Isolation and promoter characterization of barley gene *ltr1* encoding trypsin inhibitor BTI-CMe: differential activity in wild-type and mutant *lys3a* endosperm

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The gene *ltr1*, encoding trypsin inhibitor BTI-CMe, has been obtained from a genomic library of *Hordeum vulgare* L. The gene has no introns and presents in its 5'-upstream region 605 bp that are homologous to the long terminal repeats (LTR) of the 'copia-like' retro-transposon Bare-1. Functional analysis of the *ltr1* promoter by transient expression in protoplasts derived from different barley tissues, has shown that in this system the *ltr1* promoter retains its endosperm specificity and the *trans-regulation* mediated by the *Lys3a* gene. The proximal promoter extending 343 bp upstream of the translation initiation ATO codon is sufficient to confer full *GUS* expression and for endosperm specificity. In protoplasts derived from the *lys3a* mutant, Ris₁, 1508, *GUS* activity was less than 5% of that obtained with the same constructs in the protoplasts of wild-type Bomi from which it derives. Gel retardation experiments, after incubation with proteins obtained from both types of endosperm nuclei, also show differential patterns. Possible reasons for these differences are discussed.

Introduction

Trypsin inhibitor BTI-CMe from barley endosperm is encoded by the gene *ltr1*, which is expressed during the first half of endosperm development [31]. In the high lysine mutant Ris01508, both the *ltr1* mRNA and the protein are present at <5% the levels found in its wild-type cv. Bomi [22, 31]. This mutation affects locus *Lys3a* on chromosome 5H [18]. The *Lys3a* locus also regulates *in trans* the activity of other barley endosperm-specific genes, including those encoding B- and C-hordeins, α -amylase and protein Z [1, 8, 14, 19, 20]. To understand the regulatory properties of the *Lys3a* gene it is of interest to characterize the promoters of genes under its control.

Functional analysis of genes from recalcitrant species can still not be achieved by stable transfor-

mation of these species with appropriate truncated promoter fusions. Although an increasing number of transformation protocols are being reported for cereals [2, 4, 42, 43], these are still difficult and time consuming. For this reason, monocotyledonous promoters have been often analysed in tobacco. Whereas temporal and spatial specificities were retained by some promoters in the alien background [27, 30], abnormal expression patterns have often been observed [7, 10, 28, 35]. An alternative approach is the transient transformation of homologous protoplasts, provided that reliable preparations can be readily obtained. This is the case of barley, where procedures for the reproducible isolation of viable endosperm protoplasts and for their transfection have been recently developed [5, 6, 23].

We report here cloning of *ltr1* gene and the func-

tional characterization of its promoter by transient expression in barley protoplasts, where it retains its two major features: endosperm specificity and *Lys3a*-mediated expression.

Materials and methods

Plant material

Barley (*Hordeum vulgare*) cv. Borni and its high-lysine mutant Ris01508 were germinated in the dark, vernalized at 4 °C for four weeks and grown in greenhouse at 18 °C under constant illumination.

Screening of a genomic library

A barley cv. Villa genomic DNA library in λ EMBL4 [7] was screened by standard procedures [32] using the insert in the cDNA clone for trypsin inhibitor BTI-CMe, λ ACMe, as a probe [31]. Prehybridization was at 65 °C for 2 h, in 5x SSPE, 5x Denhardt's solution, 0.1% SDS, 100 μ g/ml dextran sulphate and 100 μ g/ml salmon sperm DNA. Hybridization was done for 16 h at the same temperature. The membranes were washed twice with 2x SSPE, 0.1% SDS for 10 min at room temperature, followed by washes at 65 °C with 1x SSPE, 0.1% SDS for 15 min and 0.1x SSPE, 0.1% SDS for 10 min. One positive clone, λ AgCMe, was selected for further study, and an appropriate subfragment of the insert in that clone was subcloned into pBluescript SK(-) vector. Series of overlapping fragments of the insert were prepared by exonuclease restriction and digestion with endonuclease III [32], and sequenced by the Sanger dideoxy-chain termination method [33].

PCR amplification of proximal promoters

The proximal promoter regions of the *ltr1* gene in the barley cvs. Borni and Ris01508 were obtained by PCR amplification using *Taq* polymerase, 250 ng of the corresponding genomic DNA prepared by the cetyl trimethyl ammonium bromide (CTAB) method [40] and 25 pmol of the oligonucleotides 5'-ACTCATGCCAAGCACAC-3' (2088 to 2104 in λ AgCMe, Fig. 1) and 5'-AGAATGGCGAGCATGAC-3' (2446 to 2430 in λ AgCMe, Fig. 1). After 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, a unique product of 360 bp was obtained with both DNAs. This PCR-product was gel purified and cloned

in the M13mp18 phage vector for nucleotide sequencing [32, 33].

