Trypsin/α-Amylase Inhibitors and Thionins: Possible Defence Proteins from Barley

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Plant storage tissues in general should be good substrates for a wide range of organisms on the basis of their gross biochemical compositions. However, only a few specific taxa are able to feed on each type. One possible reason for this is that they have proteins and other molecules that are toxic or inhibitory towards heterologous systems. In the case of barley endosperm, over 10% of the total protein is represented by protein families that fall within this description. A substantial fraction of the albumins and globulins of barley belong to two of these families, the trypsin/α-amylase inhibitors and the thionins, which are the subject of the present chapter.

The first family includes inhibitors of serine proteases and of heterologous α-amylases. Some members of this family can be selectively extracted with chloroform/methanol mixtures and have been designated CM-proteins. Their apparent molecular weights are in the 12–16 kDa range. The α-amylase inhibitors can be classified according to their degree of aggregation into monomeric, dimeric and tetrameric forms. No members of this family have been found in tissues other than endosperm, although this has not been investigated in detail. These inhibitors have been found in other species of the Poaceae, including wheat, where they have been extensively studied.

The thionins are polypeptides of about 5 kDa, which have three or four disulphide bridges and are toxic to plant pathogenic micro-organisms. Although they are very abundant in endosperm, different types are found in other tissues. Members of this family have been identified in a few but wide-ranging taxa.

Comprehensive reviews of work on the trypsin/α-amylase inhibitors (García-Olmedo *et al.*, 1987) and on the thionins (García-Olmedo *et al.*, 1989, 1991) have been published recently. This chapter focuses on recent developments concerning the two protein families in barley and related species, with special emphasis on their possible role as defence proteins.

Trypsin/α-amylase inhibitors

Inhibitor types

The first member of this protein family in barley was reported by Mikola & Suolinna (1969), who isolated a 14.1 kDa protein which was active against trypsin and inactive against the endogenous proteinases from green malt. Antibodies raised against this protein did not react with inhibitors from barley embryo or from wheat endosperm (Mikola & Kirsi, 1972). A similar or identical inhibitor was purified by Boisen (1976) from a different genetic stock. The inhibitor was sequenced by Odani *et al.* (1983a), who found that it was homologous to a monomeric α-amylase inhibitor from wheat which had been previously sequenced by Kashlan & Richardson (1981). Two chloroform/methanol soluble proteins (CM-proteins) from barley endosperm, designated CMe and CMc, were found to have antitrypsin activity (Lazaro *et al.*, 1985; Barber *et al.*, 1986a). The first of these proteins (BTI-CMe) was found to be identical to the previously described trypsin inhibitor of Mikola & Suolinna (1969).

A tetrameric α-amylase inhibitor was characterized in barley endosperm by Sanchez-Monge *et al.* (1986b), who also described the presence of dimeric and monomeric inhibitors in this tissue. The subunits of the tetrameric inhibitor were identified as the previously described proteins CMa, CMb and CMd, which were known to be homologous to the previously characterized α-amylase inhibitors (Salcedo *et al.*, 1982; Shewry *et al.*, 1984; Lazaro *et al.*, 1985; Barber *et al.*, 1986a, b). The complete sequence of subunit CMd has been deduced from the corresponding cloned cDNA (Paz-Ares *et al.*, 1986; Halford *et al.*, 1988).

A dimeric α-amylase inhibitor from barley and its corresponding cDNA have been reported by Lazaro *et al.* (1988b) and a monomeric inhibitor was identified as the major barley allergen reacting with sera from patients suffering baker's asthma (Barber *et al*;, 1989; Sanchez-Monge *et al.*, unpublished). A number of cDNA clones encoding members of this family have been identified, but the corresponding proteins have not been characterized yet.

Proteins corresponding to the trypsin inhibitors and to the subunits of the tetrameric α-amylase inhibitor of *Hordeum vulgare* have been identified in *Hordeum spontaneum* and their allelic relationships have been established by genetic analysis (Salcedo *et al.*, 1984; Molina-Cano *et al.*, 1987). A similar study has been carried out for the subunits of the tetrameric inhibitor in *Hordeum chilense* (Sanchez-Monge *et al.*, 1987).

This protein family has been investigated for half a century in wheat, where all the inhibitor types seem to be present (for reviews, see Buonocore *et al.*, 1977; García-Olmedo *et al.*, 1987; Silano, 1987; Sanchez-Monge *et al.*, 1988). In addition, members of this family have been identified in rye (Lyons *et al.*, 1987), in maize (Mahoney *et al.*, 1984) and in finger-millet (Campos & Richardson, 1983).

Sequences of members of this family in barley are aligned with total or partial sequences from the corresponding proteins in other species in Fig. 16.1. Three domains, which correspond approximately to those proposed by Kreis *et al.* (1985) for the trypsin inhibitor CMe, have been used in the alignment. The reactive site of this inhibitor, Pro–Arg–Leu (P–R–L), is located at the C-terminal border of the first domain, a region which is quite variable throughout the family.

