THE THIONINS: A PROTEIN FAMILY THAT INCLUDES PUROTHIONINS, VISCOTOXINS AND CRAMBINS

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DIFFERENT NAMES FOR PROTEINS OF A SINGLE FAMILY

Almost half a century ago, a crystalline protein material was obtained from lipid extracts of wheat endosperm and designated "purothionin" (πυρωσ, wheat; θειων , sulphur) on account of its high sulphur content (Balls et al., 1942a,b). This material, which was thought to be the oxidized form of a powerful oxidation-reduction system, was found to have bactericidal and fungicidal properties (Stuart and Harris, 1942), to inhibit fermentation of wheat mashes (Balls and Harris, 1944), and to be toxic to laboratory animals (Coulson et al., 1942).

The toxic properties of mistletoes, which were responsible for a famous death in Nordic mythology, can be ascribed to different components, including the toxic lectin viscumin (Olsnes et al., 1982) and a mixture of small basic proteins, which were first isolated by Winterfeld and Bijl (1949) and designated "viscotoxin". The viscotoxins produce hypotension and other effects at sublethal doses (Samuelsson, 1974).

In the course of a compositional study on the seeds of the Abyssinian cabbage (*Crambe abyssinica*), a high-sulphur crystalline

protein was obtained from the aqueous acetone extracts and designated "crambin" (Van Etten et al., 1965).

As amino acid sequence data for several purothionin-like proteins, as well as for different viscotoxins and crambin variants, have been obtained, it has become evident, as will be discussed later, that all these variously-named proteins are indeed unequivocally members of a single protein family. We propose the general designation of "thionins" for all these proteins, in reiteration of previous initiatives (Hernandez-Lucas et al., 1978; Vernon et al., 1985).

The thionins have become the subject of intensive structural studies, both in crystalline form and in solution, as they are excellent model systems in the development and refinement of novel methods for the elucidation of the three-dimensional structure of macromolecules. The application of recombinant DNA and other techniques to the study of this protein family has led to recent advances in our knowledge of different aspects of its molecular biology, such as synthesis and deposition, genetic control and gene structure, *in vitro* activities and possible metabolic role(s), etc. In this review, we will summarize all these research topics concerning thionins.

DISTRIBUTION, VARIATION AND EVOLUTION

Work on the characterization of purothionin, the crystalline protein obtained by Balls and co-workers, was discontinued for about 25 years, until it was shown that the material from hexaploid wheat was heterogenous, yielding two closely-migrating bands in electrophoresis (Nimmo et al., 1968; Fisher et al., 1968; García-Olmedo et al., 1968). In hexaploid and tetraploid wheat cultivars, the two electrophoretic components were consistently present, but the overall yield of purothionin in the lipid extracts was consistently higher in the hexaploid *Triticum aestivum* than in the tetraploid *T. turgidum* (García-Olmedo et al., 1968). In a survey of 22 diploid, tetraploid and hexaploid species of the *Aegilops-Triticum* group, the presence of thionins was demonstrated in the endosperms of all of them, and their electrophoretic mobilities in diploid and tetraploid species suggested that at least one variant per diploid genome complement should be present in hexaploid wheat (Carbonero and García-Olmedo, 1969). Comparison of the amino acid compositions of the purified variants from diploid, tetraploid and hexaploid wheats, as well as a genetic analysis, confirmed the existence of three different thionins in the endosperm of hexaploid wheat (Fernandez de Caleya et al., 1976). The exact nature of these variants was corroborated by the determination of the amino acid sequences (Ohtani et al., 1975, 1977; Mak and Jones, 1976; Jones and Mak, 1977, 1983; Jones et al., 1982).

At least two thionin components, designated α and β-hordothionin, were found in the endosperm of barley (*Hordeum vulgare*) by Redman and Fisher (1969). The amino acid sequence of the α-variant reported by

Ozaki et al. (1980) differed from that proposed by Mak (1975) at three positions and had been thought to be in error by Lecomte et al. (1982b). However, the nucleotide sequences of the corresponding cDNA and genomic DNA clones (Ponz et al., 1986; Rodriguez-Palenzuela et al., 1988) fully support the sequence of Ozaki et al. (1980). The sequence determined for the β-variant by Mak (1975) also differs at four positions (three of which were also discrepant in the α-variant) from that deduced from the nucleotide sequences of two independent cDNA clones (Hernandez-Lucas et al., 1986).

Two thionin variants from oat endosperm (*Avena sativa*) have been sequenced by Bekes and Lasztity (1981). A protein with the size, amino acid composition and lipid-binding properties of a thionin has been reported by Hernandez-Lucas et al. (1978) in rye endosperm (*Secale cereale*), but its sequence has not been determined. A thionin-like protein from corn grains (*Zea mays*) was thought not to be structurally homologous to the thionins (Jones and Cooper, 1980).

The viscotoxin described in the leaves and stems of European mistletoe (*Viscum album*, Loranthaceae) by Winterfeld and Bijl (1949) was found to be a mixture of closely related components, when research on the subject was resumed by Samuelsson and co-workers (Samuelsson, 1961, 1966, 1974; Samuelsson et al., 1968; Samuelsson and Pettersson, 1970, 1971a,b; Samuelsson and Jayawardene, 1974). Similar toxins, such as phoratoxins A and B from *Phoradendron tomentosum* (Mellstrand, 1974; Mellstrand and Samuelsson, 1973, 1974; Thurnberg, 1983), denclatoxin B from *Dendrophtora clavata* (Samuelsson and Pettersson, 1977) and ligatoxin A from *Phoradendron liga* (Thurnberg and Samuelsson, 1982), were also characterized within the Loranthaceae. More recently, additional leaf thionins have been identified in *Pyrularia pubera*, a parasitic plant from the Santalaceae (Vernon et al., 1985), and in barley (Böhlmann and Apel, 1987; Gausing, 1987).

The crambin reported by Van Etten et al. (1965) was found to be a mixture of two variants, whose primary structures were homologous to the thionins and the viscotoxins (Teeter et al., 1981; Vermeulen et al., 1987).

Thionin-like proteins have been reported to be present in tomato, mango, papaya, and walnut (Daley and Theriot, 1987), but their possible homology to the thionins has not yet been investigated.

The amino acid sequences currently available from direct protein sequencing, or deduced from the nucleotide sequences of the corresponding cloned DNAs, have been aligned as shown in Figure 1, following a recently described multiple-alignment computer programme (Feng and Doolittle, 1987). Two unrooted phylogenetic trees have been deduced (Figure 2A,B), one following the criteria of Feng and Doolittle (1987) and the other based on the protein sequence parsimony method developed by J. Felstestein (Univ. of Washington, USA). Although they differ in minor details, both trees allow the same general conclusions. Barley leaf thionins are closer to a leaf

