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Cloning of cDNA, expression, and chromosomal location of genes encoding the three types of subunits of the barley tetrameric inhibitor of insect α-amylase

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Abstract

Three cDNA clones from barley developing endosperm, corresponding to proteins BTAI-CMa, BTAI-CMb and BTAI-CMd, which are the three types of subunits of the tetrameric inhibitor of insect α-amylases, have been identified and sequenced. The deduced amino acid sequence of BTAI-CMb corresponds to the CM16/CM17 type of subunit in wheat (92/90% identical residues) and has one putative N-glycosylation site (NLT) and a possible kinase-C phosphorylation site (SCR). The BTAI-CMa sequence differs at four amino acid residues from a previously reported one from cv. Bomi and the sequence deduced for BTAI-CMd completes (11 N-terminal residues) and confirms previously available data. The gene for BTAI-CMa (*Iat1*) is located in the β arm of barley chromosome 7H (syn.1), while genes for both BTAI-CMb (*Iat2*) and BTAI-CMd (*Iat3*) are in the long arm of chromosome 4H. The three genes are expressed in endosperm and their mRNAs are not detected in the other tissues tested, except *Iat1*, which seems to be expressed at a low level in coleoptile and roots, where it is switched off by 50 μM methyl jasmonate.

Introduction

In barley endosperm, over 10% of the total protein is represented by protein families that are either toxic or inhibitory towards heterologous systems and consequently may have a defence role. A substantial fraction of these proteins belongs to a single multigene family of trypsin/α-amylase inhibitors whose members are scattered over several chromosomes. We have character-

The nucleotide sequence data reported will appear in the EMBL GenBank and DDBJ Nucleotide Sequences Databases under the accession numbers X69937 (BTAI-CMa), X69938 (BTAI-CMb) and X69939 (BTAI-CMd).

ized over 20 different inhibitors of this family in wheat and barley. Those that inhibit α-amylase can be either monomeric, homodimeric or heterotetrameric, whereas the trypsin inhibitors are monomeric. The molecular masses of the different subunits are in the 12–16 kDa range [for recent reviews see 3, 8].

The tetrameric α-amylase inhibitor from barley is composed by three different proteins, BTAI-CMa, BTAI-CMb and BTAI-CMd, in a 1:1:2 ratio. This inhibitor is active against the α-amylase from the larvae of the storage insect pest *Tenebrio molitor*, but inactive against both the endogenous barley α-amylase and that from human saliva. Subunits CMb and CMd have no inhibitory activity by themselves, while CMa alone retains some of the activity of the reconstituted tetramer [2, 3, 25, 29].

The complete sequences of the wheat tetramer subunits have been deduced from the nucleotide sequences of their cloned cDNAs [7, 9, 15]. The inhibitory activities of different '*in vitro*' reconstituted wheat tetramers have been shown to fit a model for alloploid heterosis at the molecular level [11].

In barley, the complete amino acid sequence of the precursor of BTAI-CMa has been deduced from its cDNA [21] and two incomplete cDNA sequences of BTAI-CMd are known [12, 20], while only the N-terminal sequence of the mature protein is available for BTAI-CMb [2]. This protein is of special interest because it has been shown that it exists both in a glycosylated and in a unglycosylated form and that the glycosylated protein, CMb*, is one of the most potent allergens associated with baker's asthma [24].

We report here the complete sequence of the precursor for the BTAI-CMb subunit of the barley tetrameric inhibitor, deduced from a cDNA clone. Previously available sequence data about the other two subunits, BTAI-CMa and BTAI-CMd have been completed and essentially confirmed. The localization of the genes for the three subunits in chromosome arms 7Hβ (*Iat1*) and 4HL (*Iat2* and *Iat3*), as well as their tissue-specific expression, are also reported.

