A constitutive cystatin-encoding gene from barley

(*Icy*) responds differentially to abiotic stimuli

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A barley cDNA clone encoding a cysteine proteinase inhibitor was characterized. The deduced amino acid sequence of this barley cystatin (Hv-CPI) contains the motif QXVXG conserved among members of the cystatin superfamily. The gene (*Icy*), located on chromosome 2, was expressed in embryos, developing endosperms, leaves and roots as assessed by northern blot analysis. Western blot analysis detected a slightly retarded band in leaves that was not present in roots or seeds. In these two organs a more precise location of Hv-CPI was done by immuno-histochemical analysis, with polyclonal antibodies raised against the recombinant CPI protein expressed in *Escherichia coli*. This protein efficiently inhibited papain (K_i 2.0×10^{-8} M) and ficin (K_i 2.2×10^{-8} M) and, to a lesser extent, chymopapain (K_i 1.6×10^{-7} M) and was inactive against bromelain. The *Icy* mRNA expression in vegetative tissues increased in response to anaerobiosis, dark and cold shock (6 °C).

Abbreviations: BANA, *N*-benzoyl-DL-arginine- β -naphthylamide; BSA, bovine serum albumin; CEW, cystatin from chicken egg white; daf, days after flowering; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PhyCys, phytocystatin; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

Introduction

Cystatins are a group of proteins specifically inhibiting cysteine proteinases that have been identified in vertebrates, insects and plants. The plant cystatins or phytocystatins (PhyCys) comprise more than 30 members from different monocotyledonous and dicotyledonous species and cluster in a major evolutionary tree branch of the cystatin superfamily of proteins (Margis *et al.*, 1998). Besides the reactive site motif QXVXG, common to all plant and animal cystatins, the PhyCys

are characterized by the absence of disulfide bonds, the presence of a specific consensus sequence on a predictable N-terminal α -helix and a molecular mass in the 12–16 kDa range. PhyCys have been purified mainly from seeds (Abe *et al.*, 1987b; Hirashiki *et al.*, 1990; Abe and Arai, 1991; Ievleva *et al.*, 1995; Kouzuma *et al.*, 1996; Li *et al.*, 1996; Ojima *et al.*, 1997; Brzin *et al.*, 1998; Pernas *et al.*, 1998), but they have been also detected in other tissues such as tubers (Waldron *et al.*, 1993), leaves (Zhao *et al.*, 1996), fruit (Kimura *et al.*, 1995), pollen (Rogers *et al.*, 1993) and floral buds (Lim *et al.*, 1996).

Cystatins from rice, maize and soybean are particularly well characterized. Two isoforms with different

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under accession number Y12068.

In this paper, we report the characterization of a cDNA clone from a developing barley endosperm library that encodes a PhyCys (Hv-CPI). The corresponding gene (*Icy*), located on chromosome 2, is ubiquitously expressed as assessed by northern and western blots. In seeds and roots, a more precise localization of Hv-CPI was done by immuno-histochemical analysis, using polyclonal antibodies raised against the recombinant CPI protein expressed in *E. coli*. The *Icy* mRNA is induced in response to anaerobiosis and cold shock (6 °C) and is depressed by light. The recombinant Hv-CPI protein is an effective inhibitor of several plant cysteine proteinases such as papain, ficin and chymopapain, but does not inhibit bromelain.

Developing endosperms from barley (*Hordeum vulgare* L. cv. Bomi), collected between 5 and 14 days after flowering (daf), were the source of the poly(A)⁺ RNA used to generate a λ ZAPII cDNA library (Mena *et al.*, 1998) with the Uni-Zap XR vector system (Stratagene).

