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Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants

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A novel class of highly abundant polypeptides with antifungal activity has been detected in cell walls of barley leaves. Similar polypeptides known as thionins occur not only in monocotyledonous but also in various dictoyledonous plants. The leaf-specific thionins of barley are encoded by a complex multigene family, which consists of at least 50–100 members per haploid genome. All of these genes are confined to chromosome 6. The toxicity of these thionins for plant pathogenic fungi and the fact that their synthesis can also be triggered by pathogens strongly suggest that thionins are a naturally occurring, inducible plant protein possibly involved in the mechanism of plant defence against microbial infections.
Key words: cell wall protein/disease resistance/plant pathogen/thionin/plant defence/antifungal activity

Introduction

Essentially every cell of higher plants is encased in a cellulosic wall. During recent years it has been demonstrated that cell walls do not only function in maintaining the structural integrity of a given cell but at the same time they play an active role in a number of other processes as well, e.g. in intercellular communication (Clarke *et al.*, 1985), in the defence against intruding pathogens (Bailey, 1986), in the transport of metabolites (Gunning and Pate, 1974) and in the release of oligosaccharide fragments which may act as a second messenger and regulate a diversity of reactions within the plant cell (McNeil *et al.*, 1984).

In addition to polysaccharides, proteins have been identified as the second largest group of cell wall components. Among these, hydroxyproline- and glycine-rich glycoproteins are the most prominent ones which are assumed to play a role in the structure of plant cell walls and may therefore be important in regulating growth (Condit and Meagher, 1986; Varner, 1987). Another rather heterogeneous group of cell wall-specific proteins consists of a complex mixture of enzyme proteins most of which are involved in the metabolism of cell wall polysaccharides (Pierrot and van Wielink, 1977; Huber and Nevins, 1981). Even though the cell wall is generally assumed to play an important role in the resistance of plants against microbial attack (Bailey, 1986), only a few of its protein constituents have as yet been implicated to be actively involved in the defence mechanism (McNeil *et al.*, 1984). Most of the putative defence related proteins known so far have been shown to be exported out of the cell and to accumulate in the intercellular space (van Loon, 1985; Legrand *et al.*, 1987).

In this report we describe a novel class of highly abundant polypeptides with antifungal activity which is present within the cell wall of barley leaf cells. Similar polypeptides known as thionins occur not only in monocotyledonous but also in various dicotyledonous plants (Ramshaw, 1982). Thus, among higher plants these antifungal proteins may represent an important major subfraction of cell wall proteins whose biological significance has remained unnoticed so far.

Results

Localization of leaf-specific thionins

In cereals the occurrence of thionins had been thought to be confined to seeds (Wada, 1982). In subsequent work with barley, however, we (Steinmüller *et al.*, 1986; Bohlmann and Apel, 1987) and others (Gausing, 1987) have demonstrated that in addition to the endosperm-specific hordothionin mRNA a closely related but distinct second group of thionin mRNAs is present in the leaf during early seedling development, whose concentration rapidly declines upon illumination. The mRNA codes for a high mol. wt thionin precursor which is composed of three different sequence domains encoding a signal sequence, a thionin part and an acidic protein (Figure 1).

To locate the leaf-specific thionins of barley an antiserum was raised against a fusion protein of *Escherichia coli* β-galactosidase and the 15 000 M_r precursor polypeptide of leaf-specific thionins. A 444 bp *Nco*I subfragment of the cDNA of clone DB4 (Figure 1) was inserted into the expression vector pUR 291 (Rüther and Müller-Hill, 1983). The antiserum against the isolated fusion protein (Bohlmann and Apel, 1987) was allowed to react with ultrathin sections of Lowicryl-embedded leaf material of etiolated barley seedlings. After the samples had been processed with protein A

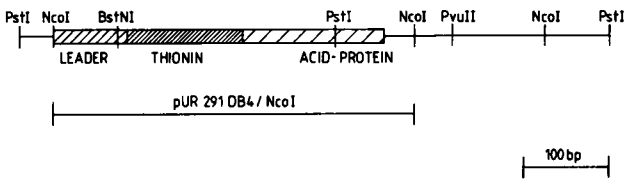

Fig. 1. Physical map of the cDNA of clone DB4. The 444 bp *Nco*I fragment of the cDNA, which contains the putative signal sequence, the thionin part and the acid protein, was inserted into the expression vector pUR291 (Rüther and Müller-Hill, 1983) and expressed by inducing the transcription of the *lacZ* gene. The resulting fusion protein was isolated and used for the production of antiserum.