Materials and methods

Biological material

Developing barley endosperm from *Hordeum vulgare* L. cv. Abyssinian 2231, collected at 14 days after pollination, was the source of the poly(A)$^+$ mRNA for the construction of the cDNA library in the lambda vector NM1149. Hexaploid wheat, *Triticum aestivum* L. cv. Chinese Spring, diploid barley, *Hordeum vulgare* L. cv. Bétzès, and the disomic addition lines of chromosomes 2H, 3H, 4H, 5H (formerly 7), 6H and 7H (formerly 1) from barley cv. Bétzès on a wheat cv. Chinese Spring background were obtained from K. W. Shepherd and A. K. M. R. Islam [28]. The ditelosomic addition lines corresponding to chromosomes 4H and 7H were also provided by these authors: 4HS (short arm of chromosome 4H), 4HL (long arm of 4H); 7Hα (probably long arm of 7H), 7Hβ (probably short arm of 7H). These lines were used for the chromosomal location of the genes.

Screening of the CDNA library

The cDNA library was screened under non-stringent conditions (60 °C) using a mixture of the inserts in clones pCT1, pCT2 and pCT3 [7], which encoded the subunits of the wheat tetrameric inhibitor. To complete the 5′ end of the cDNA corresponding to the BTAI-CMd subunit, PCR amplification was carried out [4], using cDNA from developing barley endosperm as template with primers 5′-ATGGCGTGCAAGTCC-3′ and 5′-GCATACATGCACACCAC-3′ derived respectively from the sequence of the first nucleotides of the signal peptide region of its wheat homeologue cDNA, pCT1 [7] and an appropriate region of the incomplete BTAI-CMd cDNA [12, 20]. About 100 ng of cDNA were added to 50 μl of a PCR cocktail consisting of 1 × PCR buffer (Promega), 1.6 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 60 ng of each primer and 1.2 units of *Taq* DNA polymerase (Promega). Thirty cycles of amplification

were carried out using a step programme (95 °C, 1 min; 52 °C, 2 min; 72 °C, 2 min), followed by a 5-min extension at 72 °C. The amplified DNA was cloned into the *Sma* I site of vector M13mp18 and the recombinants screened for positive clones.

Nucleotide Sequencing

The cDNAs cloned into the M13mp18–19 vectors were sequenced by the dideoxy chain termination method [26] using a sequenase kit (United States Biochemical) according to the manufacturer's instructions. Sequencing reactions used [α^{35}S]dATP (1000 Ci/mmol) as label and the products were separated on 8% polyacrylamide, 9 M urea 'wedge' gels. The sequences were analysed with the Beckman Microgenie software and the EMBNet/CNB computer facilities.

Southern blot analysis

Total DNAs were prepared from leaves of barley, wheat and wheat/barley addition lines after 10–15 days of germination. Green tissue was ground under liquid N_2 and extracted according to Sharp *et al.* [27]. After complete digestion with the appropriate endonuclease, DNA was separated on 0.8% agarose gels. Southern blotting on Nylon membranes (GeneScreen Plus, Dupont), hybridization (0.5 M Na_2HPO_4, 1% SDS, 100 μg/ml salmon sperm DNA, pH 7.2, at 65 °C) and high-stringency washings (2 × SSC, 1 × SSC, and 0.1 × SSC with 0.1% SDS, for 15 min each, at 65 °C) were performed by standard procedures [16]. The inserts of the clones used as probes were ^{32}P-labelled by the multiprime labelling method [6].

Northern blot analysis

RNA was purified from frozen tissues (developing endosperms, young roots and leaves) by phenol/chloroform extraction followed by lithium chloride precipitation [14]. Total denatured RNA was electrophoresed through 5% formaldehyde agarose gels and blotted into Nylon membranes (Hybond N, Amersham). Hybridization and washings were performed following standard procedures [16]. Young barley plants grown for 7 days at room temperature were treated with 0.1 mM abscisic acid, 1mM sodium salycilate, and 50 μM methyl jasmonate. Aqueous solutions of these compounds were sprayed over the entire plant every six hours. Roots were excised from leaves at 24, 48 and 72 h and frozen in liquid N_2. RNA was extracted from these tissues as previously indicated [14].