Primer extension analysis

A 17-mer synthetic oligonucleotide, 5'-AGAATGGCGAGCATGAC-3' (2446 to 2430 in λ AgCMe, Fig. 1), complementary to the coding strand near the ATG-translation initiation codon, was used to prime the reverse transcriptase reaction, using 5 μ g of poly(A)+ RNA which had been heated at 90 °C for 3 min and cooled down to room temperature during 30 min. Reaction conditions with reverse transcriptase were as previously described [32].

Plasmid constructs for transient expression

A series of *ltr1* promoter fragments were obtained spanning in its 3' end from the first *HaeIII* restriction site after the ATG translation initiation codon (position 2422 in λ AgCMe, Fig. 1) to the following restriction sites in their 5' ends: *Sall* for construct -2369, *KpnI* for -1969, *HaeIII* for -816; *SphI* for -343 and *PvuII* for -83 (numbering refers to the distance to the translation initiation codon, see Fig. 1). These fragments were subcloned into the *SmaI* site of the polylinker in pHW9, a β -glucuronidase coding region (GUS) containing plasmid, producing an in-phage fusion with the reporter gene [16].

Constructs -289, -241, -211, -179 and -113 (Fig. 1) were prepared from the -343 by treatment with exonuclease III. The 5' ends of all the constructs were established by DNA sequencing [33].

Transient gene expression in protoplasts

Protoplasts were isolated from developing endosperm (10-15 days after pollination, dap), mature aleurone, young leaves and roots from barley cvs. Borni and Ris01508, following procedures developed in our lab [5, 6]. Plasmid DNA (30 μ g per 10^6 protoplasts) was transfected into endosperm and aleurone protoplasts, using the polyethylene glycol (PEG) method [6]. Protoplasts derived from leaves and roots were electroporated [5] with 20 μ g of plasmid DNA per 10^6 protoplasts, using a Electro Cell Manipulator 600 BTX (BTX electroporation system) at 625 V/cm (leaves: 200 μ F, 10-15 ms; roots: 500 μ F, 20-25 ms). Transfected protoplasts were incubated for 22 h at 27 °C in the dark until sampling for GUS determination [16].