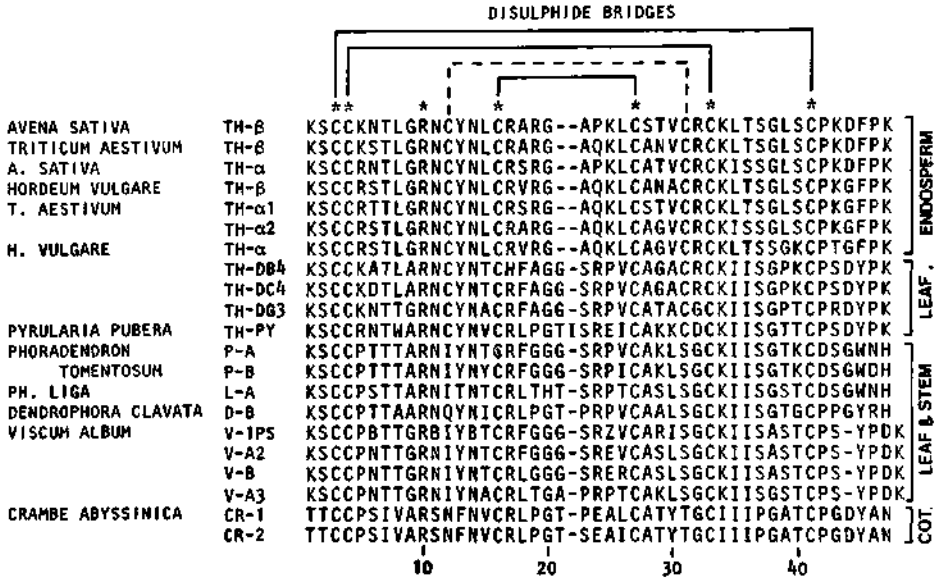


Figure 1. Alignment of currently available amino acid sequences of members of the thionins family. The multiple alignment criteria of Feng and Doolittle (1987) have been followed. Sources for the sequences are the following: T. aestivum, *wheat endosperm, Ohtani et al. (1975, 1977), Mak and Jones (1976), Jones and Mak (1977), and Jones et al. (1982).* H. vulgare, *barley endosperm, Ozaki et al. (1980), Ponz et al. (1986), and Hernandez-Lucas et al. (1986).* A. sativa, *oats endosperm, Bekes and Latztity (1981).* Pyrularia pubera, *leaves, Vernon et al. (1985).* H. vulgare, *leaves, Gausing (1987), and Böhlmann and Apel (1987).* Viscum album, *mistletoe, Samuelsson (1961, 1966, 1974), Samuelsson et al. (1968), Samuelsson and Pettersson (1970), and Samuelsson and Jayarvardene (1974).* Phoradendron tomentosum, *Mellstrand (1974) and Mellstrand and Samuelsson (1973, 1974).* Dendrophtora clavata, *Samuelsson and Pettersson (1977).* Phoradendron liga, *Thurnberg and Samuelsson (1982).* Crambe abyssinica, *Abyssinian cabbage, Teeter et al. (1981) and Vermeulen et al. (1987). Invariant positions are indicated with stars (*). The disulphide bridge indicated with discontinuous line is not common to all sequences.*

thionin characterized in a dicot (*Pyrularia pubera*, Santalaceae) than to those present in the endosperm of cereals, including barley, which means that tissue specialization probably took place before the monocot/dicot evolutionary diversification. The Pyrularia/barley group of leaf thionins occupies an intermediate position between the cereal endosperm group and that of the mistletoe toxins, although they are closer to the endosperm type, with which they share one extra disulphide bridge (Figure 1). This is noteworthy because *P. pubera* (family Santalaceae) belongs to the order Santalales, to which family Loranthaceae also belongs, and is also a parasitic plant. The crambins from the cotyledons of *Crambe abyssinica* (Cruciferae), although quite distant, are closer to the viscotoxin group than to the other groups.

STRUCTURAL STUDIES

Thionins are particularly suited as model systems for structural studies because they are easy to prepare and to crystallize in good yield, there are many genetic variants with known sequence available, they are small and rigidly folded by disulphide bridges, and they form highly ordered crystals. Thus, an X-ray diffraction method based on the anomalous scattering of sulphur was specifically developed to solve the structure of crambin (Hendrickson and Teeter, 1981). This protein was used to test the utility of molecular dynamics with interproton distance restraints for structure determination (Brünger et al., 1986; Clore et al., 1986a,b), and to show that structures obtained for a protein in solution with NMR data can be used to solve the crystal structure of the same protein by molecular replacement (Brünger et al., 1987).

The sequences aligned in Figure 1 have either six or eight cysteines which form three or four disulphide bridges as indicated in Figure 1. Their positions were first determined by enzymatic degradation and sequencing methods (Samuelsson and Pettersson, 1971a; Hase et al., 1978), and then confirmed in the course of the characterization of the three-dimensional structures. The first structure to be resolved was that of crambin, the thionin from the cotyledons of Abyssinian cabbage, whose crystals diffract X-rays strongly at a remarkable high angle (Teeter and Hendrickson, 1979). The structure was determined directly from the anomalous scattering of sulphur to a resolution of 1.5 Å (Hendrickson and Teeter, 1981) and refined to 0.945 Å (Teeter, 1984). Crambin has the shape of the Greek capital letter gamma (Γ), as represented in Figure 3. The stem of the Γ is an antiparallel pair of helices and the cross-arm consists of two antiparallel β-strands, an irregular strand and a classic β-turn. The surface of crambin has an amphipathic character. The six charged groups (1 NH_3^+, 10 Arg, 17 Arg, 23 Glu, 43 Asp and 46 COO^-), as well as several other hydrophilic side chains, are segregated from what otherwise is largely a hydrophobic molecular surface (Hendrickson and