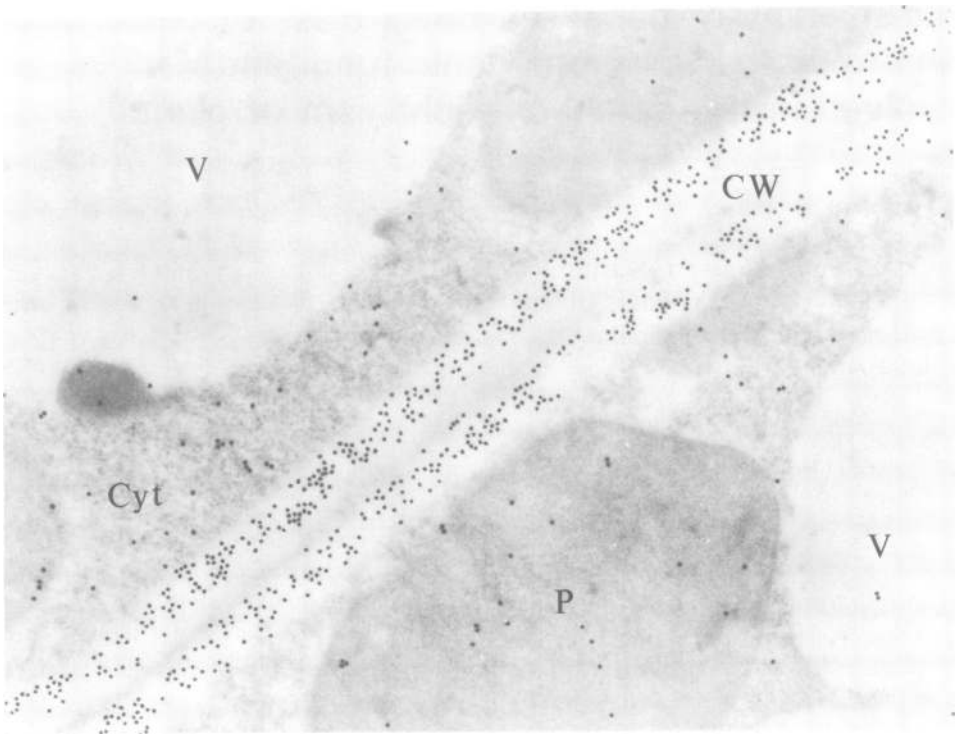

Fig. 2. Localization of thionins in leaves of dark-grown barley (*Hordeum vulgare*). Sections of Lowicryl-embedded tissue slices were prepared and allowed to react with the antiserum directed against the fusion protein, and subsequently treated with 20 nm protein A gold as described by Dehesh *et al.* (1986). CW = cell wall, Cyt = cytoplasm, P = plastid, V = vacuole.