Poly(A)⁺ RNA was obtained from developing barley endosperm and reverse-transcribed to cDNA with the primer 5'-TCGACTCTAGAGGATCCGAATTCAAGC(T)₁₅-3' and the polyAT tract kit (Promega). The cDNA was then PCR-amplified using as sense primer the oligonucleotide 5'-TCGCCCCGCTTCGCCGTC-3', derived from a conserved sequence in the coding region of the rice and maize cystatin cDNA clones

(Abe *et al.*, 1987a, 1992; Kondo *et al.*, 1990; see Figure 1) and as antisense primer the oligonucleotide 5'-CTGCAGGTCCGACTCTAGAGGATCCG-3', partially coincident with that used to prime cDNA synthesis. The reaction product was electrophoresed in 1% agarose gel, blotted onto a Hybond-N membrane (Amersham) and checked to hybridize with the insert of a cystatin cDNA clone from maize (CC-I), kindly provided by M. Abe. This amplified DNA fragment was cloned into the *Sma*I restriction site of the pUC18 vector and used as a homologous probe in the screening of the cDNA library. Positive cDNA clones were recovered in the pBluescript SK vector after *in vivo* excision.

Nucleotide sequences were determined with the ABI PRISM 377 dye terminator sequencing system and the ABI PRISM 377 DNA sequence analyser (Perkin Elmer-Applied Biosystems). DNA and deduced protein sequence analyses were done with the Gene Bee-Net Ver 1.0 (www.genebee.msu.su) computer facilities.

Southern and northern blot analysis

Total DNA was prepared from leaves of barley, wheat and wheat/barley addition lines after 7 days of germination (Sambrook *et al.*, 1989). DNA was restricted with appropriate endonucleases, separated on 0.8% agarose electrophoretic gels and transferred onto Hybond N membranes (Amersham). Hybridization was performed under stringent conditions following standard procedures (Sambrook *et al.*, 1989) with a specific barley cystatin probe of 198 nt derived from the 3' end of the cDNA clone (Figure 1).

RNA was purified from frozen tissues (developing endosperms, embryos, young roots and leaves) by phenol/chloroform extraction, followed by precipitation with 3 M LiCl (Lagrimini *et al.*, 1987). Total denatured RNA was electrophoresed in 0.8% agarose gels containing 5% formaldehyde, and blotted onto Hybond N membranes (Amersham). Hybridization and washings were done under stringent conditions, following standard procedures (Sambrook *et al.*, 1989). The same ³²P-labelled probe previously described for Southern blot analysis was used in northern blots.

To study the effect of anaerobiosis, young barley plants grown for 4 days at 25 °C were submerged in 10 mM Tris buffer pH 8 for 24 h. The cold shock treatment was performed on 4-day old plants grown at 25 °C, incubated for an additional 24 h period at

6 °C, under a normal 16:8 (light/dark) photoperiod. To assess the effect of light, 4-day old dark-grown seedlings were illuminated with continuous white light (Osram 18W/19 Tages light; 1200 lux) for 24 h at 25 °C and from seedlings kept in the dark for the same time. As controls, plants were grown under a 16:8 (light/dark or dark/light) photoperiod under identical light and incubation temperature conditions. After the different treatments, samples were harvested and frozen in liquid nitrogen until RNA extraction, as previously indicated. Quantification of radioactive signal on northern blots was carried out by densitometry of the filters with a Scanner UMAX (UC-840) Data System and the NIH computer program.

Expression and purification of a recombinant cystatin protein in E. coli

A 427 bp fragment, spanning the longest ORF (positions 98 to 525 in Figure 1) was amplified by PCR. The oligonucleotides used as primers were the forward primer 5'-CTTAGGATCCCATATGCCCGAAGCG-GCGCAT-3', which incorporated a *Bam*HI restriction site (underlined) upstream of the ATG initiation codon (doubly underlined) and the reverse primer 5'-CAGGTACCGTCGACATTACGCCAGTATCC-3', which added a tail with *Kpn*I (underlined) restriction site at the 3' end of the amplified fragment. The final PCR product was inserted in frame into the fusion expression vector pRSETA (Invitrogen), previously restricted with *Bam*HI and *Kpn*I endonucleases.

E. coli BL21 (DE3) pLysS cells containing the recombinant plasmid were grown at 37 °C to an OD₆₀₀ of ca. 0.4 and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h. The resulting fusion protein with a histidine tag was purified using a His-Bind Resin (Novagen) and the protein eluted from the Ni²⁺ column, following the manufacturer's instructions.