In addition to the homology relationships shown in Fig. 16.1, weaker and more elusive relationships have been proposed

between this family and the 2S storage proteins from dicots (Odani *et al.*, 1983c; Shewry *et al.*, 1984), with dispersed sulphur-rich domains from cereal prolamins (Kreis *et al.*, 1985), and even with the Kazal secretory trypsin inhibitor from bovine pancreas (Odani *et al.*, 1983b).

Genomic distribution of inhibitor genes in barley and related species

The α-amylase/trypsin inhibitors are encoded by a multigene family which is dispersed over several chromosomes, both in barley and in wheat. Present knowledge of the genomic organization of this family is summarized in Table 16.1. A total of 24 members of the family have been identified in barley, wheat and rye. These can be grouped into nine types according to sequence similarity and/or *in vitro* activity. All but four of these have been characterized at the protein level and their *in vitro* activities have been determined except for protein WTI from *Triticum monococcum*, whose sequence is quite close to BTI-CMe.

Table 16.1. Genomic distribution of genes encoding α-amylase/trypsin inhibitors

Inhibitory activity (aggregation)	Protein (syn.)	Clones	Gene locus (syn.)	Chromosome no. genome (arm)
α-amylase (monomeric)	BMAI-1 (Horv1)		*Iam1*	?
	WMAI-1 (0.28)	pUP-28	Imha-D1	6D(S) ab
	WMAI-2		Imha-B1	6B(S) c
α-amylase (dimeric)	BDAI-1	pUP-44	*Iad1*	?
	WDAI-1 (0.53)		Idha-B1-1	3B(S) abd
	WDAI-3		Idha-B1-2	3B(S) e
	WDAI-2 (0.19)		Idha-B1	3D(S) abd
α-amylase (tetrameric)				
1st subunit	BTAI-CMa		*Iat1*	1(7H) f
	WTAI-CM2		Itha-B1	7B(S) adgh
	WTAI-CM1	pCT-2	Itha-D1	7D(S) adgh
	RAI		Itha-R1	?R
2nd subunit	BTAI-CMb		*Iat2*	4(4H) f
	WTAI-CM16	pCT-3	Itha-B2	4B(S) adh
	WTAI-CM17		Itha-D2	4D(S) adh
3rd subunit (2 copies)	BTAI-CMd	pUP-38	*Iat3*	4(4H) f
	WTAI-CM3B	pCT-1	Itha-B3	4B(S) adh
	WTAI-CM3D		Itha-D3	?D
Trypsin	BTI-CMe	λcCMe λgCMe	*Itr1*	3(3H) fij
	WTI?			?A
	?	pCT-4		?A, B, D?
	RTI		Itr-R1	3R k
Trypsin	BTI-CMc		*Itr2*	1(7H) f
Unknown	?	pUP-23	*Iah2*	?
	?	pCT-88	?	?A, B, D?
Unknown	?	pUP-13	*Iah1*	?

(a) Fra-Mon *et al.*, 1984; (b) Sánchez-Monge *et al.*, 1986a; (c) Gomez *et al.*, unpublished; (d) Aragoncillo *et al.*, 1975; (e) Sanchez-Monge *et al.*, 1989; (f) Salcedo *et al.*, 1984; (g) García-Olmedo & Carbonero, 1970; (h) García-Maroto *et al.*, 1990; (i) Hejgaard *et al.*, 1984a; (j) Rodriguez-Palenzuela *et al.*, 1989; (k) Hejgaard *et al.*, 1984b.