Results

Characterization of cDNA clones

Two positive clones that differed in their restriction maps were selected from the barley endosperm cDNA library, using as probe a mixture of cloned cDNAs encoding the three subunits of the wheat tetrameric inhibitor of insect α-amylase [7]. The longest open reading frames in the nucleotide sequences of the inserts in the two clones respectively included regions encoding the known N-terminal sequences of the previously purified proteins BTAI-CMa and BTAI-CMb, two of the three components of the barley tetrameric inhibitor of α-amylase [2]. The deduced amino acid sequence of BTAI-CMa from cv. Abyssinian reported here (accession number X69937) differs at four positions with respect to that of the same protein in cv. Bomi [21] and these differences imply a different net charge for the two proteins, which is of interest because no charge variants have been found for this protein in previous surveys [22, 18]. The nucleotide sequence encoding subunit BTAI-CMb (accession number X69938) had not been previously reported. A possible kinase-C phosphorylation site (SRC) and one putative N-glycosylation site (NLT) for BTAI-CMb can be identified in the deduced amino acid sequence (Fig. 1). Truncated cDNA clones corresponding to BTAI-CMd, the third and most hy-

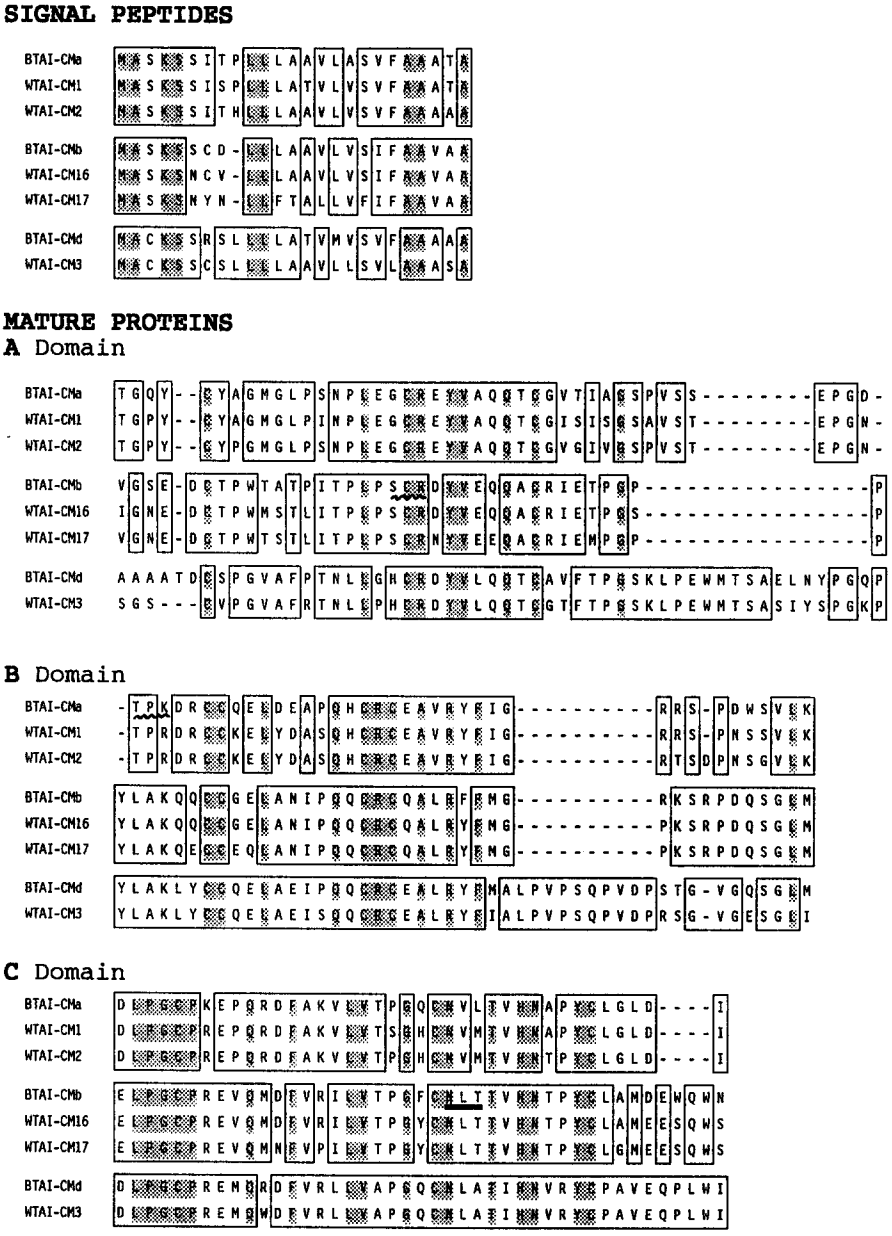

Fig. 1. Alignment of the deduced amino acid sequences of the precursors for the barley subunits of the tetrameric inhibitor of α-amylase (BTAI-CMa, BTAI-CMb and BTAI-CMd) and comparison with the wheat tetrameric amylase inhibitor (WTAI) homeologues CM1, CM2, CM16, CM17 and CM3 [7, 9, 15]. The sequences of the mature proteins are divided into three domains (A, B and C) following the criteria of Kreis *et al.* [13]. Identical residues between proteins belonging to the same subfamily are boxed and coincident residues in the eight members compared are shaded. Possible phosphorylation sites (TPK and SCR) in BTAI-CMa and BTAI-CMb are indicated by wavy lines, and the putative N-glycosylation site (NLT) is marked with a double underline.

drophobic component of the tetrameric inhibitor, had been previously reported by us and others [12, 20]. The coding sequence was completed up to the initial methionine of the signal peptide by a PCR strategy (accession number X69939). Three independently generated clones were se-