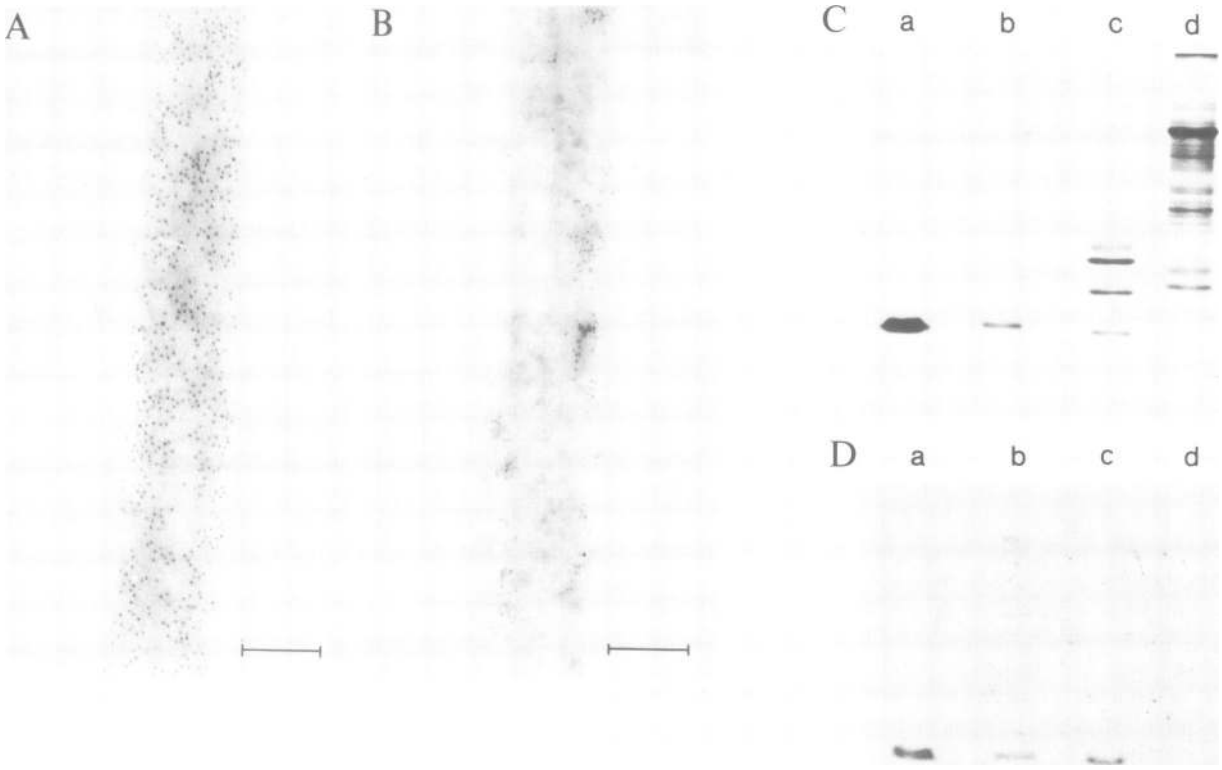

Fig. 3. The extraction of thionins from isolated cell walls of barley leaves. (**A** and **B**) Immuno-gold-labelling of thionins before (A) or after (B) the extraction of isolated cell walls with LiCl. (**C** and **D**) Electrophoretic analysis of total water-soluble cellular protein of leaf homogenates (d), extracted protein of washed cell walls prior to (c) or after sonication (b) and cell wall-extracted thionins after purification by HPLC chromatography (a). After separation on a SDS 12–20% polyacrylamide gradient gel (Laemmli, 1970) proteins were stained with Coomassie blue (C) or blotted onto nitrocellulose and detected immunologically using the antiserum against the fusion protein (D). Comparison of the results clearly demonstrates that leaf-specific thionins represent a major subfraction of cell wall proteins of barley. Bar = 0.2 μm.

gold particles, the antigens were found exclusively in the cell wall (Figure 2).

It had been shown earlier that β-galactosidase of plants may be present in the cell wall (Corchete and Guerra, 1987). Thus, it was important to exclude a possible immuno-cross-reaction of this plant protein with the antiserum raised against the fusion protein. Two different controls were used to confirm the presumptive localization of leaf-specific thionins in cell walls. Firstly, control sera were raised against purified *E.coli* β-galactosidase or against another fusion protein of *E.coli*, β-galactosidase and the *gal* repressor (von Wilcken-Bergmann *et al.*, 1983) and were used for the immuno-gold-labelling of ultrathin sections of barley leaves. However, none of these sera led to a labelling of cellular compartments (data not shown). Secondly, cell walls were isolated, sonicated, washed extensively and subsequently extracted with 1.5 M LiCl. As shown by immuno-gold-labelling in Figure 3A and B, most of the immunoreactive material had been extracted from the isolated cell walls. When the various cell wall protein fractions were analysed electrophoretically and by immunoblotting it became evident that cytoplasmic debris had been removed from isolated cell walls of barley after sonication. The only major protein which could be extracted from these purified cell walls with LiCl was a low mol. wt polypeptide whose mol. wt was indistinguishable from that of the 5000 M_r endosperm-specific hordothionin and which cross-reacted specifically with the antiserum raised against the fusion protein (Figure 3C and D). The identity of this polypeptide as thionin was confirmed by a partial sequence determination of the isolated cell wall polypeptide. The partial sequence for the isolated polypeptide matched the corresponding part of the consensus sequence of several thionins derived from nucleotide sequences of cDNAs and genomic DNAs (Figure 4).