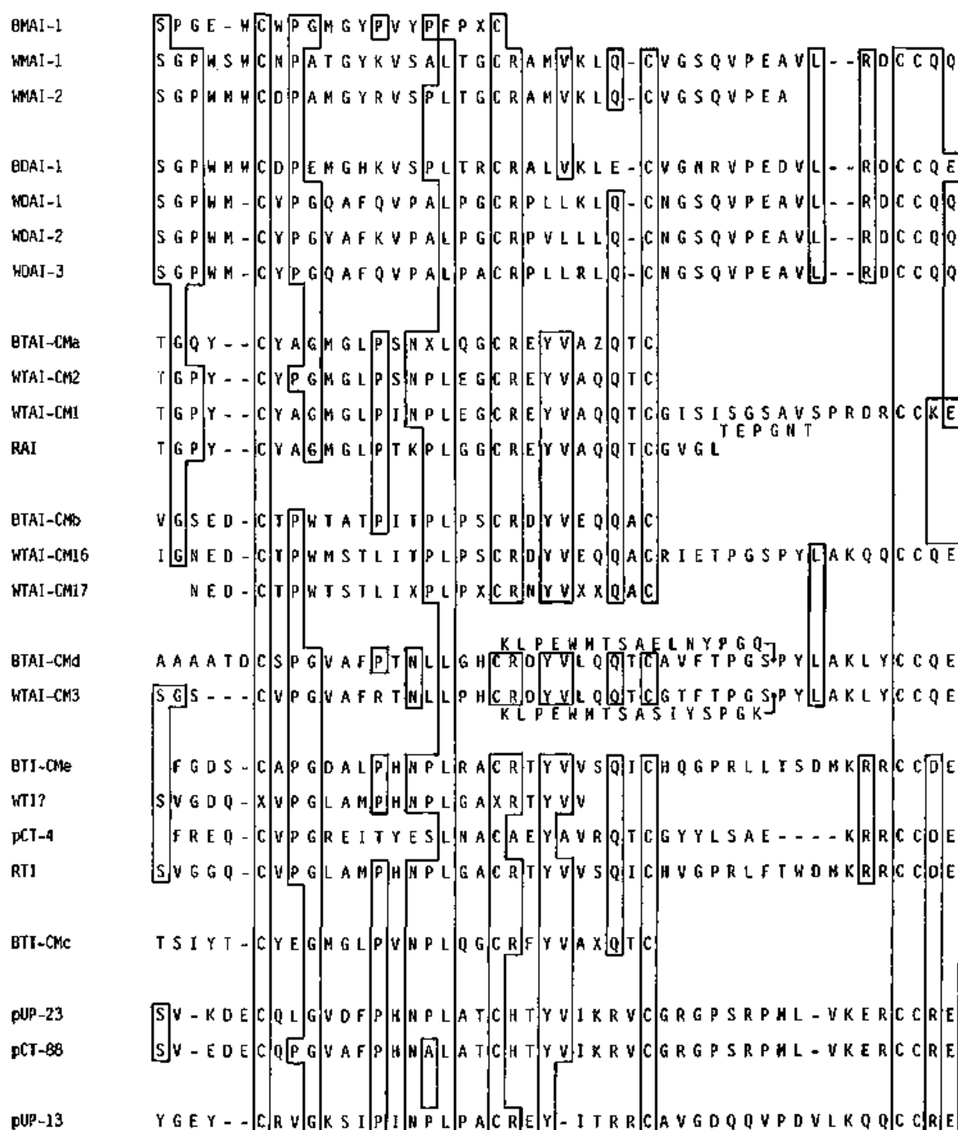

Fig. 16.1. Alignment of total or partial amino acid sequences of barley inhibitors of α-amylase and trypsin with those from wheat and rye: monomeric α-amylase inhibitors from barley (BMAI-1, ref. 1) and wheat (WMAI-1, ref. 2; WMAI-2, ref. 3); dimeric α-amylase inhibitors from barley (BDAI-1, ref. 4) and wheat (WDAI-1 and WDAI-2, ref. 5; WDAI-3, ref. 6); tetrameric α-amylase inhibitor subunits from barley (BTAI-CMa and BTAI-CMb, ref. 7; BTAI-CMd, refs 11–13), wheat (WTAI-CM2 and WTAI-CM17, ref. 8; WTAI-CM1 and WTAI-CM16, refs 8,9; WTAI-CM3, refs 9,11) and rye (RAI, ref. 10); trypsin inhibitors from barley (BTI-CMe, refs 14–16; BTI–CMc, refs 7,11), rye (RTI, ref. 10) and two related wheat proteins (WTI, ref. 11; pCT-4, ref. 17); amino acid sequences deduced from barley cDNA clones (pUP-23, ref. 18; pCT-88, ref. 17; pUP-13, ref. 12). References: (1) Barber *et al.*, 1989; (2) Kashlan & Richardson, 1981; (3) Gomez *et al.*, unpublished; (4) Lazaro *et al.*, 1988b; (5) Maeda *et al.*, 1985; (6) Sanchez-Monge *et al.*, 1989; (7) Barber *et al.*, 1986b; (8) Barber *et al.*, 1986a; (9) García-Maroto *et al.*, 1990; (10) Lyons *et al.*, 1987; (11) Shewry *et al.*, 1984; (12) Paz-Ares *et al.*, 1986;

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PROTEIN                               B DOMAIN
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WMAI-1      L A D T N N E W C R C G D L - S S H L R A V Y Q E L G V R E G K - - - E V L P G C R

BDAI-1      V A N I S N E W C R C G D L - G S H L R S V Y A A L G V G G G P - - E E V F P G C Q
WDAI-1      L A D I S - E W P R C G A L - Y S M L D S H Y K E H G V S E G Q A G T G A F P S C R
WDAI-2      L A H I S - E W C R C G A L - Y S M L D S H Y K E H G A Q E G Q A G T G A F P R C R

WTAI-CM1    L Y D A S - Q H C R C E A V R Y F I - - - - - G R R S - D P N S S V L K D L P G C P

WTAI-CM16   L A N I P - Q Q C R C Q A L R Y F M - - - - - S P K S - R P D Q S G L M E L P G C P
                                              S Q P V
BTAI-CMd    L A E I P - Q Q C R C E A L R Y F M A L P V P D P S T G N V G Q S G L M D L P G C P
WTAI-CM3    L A E I S - Q Q C R C E A L R Y F I A L P V P D P R S G N V G E S G L I D L P G C P

