with 2 immunochemically distinct proteins, protein R and S (M_r ~23 kDa; pI 9–10), which were isolated in pure form from barley grain. The proteins are homologous with thaumatin- and pathogenesis-related proteins of the PR5 family. The proteins inhibit growth of i.a. *Trichoderma viride* and *Candida albicans* in microtiter plate assays and act synergistically with barley grain chitinase C. Like maize zeamatin, protein R and S but not chitinase C retarded fungal growth in synergism with nikkomycin Z, a nucleoside-peptide inhibitor of fungal chitin synthesis. Although no inhibition of α-amylases or serine proteases could be associated with protein R or S the results indicate that the homologous maize grain bifunctional inhibitor of insect α-amylase and trypsin is very similar to or identical with maize zeamatin, which was proposed to have permeabilizing activity towards fungal membranes. Thus, in addition to the intensely sweet properties of thaumatin, multiple unrelated defense functions against insect and fungal pests can now be associated with the family of thaumatin-homologous proteins.

Amino acid sequence; Antifungal; Osmotin; Pathogenesis related; Thaumatin; *Hordeum vulgare*

1. INTRODUCTION

Various types of environmental stress induce the systemic synthesis of at least 10 families of homologous proteins with protective functions in vegetative tissues of plants [1]. These proteins, termed pathogenesis-related proteins or PR-proteins, include chitin-binding lectins, chitinases and β-1,3-glucanases which impair growth of fungal hyphae, peroxidases involved in cell wall fortification, inhibitors directed against digestive amylases or proteases of herbivorous insects as well as proteins with as yet unidentified functions. One family of PR-proteins (often called PR5 or osmotins) contains proteins of M_r ~22 kDa, which are homologous with the intensely sweet protein thaumatin from fruits of *Thaumatococcus daniellii* Benth and they are therefore called thaumatin-like or TL-proteins [1]. These proteins and their expression have been studied in some detail in tobacco [2–4] and potato [5] but their biological function is unknown. Recently a TLPR-protein was identified in barley leaves challenged with an incompatible race of mildew [6].

The recently identified seed proteins which are homologous, but not identical, with leaf PR-proteins are assumed to be involved in defense of resting and germinating plant seeds [7,8]. The only known seed TL-protein is the bifunctional α-amylase/trypsin inhibitor isolated from maize grain [9]. The specific inhibition of both α-amylase from *Tribolium* beetles and a digestive protease clearly suggested a defense role against insect pests. We have now isolated and characterized 2 immunochemically distinct members of the TL-protein family from barley grains. Neither inhibition of α-amylases nor serine proteases could be associated with these 2 proteins. However, both proteins acted synergistically with another barley grain protein as well as with nikkomycin Z in inhibition of the growth of i.a. *Trichoderma viride* and *Candida albicans* in a way similar to the recently characterized antifungal maize grain protein zeamatin [10]. During purification the 2 barley grain proteins were termed protein R and S, accidentally, in accordance with the PR-R/PR-S nomenclature used for the homologous tobacco proteins [2].

Abbreviations: PR, pathogenesis-related; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TL, thaumatin-like.

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2. MATERIALS AND METHODS

2.1. *Biological materials*

Seeds of barley (*Hordeum vulgare*, cv. 'Bomi' mutant 'Risø 1508') were used for protein purification. Larvae of *Tenebrio mollitor* and *Tribolium confusum* were obtained from the Danish Pest Infestation Laboratory, Lyngby. After 24 h starvation, they were washed with water, freeze-dried and extracted with 5 volumes of 50 mM phosphate buffer, pH 6.9, in a mortar. Following centrifugation these extracts as well as human saliva samples were precipitated with 80% acetone to remove turbidity and stored at −20°C until used in α-amylase assays. Strains of *Candida albicans*, *Trichoderma viride*, and *Fusarium oxysporum* were obtained from the Department of Biotechnology, DTH, Lyngby. All organisms were grown at 31°C on 2.4% potato dextrose broth from Difco (Detroit, USA). *Candida* cells were harvested in the midlogarithmic growth phase. Spores of the filamentous fungi were collected from cultures on agar plates after 7 days. Cells and spores were stored in 20% glycerol at −40°C. Prior to use spores were pregerminated in 40× diluted medium for 18 h.