Characterization of thionin genes

Genomic DNA fragments with thionin genes were selected from a genomic DNA library of barley cloned in the λ phage charon 35 (Loenen and Blattner, 1983), using the cDNAs as a hybridization probe. Several of these genomic sequences were characterized further by restriction mapping. The number and position of the thionin genes were determined, and in each case only one thionin gene was found on the cloned genomic DNA fragments (Figure 5A). In one of the genomic DNA fragments (BTH6) the protein-coding part plus regions upstream and downstream of the thionin gene were sequenced. The gene is interrupted by two intervening sequences (Figure 5A), but the three exons do not correspond to the three protein domains of the complete precursor (Figure 1). The nucleotide sequence of the genomic fragment is closely related but not identical to that of the cDNA of the clone DB4 (Bohlmann and Apel, 1987, see also Figure 4). Earlier work in our laboratory had demonstrated that leaf-specific thionins are encoded by a highly abundant multigene family (Steinmüller *et al.*, 1986; Bohlmann and Apel, 1987). We now used the cloned genomic sequence of the clone BTH6 and performed a reconstruction experiment (see Material and methods) to estimate the copy number of thionin genes per haploid genome of barley (Figure 5B). We calculated that at least 50−100 copies of thionin genes must exist per haploid genome. So far we have sequenced nine different cDNAs (Bohlmann and Apel, 1987) and genomic DNAs at least in part. None of these thionin sequences were identical, indicating that most of the genes are not identical copies of a master thionin gene and that many of them must be transcribed in the plant.

cDNAs of leaf-specific thionins of barley do not cross-hybridize to the genomic DNA of other cereals under conditions of high stringency (H.Bohlmann, unpublished results). This limitation in sequence similarity has been exploited for an assignment of the various members of the thionin multigene family to different barley chromosomes in barley−wheat addition lines (Islam *et al.*, 1981). The leaf-specific thionin probe of the cDNA clone DB4 did not hybridize to genomic DNA fragments of the wheat variety 'Chinese spring' while several genomic DNA fragments hybridized in samples obtained from the barley variety 'Betzes' whose chromosomes are used in the barley−wheat addition lines (Figure 5C). All of these hybridization bands were detected in the addition line with the barley chromosome 6. The other chromosomes tested did not exhibit any

a

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                                10                  20                  30                  40          46
Leaf Thionin BTH6   K S C C K D T L A R N C Y N T C R F A G G S R P V C A G A C R C K I I S G P K C P S D Y P K
Leaf Thionin DB4    * * * * * * * * * * * * * * H * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Leaf Thionin DG3    * * * * * N * T G * * * * * A * * * * * * * * * * * * T * * G * * * * * * * T * * R * * * *
Hordothionin 1      * * * * R S * * G * * * * * L * * V R * A Q K - L * * * V * * * * L T * S G * * * T G F * *
Viscotoxin A2       * * * * P N * T G * * I * * * * * * G * * * * E * * * S L S G * * * * * A S T * * * Y P D *
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b

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                                10                  20                  30                  40          46
Leaf Thionin BTH6   K S C C K D T L A R N C Y N T C R F A G G S R P V C A G A C R C K I I S G P K C P S D Y P K
Cell Wall Thionin   ? ? ? ? * * * * * * * ? * * * ? ? * * * * * K * * ? * * * ? ? ? * * *
                                Q V       L   P   I                       A       V
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Fig. 4. (A) The amino acid sequences of different leaf-specific thionin variants of barley derived from nucleotide sequences of the cDNAs DB4 and DG3 (Bohlmann and Apel, 1987) and the genomic DNA BTH6. For comparison, the amino acid sequences of a seed-specific thionin (Hordothionin 1) of barley (Ozaki *et al.*, 1980) and the thionin (Viscotoxin A2) of the dicotyledonous plant *Viscum album* (Olson and Samuelsson, 1972) are included. The sequences of the clones DB4 and DG3 and of hordothionin 1 and viscotoxin A2 are shown below that of BTH6 with only the amino acid differences indicated. (B) A comparison of the amino acid sequence of barley leaf thionin as predicted from the nucleotide sequence of the thionin gene of the genomic DNA clone BTH6 with the partial amino acid sequence determined for thionins which had been extracted from cell walls of etiolated barley leaves and purified by HPLC as described in Materials and methods. Cysteine residues had not been determined. The question marks in the protein sequence indicate residues where no clear signal was obtained during the sequence analysis. Amino acids which are identical in both sequences are marked by an asterisk. At some positions more than one amino acid was found indicating that the fraction analysed contains more than one sequence variant of thionin.