figure 2

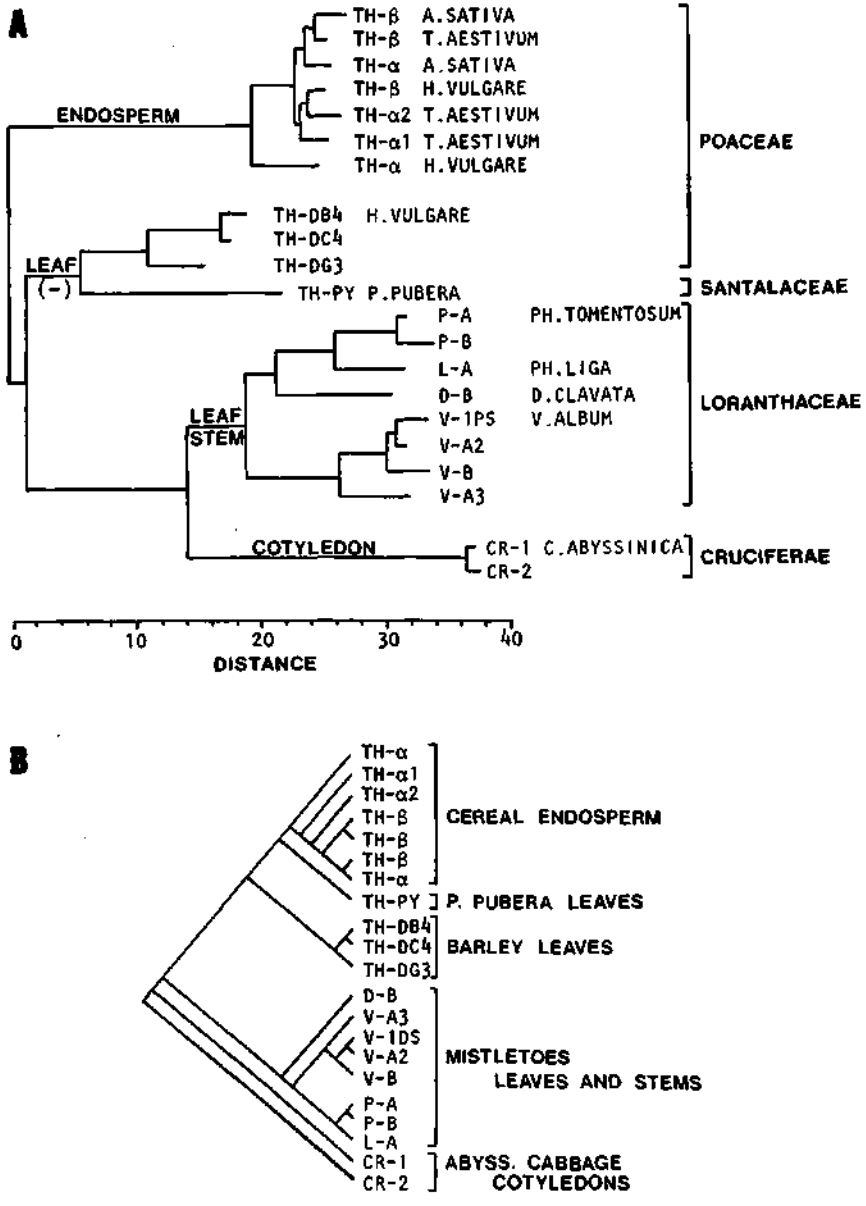

A
TH-β A.SATIVA
TH-β T.AESTIVUM
TH-α A.SATIVA
TH-β H.VULGARE
TH-α2 T.AESTIVUM
TH-α1 T.AESTIVUM
TH-α H.VULGARE
POACEAE
ENDOSPERM
TH-DB4 H.VULGARE
TH-DC4
TH-DG3
LEAF
(-)
TH-PY P.PUBERA
SANTALACEAE
P-A PH.TOMENTOSUM
P-B
L-A PH.LIGA
D-B D.CLAVATA
V-1PS V.ALBUM
LORANTHACEAE
LEAF
STEM
V-A2
V-B
V-A3
COTYLEDON
CR-1 C.ABYSSINICA
CR-2
CRUCIFERAE
0
10
20
30
40
DISTANCE
B
TH-α
TH-α1
TH-α2
TH-β
TH-β
TH-β
TH-α
CEREAL ENDOSPERM
TH-PY
P. PUBERA LEAVES
TH-DB4
TH-DC4
TH-DG3
BARLEY LEAVES
D-B
V-A3
V-1DS
V-A2
V-B
P-A
P-B
L-A
MISTLETOES
LEAVES AND STEMS
CR-1
CR-2
ABYSS. CABBAGE
COTYLEDONS

Figure 2 (opposite). Phylogenetic trees. A) Based on the alignment in Figure 1, following Feng and Doolittle (1987). (-) indicates negative value for that distance. B) According to the PROT PARS, protein sequence parsimony method included in the program PHYLIP 3.0 from Dr. J. Felstestein (Univ. of Washington, USA).

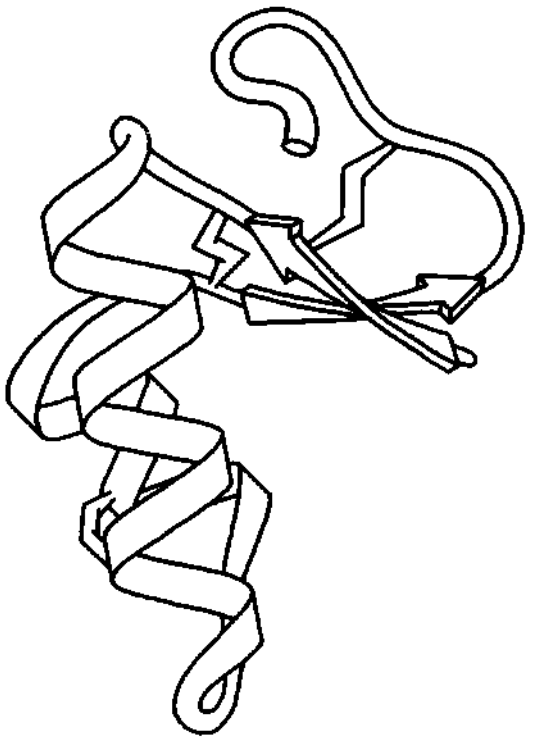


Figure 3. Schematic drawing of the backbone of crambin. This representation of crambin was drawn by Jane S. Richardson. Arrows depict β-strands. The disulphides are drawn as "lightning flashes" (reproduced from Whitlow and Teeter, 1985).

Teeter, 1981). All but one of the charged groups are clustered on the face of the inner bend, which is highly stabilized by numerous salt bridges, hydrogen bonds and other contacts, yielding a compact structure that justifies the high rigidity observed in the structural studies of the protein in solution.

Crambin in solution has been studied by a variety of methods, such as methyl ^{1}H-NMR in acetic acid at different temperatures (Lecomte et al., 1982a), aromatic ^{1}H-NMR in organic solvents (Lecomte and Llinas, 1984a,b), circular dicroism of the protein incorporated into phospholipid vesicles (Wallace et al., 1984), two-dimensional NMR (Lamerichs et al., 1988), and Raman spectroscopy (Williams and Teeter, 1984). The globular structure of the protein in solution is very similar to that in the crystals, with only slight differences. Furthermore, the structure is also similar at different pH (acetic acid or dimethylformamide), as well as in vesicles and in 60% ethanol (Llinas et al., 1980; De Marco et al., 1981; Wallace et al., 1984). The molecule seems to be quite rigid, as judged by hydrogen/deuterium exchange (Llinas et al., 1980; Lamerichs et al., 1988; Lecomte et al., 1987).

Although X-ray diffraction studies on the crystalline structure of other members of the family is under way (Teeter and Whitlow, personal communication), most of the available structural information comes from studies carried out in solution. The best characterized is the α-1 thionin from wheat endosperm (Clore et al., 1986b, 1987), whose structure closely resembles that of crambin. Although the α-1 thionin is more highly charged than crambin, the hydrophobic residues are segregated (in the exterior surfaces of the helices) from the hydrohilic ones (external surface of the angle, inferior surface of the short arm and the angle). Studies carried out with other endosperm thionins from wheat and barley (Lecomte et al., 1982b; Prendergast et al., 1984), as well as with the thionins from the mistletoe leaves (Lecomte et al., 1987; Clore et al., 1987), consistently indicate that all these proteins not only show primary structure homology but also have very similar three-dimensional structures.