Preparation and purification of antibodies

Antibodies against the Hv-CPI fusion protein, with a N-terminal histidine tag, expressed in *E. coli* were raised in rabbits by Eurogentec (Brussels) according to their standard protocols. For purification of the monospecific polyclonal antibodies, the recombinant barley cystatin expressed in *E. coli* was separated by SDS-PAGE (Laemmli, 1970), transferred onto PVDF membranes (Millipore) and blocked for 1 h with TBS, 5% w/v non-fat milk and 0.1% v/v Tween 20. After

the membrane was incubated with 5 ml of antiserum diluted 1:10 v/v, side strips were cut and developed by using the immunoblot protocol. The rest of the membrane was realigned with the stained side strips, and the region corresponding to the cystatin band was cut out and the antibodies were eluted in 1 ml of 100 mM Gly pH 2.5 for 5 min. The eluted IgG fraction was neutralized with 1 M Tris-HCl pH 7.5 and stored in aliquots at -20°C until needed. Pre-immune sera were treated in the same way.

Western blot analysis and immunolocalization

Barley crude extracts were prepared by grinding frozen barley tissues (15 dap endosperms, 7-day old roots and green and etiolated 7-day old leaves) in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM NaCl, 20 mM DTT, 1 mM PMSF, 1 $\mu\text{g}/\mu\text{l}$ leupeptin and 5 μM pepstatin. After centrifugation, protein concentration was estimated in the supernatants using a protein assay kit (BioRad) and BSA as a standard. Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred onto PVDF membranes (Millipore). Immunological staining was done with anti-Hv-CPI purified antibody (1:50 v/v) for 90 min. After several washes, the second antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase, Sigma) was added at a dilution of 1:500 v/v and the colour was developed by the reaction of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, as previously described (Guerin and Carbonero, 1997).

For immunolocalization, barley tissue was harvested from roots (3 days) and from endosperms and embryos at 15 dap, and fixed immediately in 4% w/v paraformaldehyde, included in paraffin and cut into 8 μm sections using a microtome (Jung-Autocat model, Leica). The samples were treated with xylol and hydrated in an ethanol-to-water dilution series. Immunological detection was performed by incubation with the IgG purified fraction (1:2 v/v for roots and 1:4 v/v for endosperm and embryos), followed by incubation with the second antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase, Sigma). Colour was developed as previously described (Guerin and Carbonero, 1997) After dehydration in a water-to-ethanol dilution series, slides were mounted in Entellan mounting medium (Merck), viewed and photographed under an Axiophot light microscope (Zeiss).

Inhibitory activity

Inhibitory activity of the recombinant Hv-CPI protein purified from *E. coli* was tested against papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6), bromelain (EC 3.4.22.32) and ficin (EC 3.4.22.3), essentially as described by Abe *et al.* (1992). Protein concentration was quantified by using the BioRad kit with bovine albumin as standard.

The corresponding enzyme was pre-incubated at 37°C for 10 min with the Hv-CPI fusion protein in 100 mM sodium phosphate buffer, pH 6.0, containing 10 mM EDTA, 10 mM cysteine and 0.01% v/v Brij-35. After addition of 1.5 mM BANA (*N*-benzoyl-DL-arginine- β -naphthylamide) as substrate, and incubation at 37°C for 20 min, the reaction was stopped with 2% HCl in ethanol and the colour developed by adding 0.06% w/v *p*-dimethyl-aminocinnamaldehyde in ethanol. Absorbance was measured at 540 nm after 15 min at room temperature. Inhibition was expressed as % of remaining protease activity as compared with controls without inhibitors. K_i values were determined from Dixon plots (1/v versus [I]). The commercial cystatin from chicken egg white (CEW) from Sigma was used as a positive control in the inhibition tests.