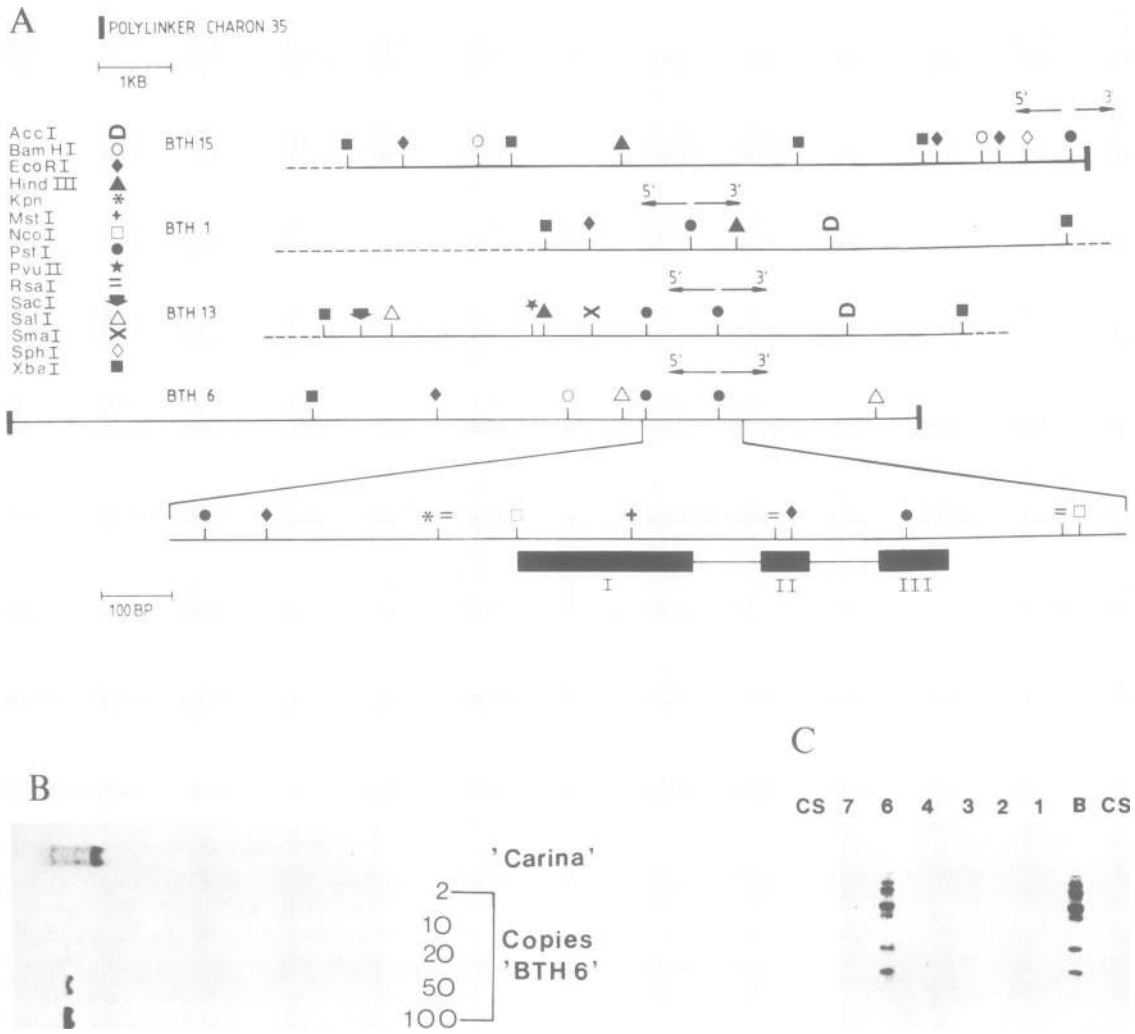

Fig. 5. Characterization and localization of the multigene family coding for leaf-specific thionins on chromosome 6 of barley (*Hordeum vulgare*). (**A**) Mapping of thionin genes on genomic DNA fragments cloned in the λ phage charon 35 (Loenen and Blattner, 1983) Arrows indicate the position and orientation of thionin genes on the cloned genomic DNA fragments. The black bars indicate the positions of the three exons. (**B**) The determination of the copy number of thionin genes by Southern blotting. From the intensity of the hybridization signal it can be estimated that a least 50–100 copies of thionin genes are present per haploid genome. (**C**) The localization of thionin genes on different barley chromosomes using barley–wheat addition lines (Islam *et al.*, 1981). DNA was analysed from the wheat variety 'chinese spring' (**CS**), the barley variety 'Betzes' (**B**) and the barley–wheat addition lines containing chromosomes 1 (**1**), 2 (**2**), 3 (**3**), 4 (**4**), 6 (**6**) and 7 (**7**) of the barley variety 'Betzes'. DNA of the various plant samples were isolated, cut with the restriction enzyme Hind III and separated electrophoretically. After transfer onto nitrocellulose the DNA fragments were hybridized with a nick-translated cDNA insert of the clone DB4. The signal demonstrates that all members of the thionin multigene family of barley are confined to chromosome 6.

detectable hybridization signal (Figure 5C). Thus, it appears that all members of the leaf-specific thionin multigene family of barley are confined to chromosome 6.