INTERACTION WITH LIPIDS

Thionins were first detected in petroleum-ether extracts of wheat flour and were thought to be lipoproteins in which the protein moiety was associated with a lecithin-like lipoid (Balls et al., 1942a). The protein could be precipitated from the lipid extract as a hydrochloride that no longer resembled a lipoid and was soluble in water and aqueous alcohols. Other proteins, such as that designated as lipid-binding-protein (Ponz et al., 1984), are also present in the petroleum-ether extract, and the characterization of the lipid components which interact with the thionin has been hindered by the lack of success in obtaining the lipid-thionin complexes free of other lipid-protein associations that exist in the petroleum-ether extract (Balls

et al., 1942a; Redman and Fisher, 1968; Hoseney et al., 1971; Fisher, 1976). Lipid-thionin complexes were converted to a petroleum-ether-insoluble, chloroform soluble form by precipitation with acetone (Fisher, 1976; Hernandez-Lucas et al., 1977a). Digalactosyl diglyceride (DGDG) was the only component of the acetone extract that was able to restore petroleum-ether solubility when added back to the chloroform-soluble form (Hernandez-Lucas et al., 1977a). The chloroform-soluble thionin preparation obtained by acetone precipitation contained phosphatidylethanolamine, phosphatidylcholine, and some unidentified polar lipids, with only traces of DGDG. The apoprotein was not soluble in chloroform by itself, so it was assumed that at least some of these lipids were required for chloroform solubility (Hernandez-Lucas et al., 1977a). When flour from the tetraploid wheat *T. turgidum* was extracted with petroleum ether supplemented with DGDG, the yield of thionin was increased significantly (Figure 4), suggesting that the differences in yield between tetraploid and hexaploid wheats reported by Garcia-Olmedo et al. (1968) were probably due to interspecific differences in the levels of DGDG (Hernandez-Lucas et al., 1977a). This was confirmed by

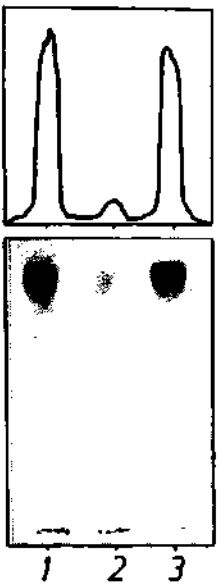


Figure 4. Yield of purothionins from T. durum *cv. "Senatore Capelli": whole flour extracted with petroleum ether + DGDG (1); whole flour extracted with petroleum ether (2); purified α- and β-purothionins (3). The amount of DGDG added was that of an equivalent weight of* T. aestivum *flour. Densitometric scanning perpendicular to electrophoretic movement (reproduced from Hernandez-Lucas et al., 1977a).*

a genetic analysis which will be discussed later (Fernandez de Caleya et al., 1976; Hernandez-Lucas et al., 1977b; Carbonero et al., 1978). Petroleum ether treatment of rye flour had repeatedly failed to extract any thionin, but when the solvent was complemented with acetone-extracted wheat lipids, a good yield of rye thionin was obtained (Hernandez-Lucas et al., 1978). The idea that DGDG was limiting the yield of thionin extracted with petroleum ether was also consistent with the fact that extraction with aqueous solvents, such as 0.05N H_2SO_4 or 1M NaCl, was more efficient than with the organic solvent (Fernandez de Caleya et al., 1976).

GENETIC CONTROL

The production of different kinds of aneuploids, especially the ditelosomics and the compensated nulli-tetrasomics of hexaploid wheat, *T. aestivum* cv. Chinese Spring, developed by Sears (1954, 1966), has been extremely useful for the location of genes encoding different types of proteins in wheat and related species (Konzak, 1977; García-Olmedo et al., 1982, 1984). Three genes (*Pur-A1*, *Pur-B1*, and *Pur-D1*), which respectively encoded the β, α-1, and α-2 thionin variants from wheat endosperm were identified in the long arms of chromosomes 1A, 1B, and 1D, through the electrophoretic analysis of the appropriate aneuploids and the characterization of the isolated proteins (Fernandez de Caleya et al., 1976). It was also observed that a gene (or genes) located in the short arm of chromosome 5D markedly affected the yield of thionins extractable by petroleum-ether, but not that obtained with aqueous solvents. This suggested that a lipid factor (or factors), genetically controlled by the short arm of chromosome 5D, was responsible for solubility in petroleum ether. This factor was identified as digalactosyl diglyceride (Hernandez-Lucas et al., 1977a), which, as predicted, was present in the petroleum-ether extracted lipids at a much lower level when the short arm of chromosome 5D was missing (Hernandez-Lucas et al., 1977b; Carbonero et al., 1978). Since Pomeranz and co-workers had previously found that bread volume was significantly decreased if "free" lipids were extracted from flour with petroleum ether and restored to its initial value if the digalactosyl diglyceride present in the extracted lipids was added back to the flour (Pomeranz, 1971), it was concluded that the observed genetic differences in free galactolipid content between tetraploid and hexaploid wheats were relevant in connection with the difference in baking quality between the two types of wheat. So far, this difference had been ascribed only to proteins contributed by the D genome (Hernandez-Lucas et al., 1977b). It was further suggested that this character could be manipulated by breeding. These observations, which are illustrated in Figure 5, have been recently followed up and confirmed (Morrison et al., 1984, 1989).

The gene encoding the endosperm thionin from rye has been located in the long arm of chromosome 1R through the electrophoretic analysis

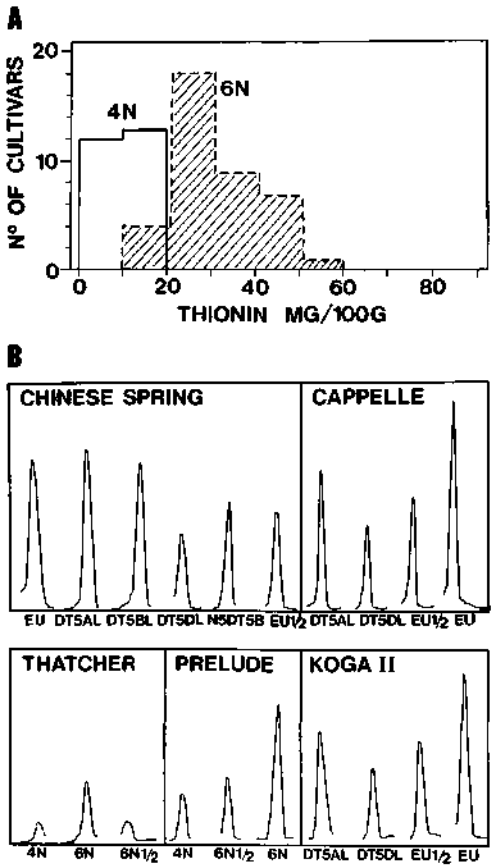


Figure 5.
A) Crude thionin (mg/100 g of tissue) present in the lipids extracted with petroleum ether from the endosperm of Triticum turgidum, *tetraploid wheat cultivars (4N) and from* T. aestivum, *hexaploid cultivars (6N), based in García-Olmedo et al. (1968). The observed difference was ascribed to genes in the short arm of chromosome 5D by Fernandez de Caleya et al. (1976).*
B) Demonstration that gene(s) in the short arm of chromosome 5D determine the level of digalactosyl diglyceride (DGDG) in the "free" lipids of wheat endosperm following the method of Hernandez-Lucas et al. (1977b). DGDG is required for solubility of thionins in petroleum ether (Hernandez-Lucas et al., 1977a) and affects baking quality (Pomeranz, 1971). Extracts corresponding to 10 mg or 5 mg (labelled 1/2) of tissue from the indicated genetic stocks were chromatographed and quantified densitometrically. Stocks are euploid (EU), ditelosomic 5AL (DT5AL, short arm of chromosome 5A missing), ditelosomic 5BL (DT5BL), ditelosomic 5DL (DT5DL); nulli 5D tetra 5B (N5DT5B). The tetraploid versions (4N) of the hexaploid cultivars Thatcher and Prelude have the D genome missing.

of the Imperial-Chinese Spring rye-wheat disomic addition lines and other aneuploids (Sanchez-Monge et al., 1979). Genes corresponding to leaf thionins from barley have been found in chromosome 6 by Böhlman et al. (1988) using Southern blotting of genomic DNA from barley-wheat addition lines obtained by Islam et al. (1975). The location of thionin loci in chromosomes of group 1 and 6, which also have in common nucleolar organizers and prolamin loci, suggests an evolutionary link between the two chromosome groups.