BTI-CMe     L S A I P - A Y C R C E A L R I I M Q G V V - T W Q G A F E G - A Y F K D S P N C P
pCT-4       L S K V P - K F C R C E V L H I L M D G
RTI         L L A I P - A Y C R C E A L R I L M D G V V - T Q Q G V F E G - G Y L K D H P N C P

pUP-23      L A A V P - D H C R C E A L R I L M D G V R - T P E G R V V E - G R L G D R R D C P
pCT-88      L A V V P - D Y C R C E A L R V L M D G V R - A E E G H V V E - G R L G D R R D C P

pUP-13      L S D L P - E S C R C - A L S I L V N G V I - T E D G S R V - - G R M E A V P R C D
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PROTEIN                               C DOMAIN
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WMAI-1      K E V M K L T A A S V P E V - - C K V P I - P N P S G D R A G V C Y G D W A A Y P D V

BDAI-1      K D V M K L L V A G V P A L - - C N V P I P N E A A G T R - G V C Y - W S A S T D T
WDAI-1      R E V V K L T A A S I T A V - - C R L P I V V D A S G D G A Y V C K D V A A Y P D A
WDAI-2      R E V V K L T A A S I T A V - - C R L P I V V D A S G D G A Y V C K D V A A Y P D A

WTAI-CM1    R E P Q R D F A K V L V T S G H C N V N T V H N A P Y - - - - - C L G - - - - L D I

WTAI-CM16   R E V Q M D F V R I L V T P G Y C N L T T V H N T P Y - - - - - C L A M E E S Q W S

BTAI-CMd    R E H Q R D F V R L L V A P G Q C N L A T I H N V R Y - - - - - C P A V E Q P L W I
WTAI-CM3    R E H Q W D F V R L L V A P G Q C N L A T I H N V R Y - - - - - C P A V E Q P L W I

BTI-CMe     R E R Q T S Y A A N I V T P Q E C N L G T I H G S A Y - - - - - C P E L Q P G Y G
RTI         R V T Q R S Y A A T L V A P Q E C N L P T I H G S P Y - - - - - C P T L Q A G Y

pUP-23      R E E Q P A F A A T L V T A A E C N L S S V Q E P G V R L V L L A D G
pCT-88      R E A Q R E F A A T L V T A A E C N L P T V S G V G S T L G A T G R W M T I E L P K

pUP-13      G E R I H S M G S Y L T A Y S E C N P - - - H N P G T P R G D - C V L F G G G S
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(13) Halford *et al.*, 1988; (14) Odani *et al.*, 1983b; (15) Lazaro *et al.*, 1985; (16) Rodriguez-Palenzuela *et al.*, 1989; (17) Carbonero *et al.*, unpublished; (18) Lazaro *et al.*, 1988a; (19) Gautier *et al.*, 1990. Domains A, B and C are indicated. Sequences have been grouped according to similarity and to their *in vitro* activities and chromosomal locations of the genes as indicated in Table 16.1.

The proteins corresponding to four of the cDNA clones have not been characterized yet, although the sequence deduced for one of them (pCT-4) can be aligned with that of the trypsin inhibitor BTI-CMe from barley.

The sequence homology data and the chromosomal location of the corresponding genes indicate that this dispersed gene family has originated both by translocation and by intrachromosomal duplication, and that most if not all of the dispersion must have occurred before the divergence of the barley genome from the diploid genomes included in allohexaploid wheat. An interesting example of intrachromosomal duplication is that of the *Iat1* locus encoding CMa and the *Itr2* locus encoding CMc on chromosome 1 (7H). The two proteins are more closely related to each other than to any other proteins encoded by the same genome. Nevertheless, BTAI-CMa, which is a subunit of the tetrameric α-amylase inhibitor, is even closer to the equivalent subunits in wheat (CM1 and CM2), which are encoded by chromosomes of group 7. In contrast, BTI-CMc is a trypsin inhibitor which shows much greater divergence from the other trypsin inhibitor, BTI-CMe [chromosome 3 (3H)], than from CMa (45% identical positions vs 78%).

The considerable sequence divergence found for this protein family within a given genome is in sharp contrast with the low allelic variability found for the individual members of the group both in barley (Salcedo *et al.*, 1984; Molina-Cano *et al.*, 1987; Sanchez-Monge *et al.*, 1988) and in wheat (García-Olmedo & García-Faure, 1969; García-Olmedo and Carbonero, 1970; Rodriguez-Loperena *et al.*, 1975; Salcedo *et al.*, 1978). In a wide-ranging survey of *Hordeum vulgare* cultivars and *Hordeum spontaneum* accessions analysed by two-dimensional electrophoresis (Salcedo *et al.*, 1984; Molina-Cano *et al.*, 1987), the following observations were made: BTAI-CMd, BTAI-CMa and BTI-CMc were essentially invariant throughout the two species (the last two proteins occurred in variant forms in one accession of *H. spontaneum* each); BTAI-CMb existed in variant forms in both species; and in the case of BTI-CMe, two variants, designated CMe2/CMe2′, were jointly inherited and co-dominantly expressed with respect to CMe (Salcedo *et al.*, 1984). Variant subunits of BTAI-CMa, BTAI-CMb and BTAI-CMd have been found in *Hordeum chilense* (Sanchez-Monge *et al.*, 1987).