Toxicity of leaf-specific thionins

Most thionins studied so far have been shown to be highly toxic for various bacteria, fungi and small animals (Stuart and Harris, 1942; Fernandez de Caleya *et al.*, 1972; Nakanishi *et al.*, 1979). The possible toxicity of leaf-specific thionins of barley was tested using the two fungi *Thievaliopsis paradoxa*, a pathogen of sugar cane (Kranz *et al.*, 1979) and *Drechslera teres*, a pathogen of barley (Hoffmann and Schmutterer, 1983). Leaf-specific thionins were extracted from isolated cell walls of barley seedlings and purified by HPLC chromatography to apparent homogeneity as revealed by SDS–PAGE. Hordothionin or leaf-specific thionin solutions, at a concentration of 5×10^{-4} M each, were applied to the test plants. Both proteins were effective in suppressing the growth of the fungus, while bovine serum albumin as a control protein had no detectable effect on the fungi if applied at the same concentration (Figure 6).

The toxicity of a cell wall-specific polypeptide suggested to us that leaf-specific thionins could possibly play an important role in the plant's defence against pathogens. If this is the case, the question arises why the high concentration of transcripts for leaf-specific thionins in dark-grown seedlings drops beyond the level of detection during illumination (Figure 7). An answer to this question was offered by experiments with plants grown under a natural day/night cycle. If these plants were exposed to stress conditions like for instance watering with a solution containing 10^{-4} M $ZnCl_2$ a rapid reaccumulation of transcripts for leaf-specific thionins occurred which reached levels more than half of the concentration found in etiolated seedlings (data not

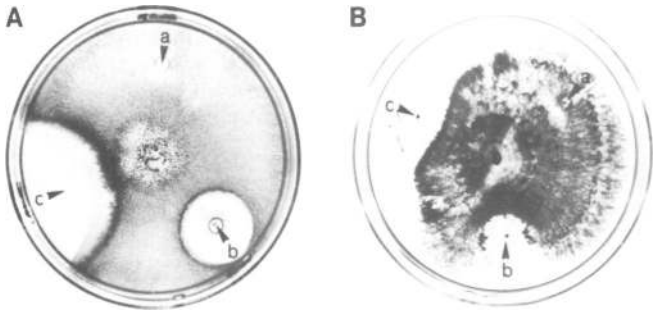

Fig. 6. Growth inhibition of the phytopathogenic fungi *T.paradoxa* (**A**) and *D.teres* (**B**) by hordothionin (b) and cell-wall extracted thionins of barley leaves (c). Bovine serum albumin (a) taken as a control did not affect the growth of the fungi.

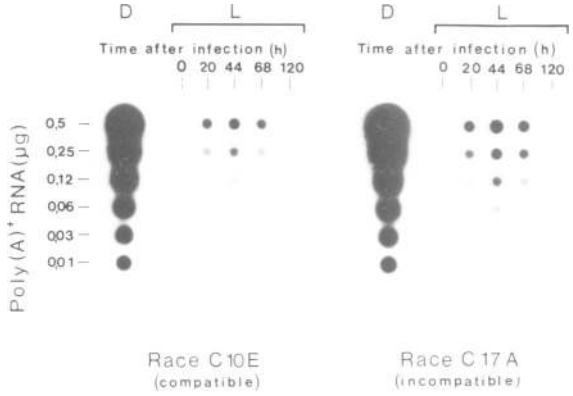

Fig. 7. The effect of different races of *E.graminis* f.sp. *hordei* on the appearance of thionin-specific mRNA in barley. Seedlings of the two barley varieties 'Emir' (compatible to race C10E and incompatible to race C17A) and 'Amsel' (compatible to race C17A and incompatible to race C10E) (W.-D.Ibenthal, personal communication) were grown under a 12 h light/12 h dark cycle for 14 days (**L**) before the leaves were inoculated with the different races of the pathogen. At various times after inoculation leaves were harvested and the poly(A)-containing RNA was isolated and applied in the indicated quantities to nitrocellulose. (**D**) Control RNA samples of untreated seedlings grown for 4 days in the dark. The dot blots were hybridized with the nick-translated cDNA insert of the clone DA6. The Figure shows the results obtained with the barley variety 'Emir'. The dot spot pattern demonstrates that upon inoculation with fungal spores the barley plants respond by a transient increase in transcript level for the leaf-specific thionins. The highest mRNA level is seen 44 h after inoculation. The response is similar in compatible and incompatible interactions of the barley cultivar with different races of the pathogen. Comparable results were obtained with the barley variety 'Amsel' (data not shown).