Results and discussion

Characterization of a Hv-CPI cDNA clone

The presence in rice and maize kernels of cysteine proteinase inhibitors led us to search for their barley counterpart. To isolate such a gene, a barley cystatin probe was generated by RT-PCR using as template for cDNA synthesis poly(A)⁺ RNA from developing (15 dap) barley endosperm. As a sense primer, for the PCR amplification, a 17-mer oligonucleotide was derived from a conserved region of cereal cystatins (ARFAV, see Figure 1), and as antisense an anchored primer at the 3' poly(A)⁺ tail. Sequencing of PCR products identified a ca. 400 bp fragment with homology to rice and maize cystatins, which was used to screen at moderate stringency a λ ZAPII cDNA library from developing barley endosperm (Mena *et al.*, 1998).

A library sample representing 80 000 plaque-forming units was plated after infection of the *E. coli* strain XL1-Blue MRF. After transfer onto Hybond N membranes and hybridization with the ³²P-labelled barley probe described above, 60 positive plaques were identified and 3 of them further purified. Among

Figure 2. Dendrogram (parsimony unrooted tree) of the deduced amino acid sequence for Hv-CPI and of related plant cystatins. The whole sequences were used for the phylogenetic analysis. Nomenclature as in Margis *et al.* (1998).

the purified clones, one containing the longest insert was selected for further characterization. The DNA sequence of this insert of 598 nt (Figure 1) contains an ORF for a protein of 107 amino acid residues with a deduced molecular mass of 11 773 Da. This protein shares with all the PhyCys described so far the glycine (Gly-12) near the NH₂ terminus, the putative reactive domain QXVXG (Q⁶³V⁶⁴V⁶⁵A⁶⁶G⁶⁷) in the central part of the molecule and the A/PW (A⁹³W⁹⁴) motif near the COOH terminus (Arai *et al.*, 1991; Margis *et al.*, 1998). The Hv-CPI protein shares with rice oryzacystatin OC-II 69.1% identical (84.1% similar) residues along the whole protein, and is also related to oryzacystatin OC-I (61.6% identical, 79.1% similar amino acid residues) and to maize cystatins, CC-I and CC-II (52.9% identical, 66.2% similar; and 50.4% identical, 68.1% similar amino acid residues, respectively). No evidence for a signal peptide could be found in a computer search (signal peptide prediction server: www.cbs.dtu.dk/services/signalP/).

The phylogenetic dendrogram (Figure 2), based on comparisons of the phytocystatins whose whole sequences (either directly obtained or deduced from cDNA clones) are in the data banks, clearly indicates that the barley Hv-CPI is closely related to the rice OC-II (Osa2 in Figure 2) and less so to the four cereal and three *Arabidopsis* cystatins and to those described in carrot, papaya and ragweed. All of these belong to a well defined branch of the parsimony unrooted tree. The cystatins from potato constitute another separate branch, and a third one includes all the other phytocystatins described so far (Figure 2).

Figure 3. Southern blot analysis. a. Total DNA from barley cv. Bomi was digested with *Eco*RI (E), *Bam*HI (B), *Xba*I (X), *Kpn*I (K) and *Sac*I (S) and hybridized with the barley *Icy*-specific probe described in Figure 1. b. Chromosomal location of the *Icy* gene in wheat Chinese Spring/barley cv. Bétzès addition lines. DNA from Bétzès barley (B), Chinese Spring wheat (W) and addition lines carrying the indicated barley chromosomes (1H, 2H, 3H, 4H, 6H, 7H) were digested with *Xba*I and hybridized, as in (a) with the *Icy*-specific probe.

Gene copy number and chromosomal location of the *Icy* gene

Total DNA from leaves of barley cv. Bomi was digested with several restriction endonucleases and analysed by Southern blot under stringent conditions, using as a specific probe the 198 bp fragment spanning from the *Eco*RI site in position 400 to the 3' end of the cDNA clone (Figure 1). A single hybridization band was observed in all cases (Figure 3a), indicating that the Hv-CPI-encoding gene (hereafter *Icy* gene) is present as a single copy in the barley genome.