quenced in order to avoid possible sequence errors due to amplification. The BTAI-CMd amino acid sequence reported here fully coincided with that reported by Paz-Ares *et al.* [20], which was 38 residues short of the mature N-terminus, and differed by one substitution (T/P at position 119) from that of Halford *et al.* [12], which lacked the first 11 residues.

In Fig. 1, the deduced amino acid sequences of the barley precursors have been aligned with their wheat equivalents [7]. The four alignment blocks correspond to the signal peptides and to the three domains proposed for the mature proteins by Kreis *et al.* [13]. No gaps were required for the alignment within each barley/wheat homologous set of subunits, except for a difference of three residues at the N-terminal of the mature proteins in the CMd/CM3 pair.

Chromosomal location of genes

The sequences encoding the three subunits of the barley inhibitor were subcloned in vector pUC18 and were used as probes to determine the chromosomal locations of the corresponding genes by Southern analysis of DNAs from the wheat/barley addition and ditelosomic addition lines (Fig. 2). High-stringency conditions were used to avoid cross-hybridization with other inhibitor genes of the same family. The CMa probe gave three bands with cv. Chinese Spring wheat DNA and one non-overlapping band of ca 3.1 kb with cv. Bétzès barley DNA when digested with the *Sac* I restriction endonuclease (Fig. 2A). The latter appeared as an extra band in the patterns of the addition lines carrying either chromosome 7H (syn. 1) or the 7Hβ ditelosomic (Fig. 2A), indicating location of the gene for BTAI-CMa (*Iat1* gene) in the β arm of chromosome 7H. In a similar manner, the CMb probe hybridized with a single non-overlapping band of the *Sac* I pattern of Bétzès barley DNA, the 4H addition, and the 4HL ditelosomic addition lines, thus showing the location of gene for BTAI-CMb (gene *Iat2)* in the long arm of chromosome 4H (Fig. 2B). The bands identified by the CMd probe in the *Eco* RI pattern, allowed mapping of the gene for BTAI-CMd (gene *Iat3*) also in the same 4HL chromosomal arm (Fig. 2C). The three barley probes hybridized