2.2. *Chemicals*

Carrier ampholytes and gel materials were obtained from Pharma-

cia (Uppsala, Sweden) and used in accordance with the manufacturer's instructions. Enzymes and their chromogenic substrates used in inhibition assays were, where not otherwise indicated, obtained from Sigma (St. Louis, USA) (substrates in brackets): Bovine trypsin (benzoyl-L-arginine-*p*-nitroanilide), bovine chymotrypsin (glutaryl-L-phenylalanine-*p*-nitroanilide), porcine pancreas elastase (succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide), subtilisin Carlsberg from NOVO-Nordic (Bagsvaerd, Denmark) (carbobenzoxy-L-phenylalanine- 2-naphtylester), *Bacillus subtilis* α-amylase from Merck (Darmstadt, Germany) and porcine pancreas α-amylase (Blue Starch from Pharmacia and *p*-nitrophenyl-α-D-maltoheptaoside). Nikkomycin Z from *Streptomyces tendae* was obtained from Calbiochem (La Jolla, USA). All chemicals used in the sequencer were from Applied Biosystems (Foster City, USA).

2.3. *Purification*

The salt soluble proteins were extracted from milled grains of 'Bomi' mutant 1508 (250 g) with sodium phosphate buffer, pH 6.0. After concentration by 30–60% saturation with ammonium sulfate the basic proteins were separated by cation exchange on a CM-Sephadex column equilibrated with 30 mM sodium citrate-phosphate buffer, pH 5.2 and eluted with a linear gradient of sodium chloride in the buffer. These initial steps have been described in detail previously [11]. The protein appearing in the first peak (termed R in [11]) was collected and concentrated in an Amicon 202 stirred cell equipped with a PM 10 membrane. The concentrate was applied on a 15 × 900 mm column of Sephadex G-50, and eluted with 0.1 M sodium chloride. The main peak eluted corresponding to an M_r around 23 kDa. This protein was collected, dialyzed and, finally, rechromatographed on a column of CM-Sephadex Cl-6B at pH 5.0. Purification of the antifungal barley grain chitinase C (protein C) and ribosome inactivating protein K has been described in detail [7,11].

2.4. *Characterization*

The methods used for amino acid sequence determination, SDS-PAGE, and detection of protease inhibitors in column eluates have been described earlier [11,12]. Preparation of monospecific rabbit antibodies and the immunochemical methods have also been described [7,11,12]. Determination of N-terminal amino acid sequences was done on an Applied Biosystems gas-phase sequencer, model 470A equipped with an on-line HPLC, model 120A. α-Amylase activity was measured with the Sigma diagnostic kit no. 576 as adapted for kinetic determinations in a microplate reader [13]. α-Amylase inhibition was measured after preincubation of the enzyme with inhibitor for 15 min at 25°C or 60 min at 0°C, pH 6.9, in the presence of 0.1% bovine serum albumin. Amylase inhibition was also measured with a Blue Starch substrate in a radial diffusion assay [14] after a similar preincubation. Inhibition of proteolytic enzymes was measured spectrophotometrically or fluorometrically as described in detail previously [12,15] after preincubation of enzyme with inhibitor for 5 min at 25°C, pH 8.2. In all assays positive inhibition control was obtained with previously purified α-amylase inhibitors from wheat and protease inhibitors from barley grain. Chitinase activity and affinity for chitin gels were estimated as described earlier [7].