The distribution of rare genetic variants of members of this protein family among Moroccan and non-Moroccan accessions of *Hordeum spontaneum* and among selections from several Moroccan landraces of *H. vulgare* and cultivars of the same species of wide-ranging origins, suggested that domestication of barley might have taken place in Morocco (Molina-Cano *et al.*, 1987).

Gene expression

The synthesis of α-amylase/trypsin inhibitors appears to precede that of the bulk of reserve proteins and starch. Thus Kirsi (1973) detected the barley trypsin inhibitor at about 5 days after pollination (DAP) and found that most was accumulated between 10 and 23 DAP and that the net amount was not affected by nitrogen fertilization. In a study of the *in vivo* and *in vitro* synthesis of these proteins in barley, Paz-Ares *et al.* (1983) concluded that synthesis peaked between 15 and 20 DAP. The inhibitors were synthesized by membrane-bound polysomes as precursors of higher apparent molecular weight (13–21 kDa) than the mature proteins (12–16 kDa). Accordingly, all cloned cDNAs corresponding to this protein family encode putative signal peptides that precede the N-terminal sequences of the mature proteins (Paz-Ares *et al.*, 1986; Halford *et al.*, 1988; Lazaro *et al.*, 1988b; García-Maroto *et al.*, 1990 and unpublished).

A significant homology has been found between at least some of these signal peptides and that of the precursor of the sweet protein thaumatin II (Lazaro *et al.*, 1988a).

The effects of high-lysine mutations on the expression of different genes from

this family in barley have been investigated (Salcedo *et al.*, 1984; Lazaro *et al.*, 1985; Rodriguez-Palenzuela *et al.*, 1989). The most notable effect concerns the gene for BTI-CMe, which is regulated in *trans* by high-lysine loci. The inhibitor is not detected in the mutant Risø 1508 (*lys 3a*) and is present at a much lower level in Hiproly (*lys*) (Salcedo *et al.*, 1984; Rodriguez-Palenzuela *et al.*, 1989). In both cases the mutations affect loci on chromosome 7 (5H), while the *Itr1* locus encoding CMe is on chromosome 3 (3H) (see Lazaro *et al.*, 1985). Steady-state levels of the corresponding mRNA in the mutant Risø 1508 are about 1% of those in the wild-type (Rodriguez-Palenzuela *et al.*, 1989). One or two copies of the gene have been estimated to be present in both the wild-type and in the mutant, and DNA restriction patterns are identical in both stocks, so neither a change in copy number nor a major rearrangement of the structural gene account for the markedly decreased expression (Rodriguez-Palenzuela *et al.*, 1989). A single dose of the wild-type allele at the regulatory *lys3a* locus restored the expression of the structural gene (Rodriguez-Palenzuela *et al.*, 1989).

The first inhibitor gene to be cloned was that coding for BTI-CMe (Royo *et al.*, unpublished). The gene has no introns and is preceded at about 2 kb upstream of the initiation codon by the 5′ end of a retroposon-like sequence. This suggests a possible dispersal mechanism responsible for the spread of inhibitor genes over several chromosomes.

The subcellular location of the different members of this family has not been fully established. In the case of α-amylase inhibitors, indirect evidence of association with starch granules has been reported (see Buonocore *et al.*, 1977). More recent immunodetection data seem to confirm this evidence, suggesting an external location of the inhibitors in these granules (Carmona *et al.*, unpublished).

Rapid disappearance of the inhibitors with the onset of germination suggests that these proteins do not play a specific role during germination (Kirsi & Mikola, 1971; Pace *et al.*, 1978).

In vitro activities

Monomeric and dimeric inhibitors show different specificities when tested against α-amylases from human saliva and from the digestive tract of the insect *Tenebrio molitor*, which have been routinely used as standard amylases in purification procedures (Fig. 16.2). It should be noted that the barley dimeric inhibitor (BDAI-1) shows different specificity from the wheat dimeric inhibitors. Not all insect α-amylases show the same specificity towards the different types of inhibitor: i.e. the enzyme from *T. molitor* is significantly more sensitive to the wheat monomeric inhibitor than to the dimeric type and the opposite is true for the enzyme from *Leptinotarsa decemlineata* (Colorado potato beetle), while in certain other cases the enzymes are about equally sensitive to both types (Gutierrez *et al.*, 1990).

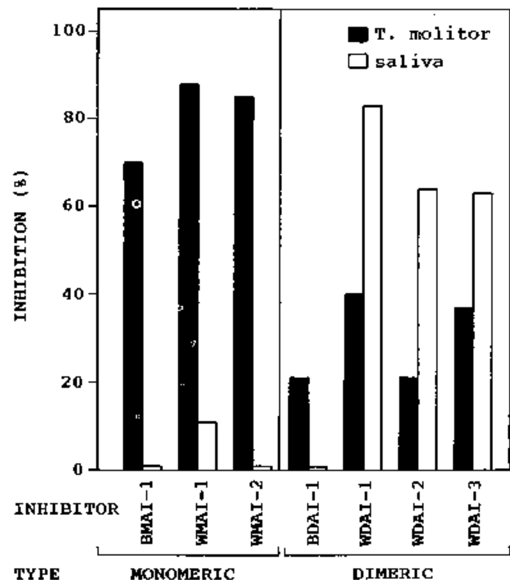

Fig. 16.2. Different specificity of monomeric and dimeric inhibitors towards α-amylases from the insect *Tenebrio molitor* and from human saliva. Inhibition was achieved by 1 μg of pure inhibitor against 1 unit of enzyme.