The chromosomal location of the *Icy* gene was determined by RFLP analysis of wheat/barley addition lines, after *Xba*I digestion and hybridization with the same specific probe used in Figure 3a. A three-band pattern was obtained for the hexaploid Chinese Spring wheat background which are indicative of three gene copies in the wheat genome (one per haploid genome),

while a single band of different mobility was found for the barley donor cv. Bétzès. The barley-specific band was detected only in the addition line corresponding to chromosome 2, indicating that the gene is located on this chromosome (Figure 3b).

Expression and abiotic induction of the *Icy* mRNA

The spatial distribution of *Icy* gene expression was examined among the major barley organs: endosperm, embryo, leaves and roots. Northern blot analysis was performed with the same specific probe as in Southern blot analysis (Figure 4). Total RNA was isolated from immature (20 daf) and mature embryos, endosperms collected at four different stages of development (10, 15, 20 and 25 daf), 7-day old roots and 7-day old leaves (Figure 4a). The barley cystatin transcripts were detected in all samples analysed but were more abundantly expressed in roots and seeds. Steady-state mRNA levels in embryos increased as maturation progressed, and in endosperms, accumulation started early (10 daf) in development. For charge checking, the same filter was hybridized with a probe corresponding to the 28S ribosomal RNA.

Response of the *Icy* gene to abiotic stresses was also investigated. As seen in Figure 4b, the level of this mRNA is induced in the dark. Its expression is also increased under anaerobiosis and during cold shock. About three-fold enhancement was observed, both in leaves and roots, after 24 h immersion in Tris buffer, and this induction was more dramatic when seedlings were incubated at 6 °C for 24 h: a six-fold increase in leaves and a close to four-fold increase in roots (Figure 4c). Although cold induction has been reported for cystatins from trees such as avocado or chestnut (Dopico *et al.*, 1993; Pernas *et al.*, 2000), this is the first time that a cold induction has been observed for those from cereals or other herbaceous plants. This is also the first time that a repression by light and an enhancement by anaerobiosis has been found in the expression of cystatin genes. Although the cold response of *Icy* was studied only in the spring cv. Bomi, it is pertinent to recall that other low-temperature-inducible genes, such as those encoding LTP4, were equally responsive in Bomi and in the winter cultivar Igri (Molina *et al.*, 1996). The functional characterization of the *Icy* promoter, which is now underway in our laboratory, will be helpful in elucidating if the abiotic inducibility of this gene depends on the *cis* motifs of the promoter and *trans*-acting factors or on the spring and winter habit of the cultivars.

Figure 4. Northern blot analysis of barley *Icy* expression. a. Total RNA (8 μ g) was extracted from 20 daf immature (iE) and from mature embryos (mE), from developing endosperms (En) at 10, 15, 20 and 25 daf, from 7-day old roots (R) and 7-day old leaves (L). The blot was first hybridized with the specific probe for *Icy* (Hv-CPI) and subsequently with a 18S ribosomal RNA for charge checking (rib). b. Total RNA (8 μ g) was extracted from leaves of 5-day old plants incubated for 24 h at 25 °C in continuous light (L), in the dark (D) or under a 16:8 dark/light (D/L) or 16:8 light/dark (L/D) photoperiod. c. Total RNA (8 μ g) was extracted from leaves (L) and roots (R) of 5-day old plants incubated for 24 h at 25 °C completely submerged in Tris buffer (An), or from leaves (L) and roots (R) of plants incubated for 24 h in the cold (6 °C).

Distribution and immunolocalization of Hv-CPI in barley

The recombinant Hv-CPI expressed in *E. coli* as a fusion protein with a histidine tail was purified as shown in Figure 5a. The induced bacterial lysate (lane 1) was fractionated into supernatant (lane 2) and pellet with inclusion bodies (not shown). The recombinant protein was purified to homogeneity from the supernatant. Proteins not retained in the Ni²⁺ column are shown in lane 3 and the eluted affinity-purified cystatin in lane 4. This protein was used to study its inhibitory