2.5. *Antifungal activity*

Inhibition of growth was measured in 96-well microtiter plates at 540 nm. Desalted inhibitory proteins were preincubated with ~10^3 cells/pre-germinated spores in 200 μl 40× diluted potato dextrose broth for 2 h at 31°C. Thereafter 50 μl 5× concentrated medium was added and the plates were read after 18–24 h at 31°C. The protein concentration (μg/ml final medium) resulting in 50% growth inhibition as measured at an absorbance of about 0.5 in the control wells was termed I_{50}. To test for synergistic effects, 2 proteins were mixed in proportion to their I_{50} values and the experimental results were compared with data calculated for additive inhibition. All results are means of at least 4 determinations. The agar gel diffusion test for synergistic growth inhibition was made exactly as described [10], except for the use of potato dextrose medium instead of carrot juice.

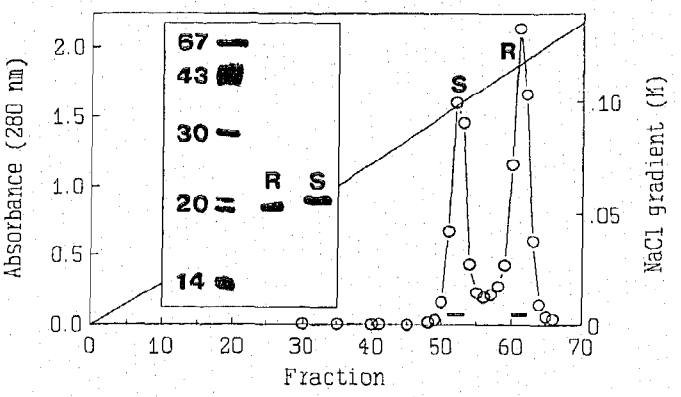

Fig. 1. Separation of proteins R and S by cation exchange chromatography. The CM-Sephadex Cl-6B column (0.9 × 28 cm) was equilibrated with 25 mM sodium acetate, pH 5.0. After protein application in the same buffer, a linear gradient of sodium chloride in the buffer was applied at 13 ml/h. Fractions of 2.3 ml were collected and pooled for characterization as indicated by horizontal bars. SDS-PAGE of the 2 protein pools under non-reducing conditions is shown (insert). Molecular mass of the reference proteins used are shown in kDa.

3. RESULTS

3.1. *Purification and physico-chemical characterization*

The final cation exchange step resulted in separation of 2 protein peaks (Fig. 1). The protein appearing at 0.11 M NaCl in the major peak was termed protein R, while the protein eluting at 0.095 M NaCl in the gradient was termed protein S.

SDS-PAGE and activity measurements showed that small amounts of protein(s) (M_r ~9 kDa) with subtilisin-inhibitory activity were removed in the fractions collected between the 2 peaks.

As judged from SDS-PAGE (Fig. 1), native cathodic PAGE and gel filtration (not shown) as well as immunological tests (Fig. 2), amino acid analyses and sequencing (Fig. 3) made on the pooled material, the combination of 2 cation exchange steps with one gel filtration resulted in highly pure preparations of the 2 proteins. Ouchterlony immunodiffusion with polyclonal rabbit antibodies towards the 2 proteins (Fig. 2a) confirmed that the antibodies were monospecific, and although the 2 proteins had very similar N-terminal sequences (Fig. 3) cross-reactivity was not observed in these tests. This reaction of non-identity was confirmed when the 2 antibody preparations were mixed with polyspecific antibodies in cathodic crossed immunoelectrophoresis of barley proteins (Fig. 2b). The one-dimensional electrophoretic mobilities demonstrate that protein S is slightly more basic than protein R, both with pI 9–10 (compare with pattern and data in [11]). SDS-PAGE with 0.1 M thioglycollic acid included in the cathode buffer gave sharp zones with identical mobility at a molecular mass of ~23 kDa for protein R and S in accordance with the value calculated from gel filtration. Under non-reducing conditions zones with slightly different mobility at ~20 kDa were obtained (Fig. 1).