r-+2ss9
 GTCGACGCATGTGTCTCATCTCATrCTGAAACGTGTGGTGTGAGACggttggaatatgccctagaggaatgataaatagtattattattattcttctgtt 100
 -S;IT-
 tcaagataatcatttattattcatgtctataattatattgaatgaaaacatagatcacatgtgtgaatacattgacgaacaatgtccttagcaagcctcta 200
 gttgctagccagttgatcaaggatagtcagggttttctgactatgtgcaagtgtgtcacttgataaactggatcacatcattaggagaatcatgtgatg 300
 gactagacccaaactatgaatgtagcatattgategtgtcattttgttctattgttttctgcgtgtcaagattttattcctatgaccatgagatcatat 400
 aactcactggtaccggaataccttgtgtgtatcaaacgtcgcaacgtaactgagtactataaagggtctctacaggtatctctgaaggtgtccgtt 500
 Kpn I
 gaggtagtatggatcaagactaggatttgcactccgtgtgacgtagaggatctcteggeccactcggtataacaacatcacacacaagccttgcaagcaa 600
 tgtgactaagtttaagtcaagggtatctgtattaccgaacgagaaaaagagA T JATTrCTT TGTAGAGCACCTCACAAC TAGACGG 700
 GCCCTGAAACACCTTACTTATAITTAGTATACAAAATGTTTGTCTGAAACAATACGTGAGTTGCAGAAAACGAAGCGACTTCGATAGACGCAGGAACA 800
 CACGATGGAACGACCACCGAGCCGAAGGACGCGCCGCGATCGGTGGT ACTAGATAATATTTrCCCCAAAGATAATG TG, CJ A gTj BCIIAT; 900
 AGAAGTACTATACATACGATACGATACGACATATCATACCAAATCGTATGCCAAATTTATCGTATATAGAGTAATTCGGTTTCTTTTTTGTCCTGGT 1000
 GGATCTGAAATAACCACTTTCTGGAAAATCTTGGAAAATTTAGGTTTTTCTGCCCGAATGAATTTAAAGGAACTTTGAAATTTGATTTTTT-TGCT GTGTGA 1100
 AAATATATATAAAGACGCTAACCTACTGACTAACGTAACCTCCCTCATGCTCGACT GATCGGTTGGGTACACTCTTTTCGCCCGCCAGGTCATGAGATTGGA 1200
 TCCTCTCGATCGGTTGCAGACATGATCTCGACTCATAAACCAAACGAGAGTTTACTTTGAAGTGCTAGATTACACTACAAAT AAACATGGAAGAGAATGAC 1300
 ATGCAGATGAAC TAGGGATAATTTCTGTTATCTCTTTTCGTAGGGTTCTTCTTTGAACCTCGACTGGCAGCATGGGATGAAGTACTCACTCGTTTGAGGGCT 1400
 CGTTCAGCTGTCAATGGAT CTAGTAGATTTCTTTTCGTAGAGCCCTCACAACAGAGCGGCCCGAAACCTTATATATCCAGTATAAAAAAT 1500
 GTCTGTCTGAAACAATACGTGAGTTTGAGGAAA CGAAAGCGGCTTCGATAGACGCAGGAAACACGAAACGAAACGCGCCGCGATGGTGGTACTAGA 1600
 Hdi/II
 TAATATTTTCCAAAAGATAATGCAJGGATGCTACTAGATTTTTT CAGAAGTACTCTGCATACGATACGATATGACATATCATACAAAATCGTATGCCA 1700
 AATATCGTATTTAGAGTTAATTATGTTTCTTT' FTTGTTCGAGGGATCTGAAATAACCGGTTTrCTGGAAAATCTTGTAAATTCAAGGTTTTTCCGCCCGA 1800
 ATGAATTTAAACAAACTTTGAATTT GATTTTTTTCAGTGTGAAAATATATATAAAGATGCTAACCTACTGACTAACGTAACCTCCCTCATGCTCGACTGA 1900
 TCGGTTGCGTAGACCTGTTCGCCCGCCAGGTCATGAGATTGGATCCTCTCGGCTGCAGACATGATCTCCACTCATAAAGCAAACGAGAGTTTACTTGAAG 2000
 TGTAGATTACACATTTACACTACGAATAAACATGGAAGAGAATG AGATGAACCGGGATGTAGCGTAGTATGATAAGATAACTCATGCCAAGC 2100
 Sph I
 r-289 r-241 r-211
 AACTGAGAAAGGTAGGAACCGAGGAATATGTTATTCTTCTACTCAACCCCACTTTT GCATTCCCTCCGATTTTTCAGCACAAGCTCCAAAT 2200
 TCTGTACGAAAAAGGTTATCAAAGCTACCCCTTTAATCAAAATTTCCATGCAGCGGCACCTTCAACAACCTAACAGAAAGCTCAGAAAGCACTTCGG 2300
 ACGATGCCAGCTGTGTGCTACTTACATTTTAC ATATATAT CATGTGTACGCCTCGCTTGTCCAAACATCCCTTCAATTTGAT ACAAATGGCGT 2400
 PiiiJ7r Hae III M A
 TCAAGTACCAAGCTTCTCTCTG CGGC' CGCGGTATGCTCGCCATTCTCGTCGCCACTGCCACCAGTTTCGGGGATTCGTGTGCTCCAGGGGATGCGTTGCC 2500
 F K Y Q L L L S A A V M L A I L V A T A T S F G D S C A P G D A L P
 ACACAACCCCTCTCAGAGCCTGCGCGACCTACGTGTCAGTCAAATCTGCCACCAAGGCCCCAGACTCTCACCCTCGGACATGAAGAGGCGGTGTTCGCAC 2600
 H N P L R A C R T Y V V S Q I C H Q G P R L L T S D M K R R C C D
 GAGCTGTGCGCCATCCCGCGTACTGTAGGTGCGAAGCGCTGCGTATCATATGCAAGGGGTAGTAACCTTGGCAGGGTGCCTTTCGAGGGTGCTACTTCA 2700
 E 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
 AGGACTGCGCCCACTGCCCTAGGGAGAGGCAAACGAGCT ACGCCGCCAACCTCGTCACCCCGCAGGAGTGCAACCTAGGGACCATCCACGGCAGCGCGTA 2800
 K D S P N C P R E R Q T S Y A A N L V T P Q E C N L G T I H G S A Y
 CTGCCCGAACTGCAGCCCGGATGGAGT GGTG' TGTATAAGT TCTAACGACTAGCTCGATCATGAATAAGCATGGT GCATCGATCAATGGACCAATC 2900
 C P E L Q P G Y G V V L
 GATTGTATGCATCTATGCCCATGTGTGATGTGTGTTAAATCGT! AATAA GAGCATCAGAGTATTTTCTAGTATGTGTCAAATGTTTGGCGGTAATCAAC 3000
 CACCGAGCATGATACCCCGAAACCTTACCCTCTCCGGTGCCGCCACCCGTGAGGCGGCGGGGAGGAATTC 3071