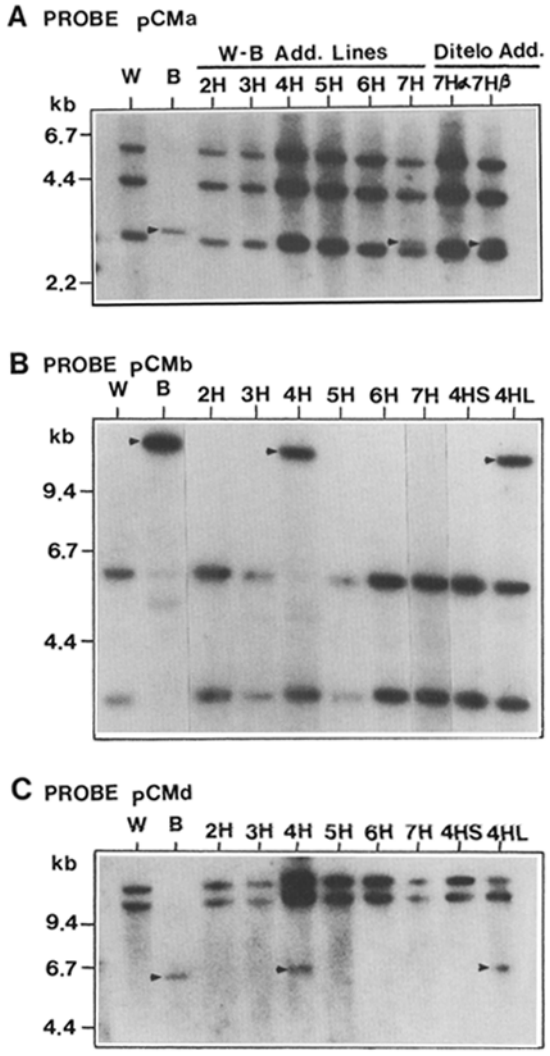

Fig. 2. Chromosomal locations of genes *Iat1*, *Iat2* and *Iat3* corresponding to the three types of subunits BTAI-CMa, BTAI-CMb and BTAI-CMd respectively, of the barley tetrameric inhibitor of α-amylase. Southern blot analysis of the following genotypes: hexaploid wheat *Triticum aestivum* cv. Chinese Spring (W); diploid barley, *Hordeum vulgare* cv. Bétzès (B); Chinese Spring wheat/Bétzès barley addition lines (2H, 3H, 4H, 5H, 6H, 7H) and ditelosomic additions lines (7Hα, 7Hβ; 4HS, 4HL). DNA digested with restriction endonuclease *Sac* I was hybridized with probes corresponding to BTAI-CMa (A) and BTAI-CMb (B) and DNA digested with *Eco* RI with probe corresponding to BTAI-CMd (C). Barley bands are indicated with arrowheads.

with wheat DNA under the stringency conditions used because of the above discussed close similarity between equivalent subunits in wheat and barley. It is to be noted that the pattern obtained with the CMb probe for addition line 4H lacked one of the wheat bands (Fig. 2B), while the same line gave the normal wheat background with probe CMd (Fig. 2C).

Expression of genes Iat1, Iat2, *and* Iat3

Analysis by northern hybridization of total RNAs from developing endosperm, the tissue from which the inhibitor subunits had been originally purified [2, 25, 29], showed the synchronous expression of the three genes during the cell proliferation stage (first half) of endosperm development (Fig. 3). Expression of these genes in other tissues and under different external stimuli was also investigated. Only gene *Iat1* (probe CMa) was found to be expressed at a low level in coleoptiles and roots (Fig. 3). Treatment with 0.1mM abscisic acid or with 1mM salicylate did neither induce the expression of genes *Iat2* and *Iat3* (data not shown), nor alter the expression of gene *Iat1* in these tissues, while 50 μM methyl jasmonate switched off the latter (Fig. 3). 3 about here