Figure 5. a. Control of the purification process of the recombinant Hv-CPI from *E. coli* cells by SDS-PAGE. 1, total protein extract from IPTG induced bacterial lysates; 2, proteins in the supernatant after discarding the pellet including inclusion bodies; 3, supernatant protein not retained in the Ni^{2+} column; 4, purified recombinant barley Hv-CPI after elution of the protein retained in the affinity column. b. Distribution of cystatin Hv-CPI in barley tissues by immunoblot analysis after SDS-PAGE of protein extracts from endosperm (E), roots (R), green leaves (gL) and etiolated leaves (eL). 80 μg of protein was loaded in each sample. Ec, 40 ng of recombinant Hv-CPI fusion protein purified from *E. coli* as control.

properties against several cysteine proteinases and for raising antibodies in rabbits for western analysis and for immunolocalization.

The cystatin distribution in barley tissues was investigated by immunoblot electrophoretic analysis (western blots), with the mono-specific polyclonal antiserum obtained as previously described. Immunoblots after SDS-PAGE of 80 μg protein extracts from the selected samples (Figure 5b) show that Hv-CPI is expressed in all tissues examined: endosperm, roots and leaves. In green leaves, the observed band has a higher apparent molecular mass (14 kDa) than that found in roots and endosperm (12 kDa); in etiolated leaves two bands appear. It remains to be investigated whether an alternative splicing occurs in green versus non-photosynthetic tissues, whether a second yet uncharacterized barley cystatin gene is expressed in leaves, and whether a post-translational modification event is responsible for the difference in electrophoretic mobility observed for the second band. The recombinant cystatin purified from *E. coli*, used as a control (lane Ec), has the mobility expected from its calculated molecular mass after including the

Table 1. K_i values of Hv-CPI from barley against papain, chymopapain and ficin.

Cystatins	K_i (M)		
	papain	chymopapain	ficin
Hv-CPI	2.0×10^{-8}	1.6×10^{-7}	2.2×10^{-8}
CC-I	2.3×10^{-8}	9.2×10^{-8}	8.3×10^{-8}
OC-I	3.2×10^{-8}	n.d.	n.d.
OC-II	8.3×10^{-7}	n.d.	n.d.

*In all cases BANA was used as substrate. Data from CC-I, OC-I and OC-II were obtained from the literature. n.d., not determined.

amino acid tail added to its NH_2 terminus to facilitate purification (16 037 Da).

The expression of the barley cystatin was further localized in different cell types within developing seeds (15 daf) and young roots (4 days old) by immunohistochemical analysis (Figure 6a–j). In developing seeds there were strong Hv-CPI signals, both in the embryo and throughout the endosperm cells that were also apparent in the assimilate-unloading tissues: the nucellar projection, vascular bundle and chalazal region (Figure 6d, e). In 4-day old roots the Hv-CPI label was detected in the rapidly dividing meristematic region and along the vascular strand, starting from about 4 mm from the root tip (Figure 6f). The pre-immune treatment gave no signal neither in endosperm or embryos, nor in roots (Figure 6g–i).

Inhibitory activity of Hv-CPI against plant cysteine proteinases

The recombinant Hv-CPI cystatin, expressed in *E. coli*, was assayed against various plant cysteine proteinases. It strongly inhibited papain and ficin and, to a lesser extent, chymopapain. K_i values of 2.0×10^{-8} M for papain, 2.2×10^{-8} M for ficin and 1.6×10^{-7} M for chymopapain were determined using BANA as substrate (Table 1). No inhibition was found against stem bromelain at the highest level tested (up to 10 $\mu\text{g}/\text{ml}$). This lack of inhibition of bromelain is common to several plant cystatins (Abe *et al.*, 1987b; Ievleva *et al.*, 1995; Ojima *et al.*, 1997). In all the experiments, the commercial inhibitor cystatin from chicken egg white (CEW) was used for comparison (Figure 7). The K_i value obtained for CEW against papain was 1.1×10^{-9} M, in agreement with data (1.5×10^{-9} M) reported by Abe *et al.* (1987b). Hv-CPI was a better inhibitor than CEW against chymopapain while the reverse was true against papain and ficin.