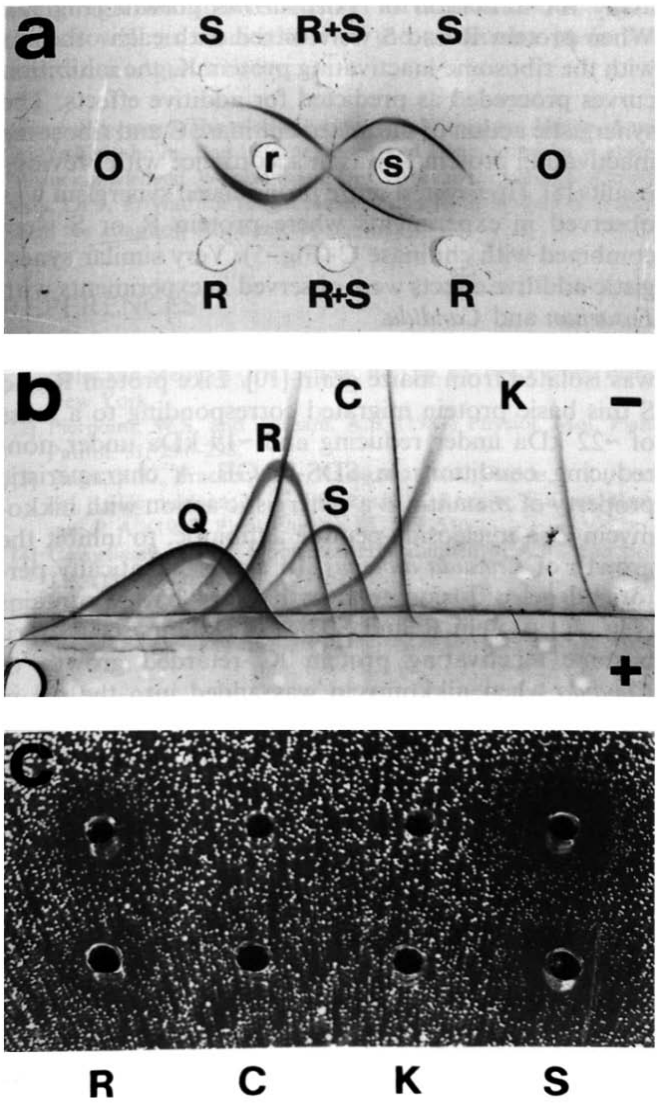

Fig. 2. Immunochemical and antifungal properties of protein R and S characterized in agarose diffusion experiments. (a) Immunodiffusion ad modum Ouchterlony. The proteins (R and S, ~5 μg) and a buffer control (O) were applied in the outer wells, and antiserum towards the 2 proteins (r and s, 10 μl) in the central wells. (b) Cathodic crossed immunoelectrophoresis of proteins in a barley 'mutant 1508' extract. A mixture of barley antibodies was used. In addition to protein R and S the precipitates of chitinase C, ribosome inactivating protein K and protein Q are indicated (see [11] for reference pattern). (c) Synergistic inhibition of *Candida* growth by barley proteins and nikkomycin Z. R, C, K and S indicate the 4 barley proteins. Amounts applied in the wells are 3 μg in the top row and 1 μg in the bottom row. The gel contained nikkomycin (0.2 μg/ml). No growth inhibition was observed in a control gel without nikkomycin.