shown). Even more important was the finding that upon inoculation with spores of the fungus *Erisyphe graminis* f.sp. *hordei*, which causes powdery mildew, barley plants responded by a rapid and transient increase in transcript level for the leaf-specific thionins. The time course of transcript accumulation and the maximum level were similar in compatible and imcompatible interactions of barley cultivars with different races of *E. graminis* f.sp. *hordei* (Figure 7).

Discussion

The toxicity of cell wall-specific thionins and the fact that their synthesis can be triggered by pathogens and under certain conditions by other stress suggests that these polypeptides may be important as an inducible defence factor which could contribute to the general resistance of barley. Similar thionins had been found in seeds of major monocotyledonous crop plants like maize, wheat and oat and also in the leaves of various dicotyledonous plants (Ramshaw, 1982). Even though the toxicity of thionins in these plants had been known for quite a long time, so far no clue to the possible function of thionins within the plant had been offered. Historically, the interest in these polypeptides stems primarily from the use of mistletoe extracts in folk medicine for treatment of neoplastic diseases (Selawry *et al.*, 1961) and from the presence in wheat flour of a substance lethal to yeast (Stuart and Harris, 1942). The fact that thionin sequences have been conserved in distantly related monocotyledonous and dicotyledonous plant families points to an essential biological function of these proteins. Further studies will have to demonstrate whether the pattern of thionin synthesis and localization observed in barley leaves is a general phenomenon in higher plants.

A remarkable feature of the genes encoding the leaf-specific thionins is their large number and their variability. The large gene number may be the inadvertant result of selection during breeding of barley. Gene amplification has been shown to occur relatively frequently in cell culture when cells with such amplification can be selected for by a relative growth advantage (Donn *et al.*, 1984; Shah *et al.*, 1986). Thus, for instance the appearance of complex multigene families encoding seed storage protein in crop plants like barley (Doll and Brown, 1979; Miflin *et al.*, 1983) may be the result of a stringent selection to which plants had been subjected during the breeding process. While leaf-specific thionins do not contribute to the nutritional value of seeds, crop plants had also been selected for vigor and disease resistance. The localization of all members of the multigene thionin family in barley on chromosome 6 indicates that during evolution these various genes have arisen from one common ancestor gene through gene duplication.

The variability of the thionin genes could be due to two different causes. Firstly, it could solely reflect the broad range of tolerance in which amino acid exchanges would have no negative impact on the functional activity of the resulting protein. Secondly, the formation of a new thionin variant could be the result of an active selection process. It is known that during the interaction of a pathogen and its host plant both try to overcome the partner's defence barrier (van der Planck, 1968). Thus, the protective effect of a defence factor in the plant can rapidly be overcome by new variants of the pathogen which eventually shift the selection pressure on the plant's side towards the formation of a new and more effective product of the defence gene (Ellingboe, 1981). If leaf-specific thionins do contribute to the plant's resistance against microbial attack, one could easily visualize that the continuous variability of pathogens in combination with a selection of more vigorous plants during the breeding process would be a strong driving force in the evolution of new thionin variants. In this case one would have to postulate that such thionin variants should also be functionally different.

The existence of cell wall-specific and tonoplast-specific thionins in barley leaf cells (U.Reimann-Philipp and K.Apel, unpublished results) would support this functional specialization. We are currently investigating in our laboratory whether or not variants within each of the two subgroups of leaf-specific thionins are selectively synthesized in response to certain environmental stimuli.

Materials and methods

Plants

Barley (*Hordeum vulgare* cv. Carina) was grown in the dark for 5 days at 25°C. Barley cultivars 'Emir' and 'Amsel' were grown for 14 days under a 12 h light/12 h dark cycle in the greenhouse before inoculating them with spores of *E.graminis*.