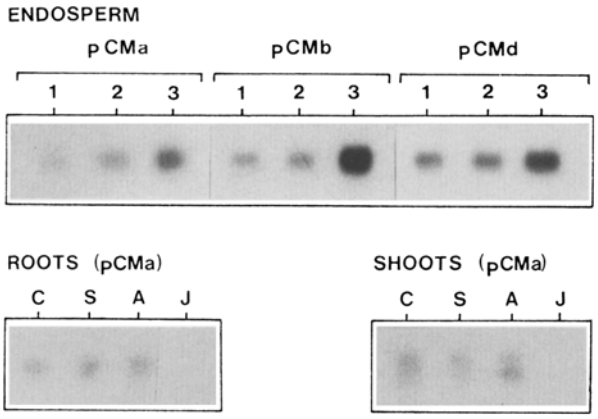

Fig. 3. Northern blot analysis of genes *Iat1*, *Iat2* and *Iat3*. 5 μg of total RNA from endosperms at different developmental stages (1 = 5 days after pollination (dap); 2 = 10 dap; 3 = 18 dap) were electrophoresed in 1% agarose gels containing 5% (v/v) formaldehyde, blotted onto nylon filters and hybridized with probes corresponding to BTAI-CMa (pCMa), BTAI-CMb (pCMb) and BTAI-CMd (pCMd). Total (15 μg per slot) RNA from young roots and shoots (7 days of germination) were hybridized with the BTAI-CMa probe (pCMa) after challenging for 72 h with the indicated chemicals: C = control; S = 1mM salycilic acid; A = 0.1 mM abscisic acid; J = 50 μM methyl jasmonate. Results were identical at 24, 48 and 72 h.

Discussion

In Table 1, all binary comparisons of the amino acid sequences of the different subunits from the wheat and barley tetrameric inhibitors whose complete sequences are known, are presented. A higher percentage of conserved residues are to be found between barley/wheat homeologues (CMa versus CM1 and CM2, 81/82% identical residues; CMb versus CM16 and CM17, 92/90% identity; CMd/CM3, 86% identity) than between any pair of the barley tetramer subunits (CMa/CMb, 49% identical residues; CMa/CMd, 50% identity; CMb/CMd, 57% identity). 1 about here

Previous data from our department [22] had

Table 1. Binary Comparisons (% Similarity and % Identity) of subunits of the tetrameric α-amylase inhibitors from wheat (CM1/CM2, CM16/CM17, CM3) and barley (CMa, CMb, CMd).

	CMb	CMd	CM1	CM2	CM3	CM16	CM17
CMa	70 49	72 50	89 81	89 82	68 47	70 50	70 46
CMb		71 57	66 48	68 50	72 56	98 92	97 90
CMd			67 43	68 46	90 86	71 60	70 54
CM1				96 92	67 46	68 49	67 47
CM2					68 47	69 50	68 47
CM3						71 57	69 51
CM16							97 91

associated protein CMa with chromosome 1 (syn. 7H) and proteins CMb and CMd with chromosome 4. We have corroborated this and assigned genes to chromosome arms. The assignment of gene *Iat1* to the β arm of chromosome 7H, together with the similar β arm location of the *Ss1* sucrose synthase gene [23], supports the equivalence of this chromosome arm (7Hβ) with the short arms of wheat chromosomes 7A, 7B and 7D [1, 17]. Location of genes *Iat2* and *Iat3* in the long arm of chromosome 4H is in agreement with that of its wheat homeologues CM16 and CM3 whose genes have been mapped within a few kilobases of each other [7] in the long arms of group-4 chromosomes [1]. The presence of closely related genes in the short arms of group-7 chromosomes and the long arms of group-4 chromosomes would be in agreement with the partial homology between these chromosome arms proposed by Naranjo *et al.* [19] based on cytogenetic evidence. The missing wheat band in the Southern pattern of the 4H disomic addition line (Fig. 2B), which is present in the 4HL and 4HS ditelosomic addition lines, implies partial or total loss of a wheat chromosome while generating the addition line or during subsequent manipulation.

The present study shows that the three genes are coordinately expressed during the proliferative stage of endosperm development. With the exception of the *Iat1* gene, which seems to be expressed at a low level in young roots and shoots, the genes are endosperm-specific. The weak signal detected with the *Iat1* probe in shoots and roots might alternatively represent weak cross-hybridization with the messenger of a not yet characterized member of the same inhibitor family. In any case, the fact that the steady-state level of this messenger is markedly decreased by methyl jasmonate is in contrast with what has been observed with other inhibitors, such as the trypsin inhibitors reported by Ryan's group [5], which are markedly induced by the same treatment.

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