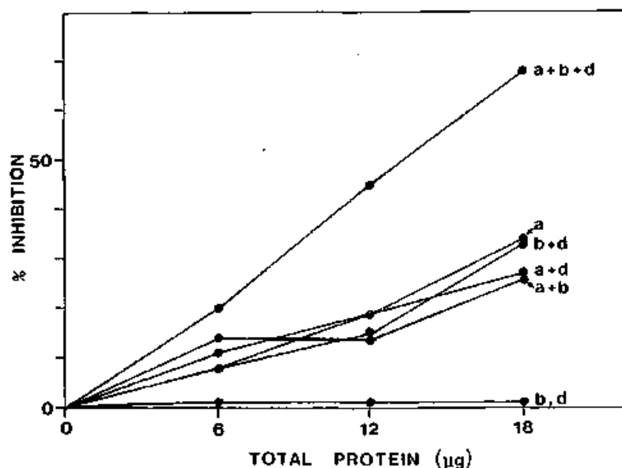

Fig. 16.3. Inhibition of α-amylase from larvae of *Tenebrio molitor* by purified proteins CMa, CMb, CMd and their mixtures. Equal amounts of each of the indicated proteins were used in the mixtures. All tests were carried out with approx. 2 units of α-amylase (1 unit defined as the amount of enzyme required to produce the reducing equivalents of 1 mg of maltose in 20 min).

The tetrameric inhibitor from barley seems to be active against the α-amylase from *T. molitor* and not against the salivary one (Sanchez-Monge *et al.*, 1986b). The subunits of this inhibitor were identified as proteins BTAI-CMa, BTAI-CMb and BTAI-CMd (two copies). Only the first protein showed some activity by itself but, as shown in Fig. 16.3, the association of the three different subunits was required for full activity (Sanchez-Monge *et al.*, 1986b). The identity of the subunits of the wheat tetrameric inhibitors was predicted on the basis of the studies of barley, and subsequently confimed by analysis of the inhibitors and by their reconstitution from the purified subunits (Gomez *et al.*, 1989).

The trypsin inhibitor BTI-CMe is inactive against chymotrypsin, papain, subtilopeptidase A, pepsin, bacterial or fungal proteinases, as well as against the endogenous proteinases from green malt (Mikola & Suolinna, 1969).

An important biological property of all these proteins is that they are the major allergens in baker's asthma, which is an important occupational disease. BMAI-1 was the first to be shown to react with sera from patients (Barber *et al.*, 1989) and subsequently subunits of both dimeric and tetrameric barley inhibitors have been identified as significant allergens (Sanchez-Monge *et al.*, unpublished). As would be expected, most of the wheat components of this family also show strong reactions (Gomez *et al.*, 1990).

Thionins

Structural types

More than 20 different thionin sequences have been determined so far, either directly by amino acid sequencing or indirectly through cDNA cloning and nucleotide sequencing (see García-Olmedo *et al.*, 1989, 1991). These sequences have been classified into five structural types (García-Olmedo *et al.*, 1991), using the algorithm of Feng & Doolittle (1987). At least three of these types, whose gross structural features are represented in Fig. 16.4, are present in barley and wheat.

Type I corresponds to that of the original purothionins isolated from wheat endosperm by Balls *et al.* (1942a, b). Two non-allelic genetic variants (α, β) of this type have been characterized in barley endosperm (Ozaki *et al.*, 1980; Hernandez-Lucas *et al.*, 1986; Ponz *et al.*, 1986). Thionins of this type have four disulphide bridges and are highly basic, with no negatively charged amino acid residues. Total chain length is 45 residues, 8 of which are in the central disulphide loop.

	CHARGES +/-	RESIDUES TOTAL/LOOP	VARIANTS IN BARLEY	TISSUE
TYPE I	10/0	45/8	α β	ENDOSPERM
TYPE II	7/1	46/9	DB4 DG3 DC4	LEAVES
TYPE V	2/2	38/9	pTT20	ENDOSPERM + ?

Fig. 16.4. Structural types of thionins present in barley.

Type II corresponds to thionins isolated from the leaves of the parasitic plant *Pyrularia pubera* (Vernon *et al.*, 1985) and from those of barley (Bohlmann & Apel, 1987; Gausing, 1987). These thionins are also quite basic, but have some negatively charged residues. Disulphide bridges are conserved and total chain length is 46–7 residues, with 9–10 in the central loop. Three different but closely related complete sequences have been deduced from the nucleotide sequences of cDNA clones in barley.