Isolation of cell walls

Leaves of dark-grown barley plants were ground to a fine powder under liquid nitrogen. Cell walls were separated from cytoplasmic debris by resuspending the leaf homogenate in 0.1 M Tris−Cl, pH 7.5, 10 mM EDTA and extensive washing with distilled water. Before extraction with 1.5 M LiCl the cell walls were sonicated for 5 min and washed again with distilled water.

Immuno-gold-labelling of thionins in isolated cell walls

Isolated cell walls which had been sonicated and washed extensively with distilled water were either extracted with 1.5 M LiCl prior to fixation or were directly fixed in 1% agar and embedded in Lowicryl. Ultrathin sections were allowed to react with antisera directed against either the fusion protein of *E.coli* β-galactosidase and the 15 000 M_r precursor of the leaf-specific thionin (Bohlmann and Apel, 1987), or the *E.coli* β-galactosidase or another fusion protein of *E.coli*, β-galactosidase and the *gal* repressor (von Wilcken-Bergmann *et al.*, 1983). The various ultrathin sections were subsequently treated with 5 or 20 nm protein A-gold particles as described by Dehesh *et al.* (1986).

Isolation of thionins

Hordothionin was isolated as described by Garcia-Olmedo *et al.* (1968). Cell wall-specific thionins were isolated by extracting purified cell walls in the presence of 1.5 M LiCl overnight at 4°C. Afterwards the solubilized protein was separated from the extracted cell wall material by centrifugation and was dialysed against distilled water for 4−5 days. Samples were lyophilized, re-dissolved in water and separated by HPLC on hydroxylapatite columns using a linear buffer gradient 45−350 mM Na phosphate, pH 7.0, containing 10 mM $CaCl_2$, for the elution of the polypeptides. After purification the protein solution was dialysed extensively against distilled water and lyophilized. The protein was either re-dissolved in SDS containing buffer, carboxymethylated and separated electrophoretically or was re-dissolved in distilled water for sequencing studies or the toxicity test.

Protein sequencing

Proteins were sequenced by the 'Normal-1' procedures on an Applied Biosystems model A477 sequenator with on-line identification of PTH-amino acids.

Toxicity test

Protein solutions were applied at a concentration of 5×10^{-4} M. *T.paradoxa* (a gift of Dr Lieberei, Braunschweig) was grown at 28°C on Petri dishes containing 0.5% potato dextrose agar. Prior to inoculation at the center of the plate, wells punched into the agar were filled with 20 μl of protein solution. After 3 days of incubation the fungus had reached the edge of the Petri dish and began to sporulate. At this time the plate was photographed. *D.teres* (a gift of Dr Ibenthal, Göttingen) was grown for 7 days at 15°C in Petri dishes in 0.6% agarose containing 10% carrot juice. After the protein solutions had been applied to the plate incubation of the fungus was continued for another 7 days before the plate was photographed.

DNA manipulations

All DNA manipulations (restriction endonuclease digestion, ligation, dephosphorylation and gel electrophoresis) were performed according to Maniatis *et al.* (1982).

Cloning of genomic DNA of barley

DNA of barley was isolated (Murray and Thompson, 1980) and digested with the restriction enzyme *Sau*3A to an average size of 15−20 kb. The DNA fragments were ligated with the purified arms of λ phage charon 35 DNA cut with *Bam*HI. A 1.4 kb DNA section of the clone BTH6 which contained the thionin gene was sequenced according to Sanger *et al.* (1977).

Determination of the gene copy number

DNA of barley (9 μg) and samples with increasing amounts of DNA of the recombinant λ phage BTH6 which were supplemented with 9 μg of calf thymus DNA were digested with the restriction enzyme *Xba*I and the resulting DNA fragments were separated electrophoretically, transferred onto nitrocellulose and probed with the nick-translated cDNA insert of the clone DA6. Relative to the applied amount of total DNA of barley the samples of the recombinant DNA corresponded to 2, 10, 20, 50 and 100 copies of thionin genes per haploid genome. The genomic *Xba*I DNA fragment of the clone BTH6 has been shown to contain only a single thionin gene (Figure 5A). Hybridization and washing of the blots were done under low stringency conditions of 65°C and 5 × SSC for hybridization and 65°C and 1 × SSC for the final washing step.

Isolation of RNA and preparation of RNA dot blots

Isolation of RNA from barley leaves and purification of poly(A)-containing RNA were carried out as described earlier (Apel and Kloppstech, 1978). Preparation of RNA dot blots and hybridization was carried out as described in Batschauer and Apel (1984).

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