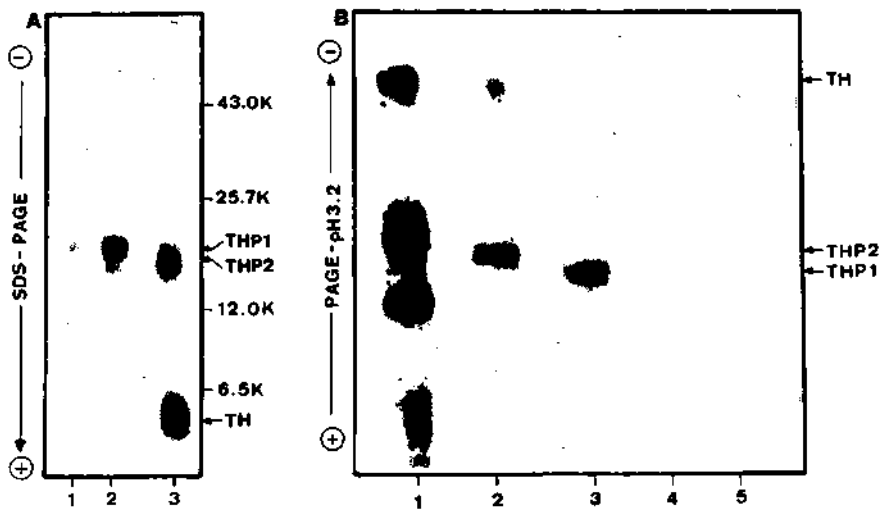


Figure 6. Comparison of in vivo *and* in vitro *products selected with monospecific antibodies and displacement of these products from the antigen-antibody complex by purified thionin.*
A) SDS-PAGE of alkylated products: (1) in vitro *precursor THP1 labelled with [^{35}S]methionine; (2) idem with [^{35}S]cysteine; (3)* in vivo *products, THP2 and TH.*
B) PAGE-pH 3.2 of reduced, non-alkylated products: (1) total in vivo *extracts; (2)* in vivo *products, THP2 and TH; (3)* in vitro *precursor THP1; (4) as in lane 2 plus 5 µg of unlabelled thionin; (5) as in lane 3 plus 5 µg of unlabelled thionin.*

SYNTHESIS AND DEPOSITION

Endosperm Thionins

Thionin accumulation in developing barley endosperm, as judged from the intensity of stained electrophoretic bands and from pulse-labelling with $[^{35}S]SO_4^{2-}$, appeared to start and to level off at earlier stages than the main reserve proteins (Ponz et al., 1983). This has been confirmed by a dot-blot hybridization analysis of the corresponding mRNA, which clearly showed a maximum concentration of messenger between 13 and 16 days after anthesis (Rodriguez-Palenzuela et al., 1988). Synthesis of these proteins, which takes place in membrane-bound polysomes (Ponz et al., 1983), is therefore specific of the cell-proliferation phase of endosperm development and ceases during the cell-enlargement phase. Using monospecific antibodies raised against the protein purified from mature endosperm, two types of precursors were identified by Ponz et al. (1983): one was detected as an *in vitro* translation product, but not *in vivo*, and the other was shown in the pulse-labelling experiments (Figure 6A,B). At least two processing steps, one co-translational and the other post-translational, were postulated on the basis of these observations.

Preliminary fractionation studies carried out both with the dry mature endosperm (Carbonero et al., 1980) and with developing tissue (Ponz et al., 1983) indicated that the thionins were in the particulate fraction in a labile association that could be disrupted by increasing the salt concentration or with non-ionic detergents. Under the conditions used by Ponz et al. (1983), the thionins roughly co-sedimented with cytochrome C reductase (NADPH, antimycin A-resistant), a marker of the endoplasmic reticulum. However, we have now observed a closer association with tryglycerides under various homogeneisation and sedimentation conditions, which suggests their location in the lipid bodies (unpublished), a matter which is currently under a more detailed examination.

Leaf Thionins

The mRNAs encoding leaf thionins, when translated *in vitro*, yield precursors that are similar to those described for the endosperm thionins (Gausing, 1987; Böhlmann and Apel, 1987). The structure of these precursors, deduced from the nucleotide sequences of the corresponding cDNAs, is also the same as that of the endosperm thionins described by Ponz et al. (1986) and Hernandez-Lucas et al. (1986). Although their possible processing in the leaves has not been investigated, it is likely that a similar pathway operates in these organs because not only the precursor structure is the same but also the amino acid residues around the cleavage points have been well conserved. Messengers of leaf thionins are specifically abundant in young ethiolated leaves and decline rapidly upon illumination or with

age (Gausing, 1987; Böhlmann and Apel 1987). They are also transiently present in older leaves when challenged with fungal pathogens and with other environmental stresses (Böhlmann et al., 1988).

Using immuno-gold localization methods, Böhlmann et al. (1988) have located thionins from barley leaves in the cell wall, which is in contrast with the observations concerning endosperm thionins.

MOLECULAR CLONING

As predicted from the work of Ponz et al. (1983), the nucleotide sequences of the cloned cDNAs corresponding to the α and β thionins from barley endosperm were found to encode precursors that were much larger than the mature protein (Ponz et al., 1986; Hernandez-Lucas et al., 1986). The deduced structures of these precursors consisted of an N-terminal signal peptide, followed by the mature protein and a C-terminal acidic protein (Figure 7). In the course of a character-