The third thionin type in barley and wheat, type V, is quite divergent from the other types: the 2nd and 8th cysteines of types I and II are missing, through point mutation and deletion, respectively, thus disrupting the 1st and 2nd disulphide bridges and potentially allowing the formation of a new bridge between the unpaired cysteines (Castagnaro *et al.*, unpublished). This new type, which is neutral, has been first identified in a cDNA library derived from developing wheat kernels and is also present in barley.

Thionin genes in barley and related species

There are 1–2 genes for type I thionins per haploid genome in barley, wheat and rye. In the last two species, these genes have been located on the long arms of the group 1 chromosomes by aneuploid analysis (Fernandez de Caleya *et al.*, 1976; Sanchez-Monge *et al.*, 1979; Rodriguez-Palenzuela *et al.*, 1988). A similar location on chromosome 5 (1H) of barley is to be expected, but direct confirmation has not been achieved by aneuploid analysis because the critical addition line has not been obtained. Type II genes have been located on chromosome 6 (6H) of barley by Southern blot analysis of DNAs from wheat–barley addition lines (Bohlman *et al.*, 1988), but there are discrepancies about their number: about 100 genes (Bohlman *et al.*, 1988) or 9–11 genes (Gausing, 1987). Type V genes have been located within a few kb of the type I genes in wheat, with only one copy per haploid genome (Castagnaro *et al.*, unpublished).

Gene expression

Barley endosperm thionins (Type I) are synthesized by membrane-bound polysomes as much larger precursors that undergo at least two processing steps: one co-translational and one post-translational (Ponz *et al.*, 1983). As predicted from these studies, the nucleotide sequences of the cDNAs corresponding to α and β thionins from barley endosperm were found to encode precursors with an N-terminal signal peptide followed by the sequence of the mature protein and that of a C-terminal acidic peptide, as shown in Fig. 16.5 (Hernandez-Lucas *et al.*, 1986; Ponz *et al.*, 1986). The same precursor structure has been subsequently found for type II (Bohlmann & Apel, 1987; Gausing, 1987) and for type V thionins (Castagnaro *et al.*, unpublished). It is therefore likely that all three types are processed in the same manner.

Thionin accumulation in developing endosperm appears to start and to level off before synthesis of the major storage proteins, with maximum steady-state concentrations of mRNAs 13–16 days after pollination (Ponz *et al.*, 1983; Rodriguez-Palenzuela *et al.*, 1988).

The final deposition site of type I thionins seems to be in the periphery of the protein bodies, based on recent immunogold detection by electron microscopy (Carmona *et al.*, unpublished) and on previous subcellular fractionation experiments (Carbonero *et al.*, 1980; Ponz *et al.*, 1983).

It was first claimed that leaf thionins were exclusively located in the cell wall, based on electron micrographs of immunogold-labelled thin sections, using an antibody raised against a fusion protein expressed in *E. coli* (Bohlmann *et al.*, 1988). Subsequently, these authors found that about 98% of the leaf thionins were intracellular (Reiman-Philipp *et al.*, 1989b). To reconcile the contradictory observations, they proposed that there were two distinct structural groups of leaf thionins: the cell wall and the intracellular ones (Reiman-Philipp *et al.*, 1989b). In our opinion, this claim was not sufficiently supported by their evidence.

The gene coding for α-thionin from barley endosperm (type I) has been cloned and its complete sequence has been published (Rodriguez-Palenzuela *et al.*, 1988). As represented in Fig. 16.5, the gene has two introns of 420 and 91 bp, respectively, both of which interrupt the sequence that corresponds to the C-terminal acidic peptide of the precursor. Although genomic clones of type II thionins have not been described in detail, it seems that they also have two introns (Bohlman *et al.*, 1988). More recently, it has been found that type V genes have the same intron organization as type I genes (Castagnaro *et al.*, unpublished).

Type I genes appear to be specifically expressed in barley endosperm during the cell proliferation phase (Rodriguez-Palenzuela *et al.*, 1988) and fusions of the corresponding promoter with the glucuronidase (GUS) reporter gene direct endosperm-specific expression in transgenic tobacco plants with the same temporal control (Fernandez *et al.*, unpublished). When fusions of the 35S promoter with the α-thionin gene (coding regions and introns) are expressed in transgenic tobacco, the introns are correctly spliced, in contrast with what happens when some genes from

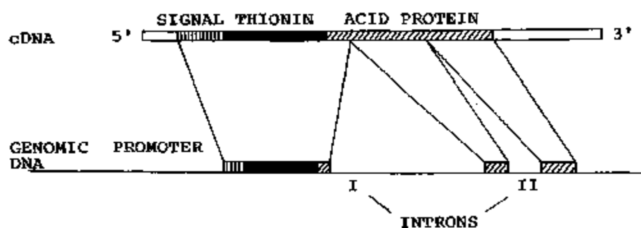

Fig. 16.5. cDNAs and genomic DNAs encoding thionin precursors.

monocotyledonous plants are expressed in a dicotyledonous species (Carmona *et al.*, unpublished).