N-terminal sequencing of 44 residues in protein R and 35 residues in protein S showed that they are homologous with proteins of the same molecular mass belonging to the TLPR-protein family (Fig. 3). Protein R and S have ~60% amino acids in identical positions in the N-terminal, and identity with the other TLPR-proteins is of the same order (~55–65%). In addition, almost all differences are conservative substitutions, e.g. the conservation of hydrophobic residues in positions 3, 5, 13,

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                          20                  40
BP-R      ATITVVNRCSYTVWPGALP GGGVRLDPGQRWALNMPAGTAGAAV
BP-S      **F**I*K*Q****AA*V*A***QK**A**T*SIXX*
BHv-1     **>NIK*N*GS*I*<>GI*V***FE*XS
MAI       *V>***<*Q*PF***>>S>*V***RQ*NR*ES*R>TA*<<<TA<RI
TPR-R     **>DI<*Q*T****>>*S*  ***RQ*NS**S*>><VNP<<VQ<RI
TOSM      **<E*R*N*P****>>ST*I***R<**R**>*V><A*P<<KN<R<
PP-C      **>DIT*<*T*P**>>*S*  ***R<**S**>*N<<VNP<<IQ<RI
THA-I     **>EI**<*<****>>*SK#***RQ*NS*ES*T><VEP<<N<GKI
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Fig. 3. N-terminal sequences of protein R and protein S – comparison with thaumatin-like proteins. BP-R, barley grain protein R; BP-S, barley grain protein S; BHv-1, barley leaf PR protein [6]; MAI, maize grain α-amylase/trypsin inhibitor [9]; TPR, tobacco leaf PR protein R [2,4]; TOSM, tobacco cell culture osmotin [3]; PP-C, potato leaf protein C [5]; THA-1, thaumatin I from fruits of *Thaumatococcus daniellii* Benth [16]. Symbols used: <, identity with BP-R; >, identity with BP-S; *, identity with BP-R and BP-S, X, Unidentified residue. A gap is introduced in position 20 of some sequences to maximize similarity. # indicates that THA-1 contains the additional sequence: GDAALD in this position.

34, and 45 (Fig. 3). Although partial, these sequence data indicate that the 2 barley grain proteins have evolved separately within a highly conserved family of homologous proteins since divergence of mono- and dicot plants. A single deletion/insertion occurs in position 20 of about half of the sequences. Close to this position only thaumatin contains an extra sequence: GDAALD, which may be of importance for the sweetness not present in the TLPR-proteins. Amino acid analyses confirmed the similarity between the 2 barley proteins and with TL-proteins from other plant species (not shown). A characteristic feature is the content of ~8 mol% cysteine in both protein R and S which corresponds to conservation of the 8 disulfide bonds present in thaumatin [16].

3.2. *Biological activity*

Two different assays were used to estimate a possible inhibition of α-amylases from *Tenebrio* and *Tribolium* larvae, human saliva, porcine pancreas and *Bacillus subtilis*. Attempts to detect protease inhibitory activity were made with pancreas elastase, trypsin and chymotrypsin and with subtilisin Carlsberg. Although, inhibitor protein/enzyme molar ratios up to about 50 were tested in all these assays no inhibitory action of protein R or S could be detected. Tests for chitinase activity or lectin-like affinity for chitin were also negative.

Antifungal activity of the 2 barley proteins was investigated in growth tests with *Trichoderma viride* (Fig. 4a) and *Candida albicans* (Fig. 4b) in microtiter plates. Under the experimental conditions used a final concentration of ~1 μg/ml of protein S and ~3 μg/ml of protein R could inhibit *Trichoderma* growth by 50% (I_{50}). Thus, the 2 proteins R and S were as potent as barley chitinase C and barley ribosome inactivating protein K, whose antifungal activities were characterized recently [8]. Similar results were obtained with the barley seed rot *Fusarium oxysporum* (not shown) and with *Candida*. The proteins R, S and K were slightly less potent inhibitors of *Candida* (I_{50} ~5 μg/ml) and chitinase C did not inhibit growth in the concentration range studied. In all