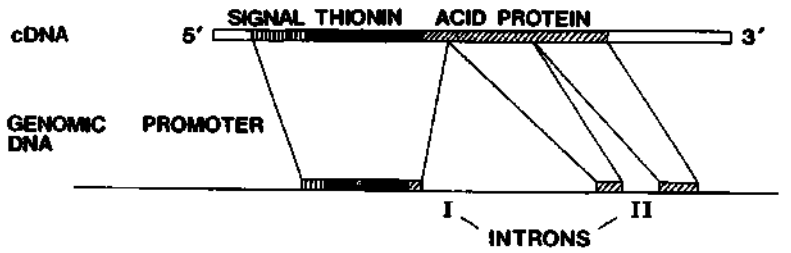
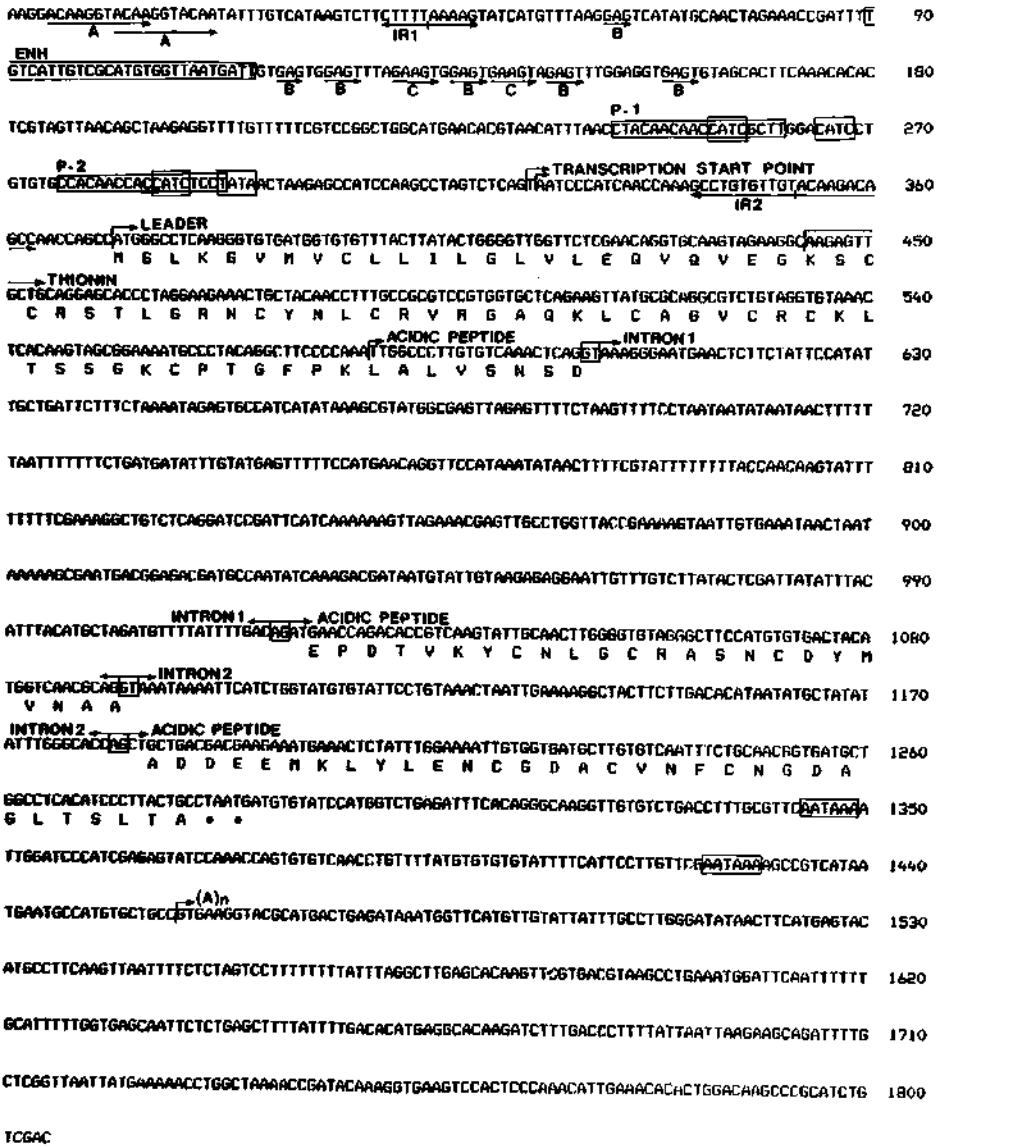


*Figure 7. Structure of the thionin precursor (Ponz et al., 1986) and of the α-thionin gene (*Hth-1*) from barley endosperm (Rodriguez-Palenzuela et al., 1988).*

Figure 8 (opposite). Nucleotide sequence and deduced amino acid sequence of the Hth-1 *gene (Rodriguez-Palenzuela et al., 1988). Short direct repeats are indicated by horizontal arrows and identified by letters (A-C). Inverted repeats (IR) are indicated by divergent horizontal arrows. TATA, CATC and AATAAA boxes are indicated. An enhancer-like sequence (ENH) and a repeated sequence (P-1/P-2), which is homologous to the coding sequence of some prolamins, are also boxed. The sequence of the posttranslationally excised, C-terminal peptide is interrupted by two introns as indicated. The one-letter amino acid symbols have been aligned with the first base of each codon.*

Figure 8



TCGAC

isation of cDNAs corresponding to abundant mRNAs present in etiolated barley leaves, Gausing (1987) and Böhlmann and Apel (1987) independently identified nucleotide sequences that included segments encoding thionin variants closely resembling the leaf thionin from *Pyrularia pubera* (Vernon et al., 1985). The complete cDNAs encoded precursors with the same structure described for those of the endosperm thionins (Ponz et al., 1986; Hernandez-Lucas et al., 1986).

The complete nucleotide sequence of the gene for the α-thionin from barley endosperm has been recently published (Rodriguez-Palenzuela et al., 1988). As shown in Figures 7 and 8, the gene has two introns, both of which interrupt the acidic protein sequence, so exons do not correspond to the structural domains of the precursor molecule (Figure 7). A preliminary report on the genomic clones of barley leaf thionins indicates that these genes have also two introns, although their precise location has not been described (Böhlmann et al., 1988). Features of the promoter region from the α-thionin gene from barley endosperm are summarized in Figure 9.

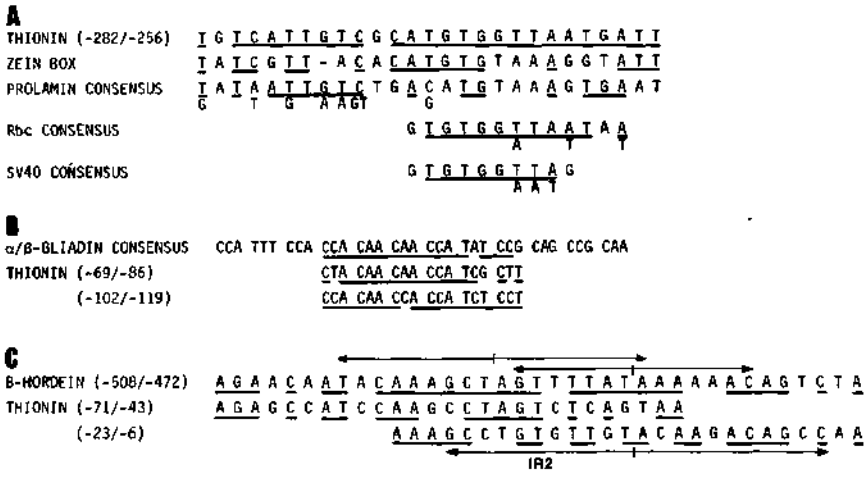


Figure 9. Homologies of the 5'-flanking region of the Hth-1 *gene with other genes (Rodriguez-Palenzuela et al., 1988).*
A) The sequence between nucleotides -282 and -256 has been aligned with the putative enhancer sequences appearing in similar positions in zein genes from maize, a consensus sequence derived from other prolamin genes, a consensus sequence from Rubisco genes and the consensus core sequence from the enhancers of SV40 virus
B) The P-1 and P-2 sequences have been aligned with a consensus sequence coding for the repetitive motive in the α/β-gliadin repetitive structural domain.
C) A sequence from the B-hordein gene starting at position -508 with respect to the first ATG codon has been aligned with the indicated thionin sequences.