Type II genes seem to be leaf-specific. They are expressed in the dark and are switched off upon illumination (Bohlman & Apel, 1987; Gausing, 1987; Reiman-Philipp *et al.*, 1989a). The inhibitory effect of light can be overcome by stress and pathogen-induced signals, as it has been shown that fungal infection induces a transient expression of the thionin genes in the leaves (Bohlman *et al.*, 1988; Ebrahim-Nesbat *et al.*, 1989) and that the chlorides of divalent cations (Mg^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}) elicit a more permanent response (Fisher *et al.*, 1989).

No information is yet available concerning the developmental expression of type V genes.

In vitro properties

The toxicity of thionins towards different kinds of organisms and to cells in culture has been investigated for several decades (for a review, see García-Olmedo *et al.*, 1991). Toxicity of thionins to plant pathogens was first reported by Fernandez de Caleya *et al.* (1972). These studies were stimulated by earlier reports concerning the antimicrobial properties of these polypeptides (Stuart & Harris, 1942; Balls & Harris, 1944). Plant pathogenic bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Erwinia* and *Corynebacterium* were found to be sensitive to wheat-endosperm thionins (type I) at concentrations ranging from 10^{-7} to 10^{-5}M, and purified genetic variants of the thionins had different activities and showed some degree of specificity (Fernandez de Caleya *et al.*, 1972). In a recent survey, the sensitivity of fungal plant pathogens to pure genetic variants of thionins from barley (types I and II) and from wheat (type I) was found to be in the 0^{-6}M–10^{-5}M range (García-Olmedo *et al.*, 1991; Molina & Fraile, unpublished). Barley thionins of types I and II were about equally effective, although for a given pathogen certain variants were somewhat more toxic than others. Bohlmann *et al.* (1988) have reported the sensitivities of two fungi, *Thielaviopsis paradoxa* and *Drechslera teres*, to both types of thionins at 5×10^{-4}M, a concentration that is orders of magnitude higher than those discussed above.

Two types of mechanism could be responsible for the toxicity of thionins: the permeabilization of the cell membrane, followed by inhibition of macromolecular synthesis (Fernandez de Caleya, 1973; Carrasco *et al.*, 1981; García-Olmedo *et al.*, 1983) or the interference with thioredoxin-mediated redox reactions (Wada & Buchanan, 1981; Johnson *et al.*, 1987).

Evidence of a possible defence role for inhibitors and thionins

Although both families are toxic or inhibitory to heterologous systems *in vitro*, their true *in vivo* functions remain unknown. In addition both show little or no intraspecific variability at given loci and their distribution is apparently limited to a few taxa, although the thionins might yet prove to be ubiquitous.

The known properties of the trypsin/α-amylase inhibitors do not lead to any obvious hypothesis about their role in plant metabolism, whereas in the case of thionins, a participation in the regulatory pathways linking light to enzyme activation/inactivation or in disulphide rearrangement mechanisms can be suggested (see García-Olmedo *et al.*, 1991). The evidence of a defence role for these proteins is increasing and merits a brief discussion here.

In the case of the inhibitors, the following aspects are to be noted: (i) different inhibitors show different specificities towards enzymes from different insects (Gutierrez *et al.*, 1990 and unpublished); (ii) considerable intra- and interspecific variation in inhibitor levels has been observed (Kirsi & Ahokas, 1983; Gomez *et al.*, 1989 and unpublished); and (iii) gene silencing has occurred in some cases (García-Maroto *et al.*, 1990). These characteristics, together with the low genetic variability at

given loci, suggest that if these proteins are involved in plant defence, they are probably related to non-host resistance rather than to more specific interactions. More direct evidence for a defence role stems from observations and experiments with insect pests. Thus, insects that are able to feed on wheat endosperm have unusually high levels of α-amylase (Silano *et al.*, 1975; Gutierrez *et al.*, 1990). High inhibitor concentrations in an artificial diet were required to affect the development of larvae of *Tribolium confusum*, a storage pest of wheat products, while quite low concentrations were effective against *Callosobruchus maculatus*, a pest of legume seeds (Gatehouse *et al.*, 1986). More recently, transgenic tobacco plants carrying the structural parts of the barley gene coding for BTI-CMe or the wheat gene coding for WMAI-1 (0.28) under the control of the 35S promoter have been found to be lethal to *Agrotis ipsilon* in a leaf-disc assay (Carbonero *et al.*, unpublished).

No true specificity has yet been found among the genetic variants of thionins with respect to pathogens, which suggests that their possible defence role would be related to the general resistance barriers. Besides their *in vitro* biocidal activity, preliminary experiments with transgenic tobacco plants carrying thionin genes under the 35S promoter were less sensitive to bacterial pathogens than the non-transformed controls (Carmona *et al.*, unpublished).

From our current knowledge of the two protein families, α-amylase/trypsin inhibitors and thionins, it can be concluded that they are potentially useful for the genetic manipulation of plant resistance to pests and pathogens, independently of whether or not they are true defence proteins.

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