Figure 6. Immunolocalization of Hv-CPI in barley seeds and roots. Transverse sections of 15 daf developing endosperms (a, d, g), longitudinal sections of 15 daf embryos (b, e, h) and longitudinal sections of 4-day old roots were treated with toluidine blue stain (a, b, c) with the IgG fraction purified from antisera raised against the recombinant cystatin produced in *E. coli* (d, e, f) and with the preimmune IgG (g, h, i). al, aleurone; n, nucellar projection; v, vascular bundle; ch, chalazal region; en, endosperm; m, meristematic region.

The K_i value for the barley Hv-CPI towards papain is of the same order as those reported for rice OC-I (3.0×10^{-8} M) and maize CC-I (2.3×10^{-8} M) and was lower than that of the rice OC-II (8.3×10^{-7} M), all of them cereal cystatins (Kondo *et al.*, 1990; Abe and Arai, 1991; Abe *et al.*, 1994). In contrast, sequence data comparison and the dendrogram in Figure 2 indicate that rice OC-II is more closely related to Hv-CPI. Other plant cystatins inhibit papain with K_i values ranging from 10^{-7} to 10^{-9} M (Kouzuma *et al.*, 1996; Brzin *et al.*, 1998) while cystatins of animal origin seemed to be more inhibitory. However, data could not be strictly compared with those of plant origin because the experimental conditions were not identical (Schwabe *et al.*, 1984).

The K_i value of Hv-CPI against chymopapain is higher than that described for cystatins from maize, CC-I (9.2×10^{-8} M; Abe and Arai, 1991) and from papaya (4.3×10^{-8} M; Song *et al.*, 1995) and two-fold lower than that from chestnut (3.7×10^{-7} M; Pernas *et al.*, 1998).

Although few data on the inhibition of phytocystatins against ficin have been reported, the K_i value of Hv-CPI towards this enzyme is about four times lower than that published for maize CC-I (8.3×10^{-8} M; Abe and Arai, 1991) and four times higher than that of chestnut (0.65×10^{-8} M; Pernas *et al.*, 1998).

Although the physiological function of Hv-CPI cannot be inferred from the data reported here, it is pertinent to consider that two different types of cystatins have been reported in rice, maize and sunflower

Figure 7. Inhibitory activity of the recombinant barley cystatin purified from *E. coli* cells (■) and of the cystatin from chicken egg white, CEW (○), expressed as % of remaining activity of the enzyme without inhibitor. Concentration of cystatins are expressed as ng or µg/ml. BANA was used as substrate, and the starting absorbance was always 0.9 at 540 nm (300 ng/ml papain; 6 µg/ml chymopapain; 1.5 µg/ml ficin).

(Kondo *et al.*, 1990; Abe *et al.*, 1992; Kouzuma *et al.*, 1998). These two types of inhibitors could be involved in different physiological roles, due to their differences in inhibitory activity towards different cysteine proteinases. Current research in our group will help to discriminate if Hv-CPI could be a regulator of endogenous gibberelin-regulated cathepsin B-like enzymes (Cejudo *et al.*, 1992) and thus be involved in germination control, and/or if it is an active inhibitor of heterologous proteinases, such as those of insect pests and pathogens, and thus could be implicated in defence.

Conclusions

1. A barley cDNA clone encoding a cysteine proteinase inhibitor (*Icy* gene) has been characterized from a developing endosperm library.
2. The *Icy* gene, located on chromosome 2, is ubiquitously expressed. In seed, mRNA expression is enhanced as maturation progresses both in endosperm and embryo.
3. Anaerobiosis and cold shock (6 °C) markedly increased steady-state levels of the mRNA in roots and leaves, while light decreased these levels in leaves.
4. Immunohistochemical analysis shows that the protein accumulates in the vascular strand and in the meristematic region of roots and throughout developing endosperm and embryo as well as in the nucellar projection, vascular bundle and chalazal region.
5. The recombinant protein is an efficient inhibitor of papain and ficin and less so of chymopapain.

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