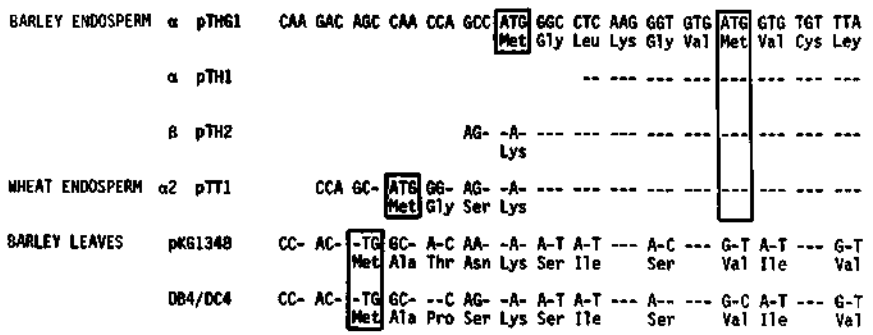


Figure 10. Translation start codon in the Hth-1 *gene. The sequence around the putative first ATG codon in the* Hth-1 *gene (pTHG) has been aligned with the corresponding regions of the α- and β-thionin cDNAs from barley (Ponz et al., 1986; Hernandez-Lucas et al., 1986), the cDNA of α2-thionin from wheat (C. Marafia, unpublished), and the cDNAs of leaf thionins (pKG1348, Gausing, 1987; DB4, DC4, Böhlmann et al., 1987). Dashes indicate identity. Met codons are boxed.*

A comparison of the deduced amino acid sequences of thionin signal peptides from wheat and barley endosperm and from barley leaves indicates considerable variability around the translational start codon (Figure 10), which is in contrast with the high conservation of the rest of the signal peptide. Binary comparisons of available nucleotide sequences, using the criteria of Li et al. (1985) for the estimation of synonymous and non-synonymous substitution rates, indicate that the core of the signal peptide is evolving at a lower rate than the sequences corresponding to the mature and the acidic proteins (Figure 11). The extreme heterogeneity around the start codon might be related to the apparent difference in subcellular location between the leaf and the endosperm thionins.

Approximately two copies per haploid genome have been estimated for barley endosperm thionins, using an α-thionin probe that cross-hybridizes with the β-thionin DNA (Rodriguez-Palenzuela et al., 1988). The number of copies of the leaf thionin genes have been variously reported at nine to eleven (Gausing, 1987) or fifty to a hundred (Böhlmann et al., 1988) per haploid genome.

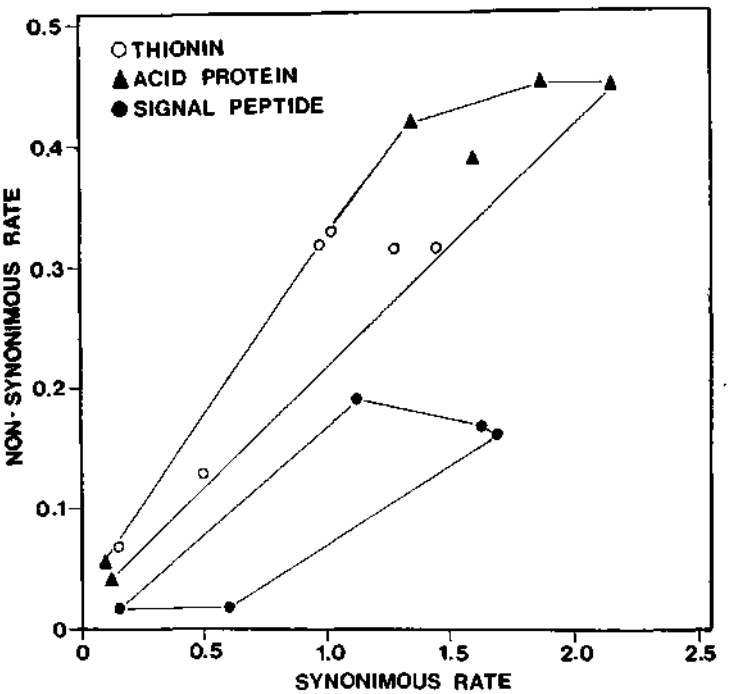


Figure 11. Non-synonymous versus synonymous substitution rates deduced from binary comparisons of available nucleotide sequences of barley endosperm and leaf cDNAs, and wheat endosperm cDNAs. Signal peptide, mature thionin, and acid C-terminal protein have been independently compared using the criteria of Li et al. (1985)

BIOLOGICAL ACTIVITIES

A variety of *in vitro* activities have been demonstrated for the thionins and, although some of these suggest possible *in vivo* roles, their real biological function remains obscure. Besides their toxicity to various organisms, which was the property that first attracted attention, other activities, such as alteration of membrane permeability, inhibition of certain enzymes and of macromolecular synthesis, or participation in redox reactions have been investigated. The possible significance of thionins in cereal-based industrial fermentations and in plant-pathogen interactions are among the practical aspects that merit special consideration.

Toxicity

The toxic properties of endosperm thionins from wheat were investigated as soon as the crystalline material was first obtained. Gram-positive bacteria and, to a lesser extent, Gram-negative ones were found to be sensitive, together with bakers yeast and some human pathogenic fungi, whereas the mycelial fungi tested were found to be insensitive (Stuart and Harris, 1942). The antimicrobial action of the crystalline protein was reversed by different phosphatides (Woolley and Krampitz, 1942). After these initial findings, the toxicity to bacteria (Fernandez de Caleya et al., 1972), to yeast (Balls and Harris, 1944; Nose and Ichikawa, 1968; Okada et al., 1970; Okada and Yoshizumi, 1970, 1973; Hernandez-Lucas et al., 1974), and to fungi (Böhlmann et al., 1988) has been further demonstrated. Toxicity of endosperm thionins to mice, guinea pigs and rabbits when the protein was injected intravenously or intraperitoneously, but not upon oral administration, was also found (Coulson et al., 1942). Cytotoxic effects on cultured mammalian cells have been reported (Nakanishi et al., 1979; Carrasco et al., 1981; Vernon et al., 1985). Insect larvae were similarly sensitive to different endosperm thionins when the proteins were administered through the hemocoel but not when incorporated in the food (Kramer et al., 1979).

Toxicity of leaf thionins from the mistletoes to higher animals has been repeatedly demonstrated. These proteins were all found to be toxic on parenteral administration to mice and cats (see Samuelsson, 1974). In sub-lethal doses they produced hypotension, bradycardia and a negative inotropic effect on the heart muscle. Intra-arterial administration, in higher doses, produced vasoconstriction in arteries of skin and skeletal muscle (see Samuelsson, 1974). Cytotoxic effects on cultured mammalian cells have also been observed (Konopa et al., 1980; Carrasco et al., 1981). In contrast, no toxic effect has been reported for the crambins present in the cotyledons from the Cruciferae.

Toxicity of wheat endosperm thionins to mice and yeast was totally lost when all the amino groups were chemically modified and partially so when the only tyrosyl group was nitrated (Wada et al., 1982).

Alteration of Membrane Permeability

Leakage of intracellular material upon exposure to thionin from wheat endosperm was demonstrated in bacteria (Fernandez de Caleya, 1973). A similar effect was described in yeast by Okada and Yoshizumi (1973), while investigating the mode of action of a toxic principle from wheat and barley that, only later, was shown to be a mixture of thionins (Ohtani et al., 1975, 1977). They further showed that this factor not only induced leakage of phosphoric acid, nucleotides, amino acids, and potassium ions, but also inhibited the incorporation of sugars. The toxic effect could be reversed by certain divalent cations, such as Ca^{2+}, Zn^{2+}, or Fe^{2+} (Okada and Yoshizumi, 1973).