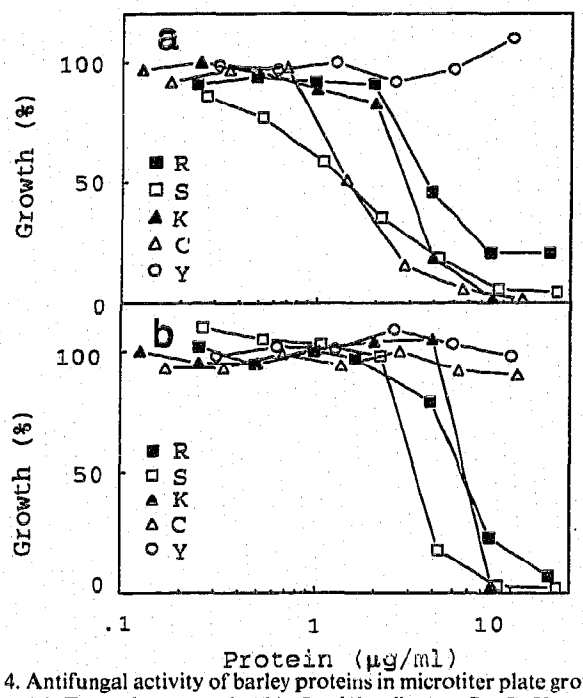

Fig. 4. Antifungal activity of barley proteins in microtiter plate growth assay. (a) *Trichoderma viride*. (b) *Candida albicans*. R, C, K and S indicate the 4 barley proteins and Y denotes yeast cytochrome *c* used as a basic protein control.

experiments protein S had a 2–3× higher activity than protein R, and ~0.3 μg/ml (<100 ng/well) was found to delay the onset of *Trichoderma* growth in the assay (Fig. 4a). Control experiments with yeast cytochrome *c* (Fig. 4) and other basic proteins confirmed that the observed inhibition patterns were not due to the general effect on cell growth found in the 10–100 μg/ml range for many basic proteins [17]

To test for synergistic interactions, the 4 proteins were mixed, 2 and 2, in ratios corresponding to the I_{50} values (Fig. 4a) and compared in the same dilution

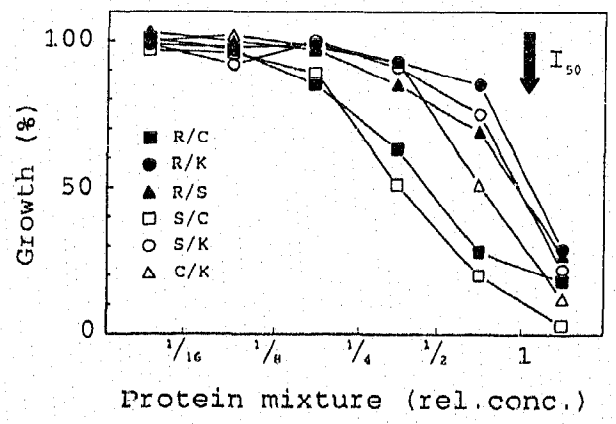

Fig. 5. Synergistic antifungal activity of barley proteins in microtiter plate assay of *Trichoderma* growth. X/X indicate mixing of the 4 proteins R, S, K and C in ratios corresponding to the I_{50} values of Fig. 4a. An arrow indicates the calculated I_{50} level for additive inhibition of growth.

assay for inhibition of *Trichoderma* growth (Fig. 5). When protein R and S were mixed with each other or with the ribosome inactivating protein K, the inhibition curves proceeded as predicted for additive effects. The synergistic action of the mixed chitinase C and ribosome inactivating protein K was in accordance with previous results [8]. However, a more pronounced synergism was observed in experiments where protein R or S were combined with chitinase C (Fig. 5). Very similar synergistic/additive effects were observed in experiments with *Fusarium* and *Candida*.