A study of the effects of endosperm thionin variants and viscotoxins on cultured mammalian cells indicated that at the minimum cytotoxic concentrations, leakage of Rb^+ and of uridine occurred. Also, concentrations of thionins that had no detectable effects on the cultured cells, allowed inhibition of translation by antibiotics, such as hygromycin B, that are not able to cross the cell plasma membrane by themselves (Carrasco et al., 1981). As in the case of yeast, Ca^{2+} and Mg^{2+} could reverse the action of thionin.

The observed effects on the contraction of smooth muscle from the uterus of the guinea pig (Coulson et al., 1942) and of the flight muscle from insects (Kramer et al., 1979), or the increased sensitivity to thionins of A31 cells infected with the Moloney strain of murine leukaemia virus (Tahara et al., 1979), are all probably related to interactions with the cell membrane.

Inhibitory Properties

Apart from a partial inhibition of the milk-clotting power of papain, possibly due to interference with essential SH-groups (Balls et al., 1942), and the inhibition of α-amylase, through competition for the Ca^{2+} ion (Jones and Meredith, 1982), no strong enzyme inhibition activity has been reported for the thionins. However, they are able to inhibit macromolecular synthesis. Nakanishi et al. (1979) reported that thionins could specifically kill cells during DNA synthesis (S phase), but had little effect during the G_0 phase, and Ishii and Imamoto (cited by Ozaki et al., 1980) demonstrated inhibition of lambda phage transcription in *Escherichia coli*. The effects of endosperm thionins and viscotoxins on the synthesis of DNA, RNA, and proteins in cultured mammalian cells have been investigated by Carrasco et al. (1981). Protein synthesis was more sensitive than RNA synthesis, which itself was more sensitive than DNA synthesis. There was a close parallel between Rb^+ leakage and inhibition of protein synthesis upon treatment with the different genetic variants tested, which suggested that the inhibition could be a direct consequence of the induced leakiness. Eucaryotic cell-free translation systems, derived from wheat germ or from rabbit reticulocytes, were inhibited by thionins, but at higher concentrations than *in vivo* (García-Olmedo et al., 1983). The inhibitory concentration varied linearly with the amount of exogenous mRNA added, which suggested a direct interaction of the toxin with the RNA (García-Olmedo et al., 1983). This would be in line with the reported interaction between DNA and viscotoxins (Woynarowski and Konopa, 1980).

Possible Participation of Thionins in Thioredoxin-Related Reactions

Thioredoxin, a hydrogen carrier protein that functions in DNA synthesis and in the transformation of sulphur metabolites, has been also found to serve as a regulatory protein in linking light to the activation of enzymes during photosynthesis (Buchanan et al., 1979). It has been shown that thionin from wheat endosperm can substitute for thioredoxin *f* from spinach chloroplasts in the dithiothreitol-linked activation of chloroplast fructose-1,6-bisphosphatase (Wada and Buchanan, 1981). Under the standard assay conditions, the thionin was only 2% as active as authentic thioredoxin *f*, a situation that could be improved by increasing the time of preincubation and the concentration of reductant. These results suggested that the thionin could be effectively reduced by thioredoxin *f* (Wada and Buchanan, 1981). This led to a more recent set of experiments which makes more plausible the *in vivo* participation of thionins in plant redox metabolism. Johnson et al. (1987) have reported a thioredoxin system, consisting of a homogeneous preparation of thioredoxin *h* and partially purified thioredoxin reductase (NADPH), which effectively reduced thionin with NADPH as the hydrogen donor. The reduced thionin, in turn, was capable of activating fructose-1,6-bisphosphatase. These results suggest a possible role of thionins as secondary thiol messengers in the redox regulation of enzymes.

Possible Implications of Thionins in Plant-Pathogen Interactions

The hypothesis that thionins might play a role in the protection of plants from pathogens was put forward by Fernandez de Caleya et al. (1972), who investigated susceptibility to wheat endosperm thionins among phytopathogenic bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Erwinia* and *Corynebacterium*. Minimal inhibitory concentrations (MIC) ranged from 1 to 540 µg/ml and the minimal bactericidal concentrations were usually twice the MICs. Purified genetic variants had different activities and showed some degree of specificity. In the example presented in Table 1, β-thionin from wheat endosperm was more active than α-thionin against *Pseudomonas solanacearum*, whereas the opposite was true against *Xanthomonas phaseoli*. More recently, Böhlmann et al. (1988) have shown that both endosperm and leaf thionins from barley are inhibitory towards two fungi, *Thievaliopsis paradoxa*, a pathogen of sugar cane, and *Drechslera teres*, a pathogen of barley, at concentrations of 5×10^{-6}M (2.5 mg/ml), a concentration that is three orders of magnitude higher than that required for the most sensitive bacteria.

The above observations, the apparent cell-wall location of leaf thionins and the finding that upon inoculation with spores of the fungus *Erysiphe 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 (Böhlmann et al., 1988), strongly suggest a role for thionins in plant protection.

Table 1. Susceptibility of *Pseudomonas solanacearum* and *Xanthomonas phaseoli* to α and β thionins from wheat endosperm

	P. solanacearum		*X. phaseoli*	
Thionin	MIC[1]	MBC[2]	MIC[1]	MBC[2]
α	3	6	6	12
β	1.5	1.5	12	25

[1]Minimum Inhibitory Concentration (MIC) in µg per ml.
[2]Minimum Bactericidal Concentration (MBC) in µg per ml.

Possible Interference with Industrial Fermentations

As early as 1895, W. Jago (cited by Okada et al., 1970) reported that certain strains of beer yeast showed a poor production of carbon dioxide in dough fermentation. In the early part of this century, a number of authors found that bottom-fermenting yeasts seemed to be more sensitive than top-fermenting ones, that the toxic substance was present in the protein fraction of wheat and barley, and that the toxicity of wheat flour could be reverted by Ca^{2+}. As already mentioned, the toxicity of thionins towards yeast was established from the start (Stuart and Harris, 1942), together with their actual ability to inhibit fermentation of wheat mashes (Balls and Harris, 1944). Yeast strains of *Saccharomyces uvarum* (syn. *carlsbergensis*) and *S. cerevisiae*, as well as wild *Saccharomyces* spp., all proved to be about equally sensitive to purified thionins (Hernandez-Lucas et al., 1974). In an independent search, Japanese workers (Nose and Ichikawa, 1968; Okada et al., 1970; Okada and Yoshizumi, 1970, 1973) undertook to purify and characterize in wheat and barley the substance(s) responsible for their toxicity towards yeast. This toxic principle was eventually found to be identical with thionin (Ohtani et al., 1975, 1977). All these observations suggest the possible interference of thionins with fermentation of mashes made out of ungerminated cereals. Additionally, Lopez-Braña and Hernandez-Lucas (1984) have demonstrated that thionins are only partially degraded during early germination and malting, and that the remaining levels are also potentially inhibitory.

PROSPECTS FOR MANIPULATION IN PLANT BREEDING

The available distribution data for this protein family suggests that it might be ubiquitous. If this is proved to be so, the potential of thionin genes as targets for manipulation in the breeding of disease resistance would depend on the specificity of natural and artificial variants, as well as in our ability to express them under different developmental and environmental situations, and in different compartments, etc. Data in Table 1 suggest that at least some degree of specificity might be associated with different genetic variants, but this matter merits further investigation.

Tobacco plants carrying genes encoding wheat and barley thionins have been already obtained in our laboratory and in that of L. Willmitzer (Berlin), using different constitutive and inducible promoters. These plants should serve as models to test some of the possible applications.

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