Recently, a new antifungal protein, called zeamatin, was isolated from maize grain [10]. Like protein R and S this basic protein migrated corresponding to a mass of ~22 kDa under reducing and ~19 kDa under non-reducing conditions in SDS-PAGE. A characteristic property of zeamatin is a synergistic action with nikkomycin Z, a nucleoside-peptide antibiotic, to inhibit the growth of *Candida albicans*. In almost identically performed gel diffusion tests with the 4 barley proteins (Fig. 2c) protein R and S, but not chitinase C or ribosome inactivating protein K, retarded growth of *Candida* when nikkomycin was added into the gel in sub-inhibitory concentrations. Similar results were obtained in tests with *Trichoderma*. Also in these experiments, protein S showed a ~3× higher inhibitory activity.

4. DISCUSSION

We have isolated 2 new immunochemically distinct barley proteins which inhibit fungal growth alone and, in a synergistic way when mixed with barley grain chitinase C . The 2 grain proteins are homologous with TL-proteins, and the chitinase with chitinases induced in plant leaves challenged with pathogens and a similar cooperative effect of these related leaf PR-proteins in antifungal defense is likely. The barley proteins R and S are very similar to maize zeamatin in many properties including the characteristic retardation of fungal growth in synergism with nikkomycin Z, an inhibitor of chitin synthesis [10]. Zeamatin was observed to permeabilize the fungal plasma membrane. The 2 barley proteins were also found to be homologous with the bifunctional maize grain α-amylase/protease inhibitor [9] suggesting that the 2 maize proteins are identical or very similar. Enzyme inhibitory properties could not be associated with protein R or S, but the inhibition of other target amylases/serine proteases cannot be excluded as basis for their antifungal activity. However, a membrane permeabilizing activity can hardly be based on inhibitory action towards insect amylases or digestive proteases suggesting that several specific defense functions, in addition to the sweet properties of thaumatin, can be associated with the conformation characteristic of the TL-family of proteins.

The 2 barley proteins can easily be isolated in rela-

tively high amounts for further structural characterization as well as elucidation of the molecular basis for their antifungal activity.

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REFERENCES

[1] Carr, J.P. and Klessig, D.F. (1989) In: Genetic Engineering: Principles and Methods (J.K. Setlow, Ed.) pp. 65–109, Plenum Press, New York.
[2] Pierpoint, W.S. and Tatham, A.S. (1987) Physiol. Mol. Plant Pathol. 31, 291–298.
[3] Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E. and Bressan, R.A. (1987) Plant Physiol. 85, 529–536.
[4] Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M. and Bol, J.F. (1986) Nature 321, 531–532.
[5] Pierpoint, W.S., Jackson, P.J. and Evans, R.M. (1990) Physiol. Mol. Plant Pathol. 36, 325–338.
[6] Bryngelsson, T. and Gréen, B. (1989) Physiol. Mol. Plant Pathol. 35, 45–52.
[7] Jacobsen, S., Mikkelsen, J.D. and Hejgaard, J. (1990) Physiol. Plant. 79, 554–562.
[8] Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) J. Biol. Chem. 266, 1564–1573.
[9] Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) Nature 327, 432–434.
[10] Roberts, W.K. and Selitrennikoff, C.P. (1990) J. Gen. Microbiol. 136, 1771–1778.
[11] Hejgaard, J. and Bjørn, S.E. (1985) Physiol. Plant. 64, 301–307.
[12] Boisen, S., Andersen, C.Y. and Hejgaard, J. (1981) Physiol. Plant. 52, 167–176.
[13] Soor, S.K. and Hincke, M.T. (1990) Anal. Biochem. 188, 187–191.
[14] Hejgaard, J. and Gibbons, G.C. (1979) Carlsberg Res. Commun. 44, 21–25.
[15] Svendsen, I., Hejgaard, J. and Chavan, J.K. (1984) Carlsberg Res. Commun. 49, 493–502.
[16] Iyengar, R.B., Smits, P., van der Ouderaa, F., van der Wel, H. and van Brouwershaven, J. (1979) Eur. J. Biochem. 96, 193–204.
[17] Yphantis, D.A., Dainko, J.L. and Schlenk, F. (1967) J. Bacteriol. 94, 1509–1515.