Fig. 1. Nucleotide sequence of the barley */r I* gene (*SaI*-*EcoRI* restriction fragment of genomic clone ,gCMe). The putative TATA box, flanked by inverted repeats, and the polyadenylation signals are boxed. The CAAAAT and CATCC motives are indicated with a double underline and a broken overline respectively. The endospem-like motif in the non-coding strand is indicated with a broken underline. Putative bZIP-binding sites in the proximal -343 promoter are underlined. The transcription start site is indicated with a downward arrowhead and the polyadenylation point with a vertical arrow. The 605 bp sequence homologous to the LTR of the BARE-1 retroposon 1261 is in lower case and the two long repeats that follow are indicated with horizontal arrows over sequences in capitals. The wavy lines show the 24 bp element present also in the *Ac* transposable element of maize [29]. Bent arrows indicate 5'-end points of deletions for promoter function analysis. Selected direct and inverted repeats in the proximal promoter region are indicated by horizontal arrows. The deduced amino acid sequence, shown by single capital letters below the translated nucleotide sequence, coincides with that reported for the cDNA [31]. Various endonuclease restriction sites are indicated.

Protein content was determined using the BioRad kit with bovine serum albumin as standard.

Preparation of nuclear extracts

Nuclei and nuclear extracts were prepared from developing barley endosperms (10-15 dap) essentially as described by Luthe and Quatrano [24]. 5-10 g of frozen tissue ground in liquid N₂ and homogenized at 4 °C for 15 min in nuclei extraction buffer (0.44 M sucrose, 2.5% Ficoll, 5.0% Dextran 40, 25 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 mM spermine, 0.5 mM spermidine and 0.5% Triton X-100) followed by filtration through cheesecloth and a 64 µm nylon mesh, and centrifugation at 4000 × *g* for 5 min. The pellet was resuspended in nuclei extraction buffer without Triton X-100, Ficoll and Dextran 40, and was layered on a discontinuous Percoll gradient (80%, 60%, 40% w/v Percoll) over a 2 M sucrose cushion. After centrifugation at 4000 × *g* for 30 min, the nuclei interface was washed in 1 ml of buffer (0.44 M sucrose, 25 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 0.2 mM PMSF), centrifuged at 4000 × *g* for 5 min and stored at -70 °C in the same buffer with 50% glycerol.

The stored nuclei were pelleted by centrifugation at 4000 × *g* for 10 min and resuspended in the lysis buffer described by Jensen *et al.* [17] for 2 h at 4 °C. Nuclear debris was removed by centrifugation (15 000 × *g*, 15 min) and the supernatant dialysed for 2 h against buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT, containing 2 µg/ml of each of the following proteinase inhibitors: antipain, leupeptin, pepstatin A and aprotinin). Aliquots were frozen in liquid N₂ and stored at -70 °C. Yields of about 7 µg protein per gram of developing barley endosperm were obtained.

Gel retardation assays

The *ltrl* gene sequence spanning from the restriction sites *SphI* at position -343 and *HaeIII* at position +30 (see Fig. 1) was digested with *SspI* and *PvuII* and the three resulting fragments were end-labelled with (α³²P)ATP using the Klenow enzyme and purified from 6% polyacrylamide gels. 0.2-0.5 µg of labelled DNA (50 000 cpm) were incubated with 15 µg of nuclear extracts and 1 µg poly dI/dC as a non-specific competitor, in 30 µl of binding buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM

EDTA, 5% glycerol. Reaction mixtures were incubated for 30 min at 25 °C and analysed after 4% polyacrylamide gel electrophoresis and autoradiography. Competition assays were carried out with the appropriate unlabelled DNA fragments [38, 39].

Results

Isolation and sequence of the ltrl gene

The screening of a genomic library from barley cv. Villa, in the λEMBL4 vector, using BTI-CMe cDNA as a probe [31], gave a positive recombinant phage, λgCMe, with an insert of about 15 kb. A *Sall/EcoRI* (internal) fragment of ca. 3 kb from this insert that hybridized with the probe was sequenced. As shown in Fig. 1, this fragment contained the entire coding and 3'-untranslated regions of the gene and 2369 bp upstream of the translation initiation codon. The gene has no introns and the coding sequence is identical to its corresponding cDNA [31]. The TATA box (at position -60) is flanked by inverted repeats, TCACAT. One of these repeats is part of an 11 bp motif, TTCA-CATTTCA, which is located in an equivalent position in the protein Z gene [1]. Sequences resembling the CATC box of prolamine promoters [9, 36] are found at positions -134 and -201. The CAAAAT motif at position -155 appears also in the barley B-hordein and protein Z promoters [1, 9, 14, 19], as well as in those of Opaque 2 and zein genes from maize [10, 11, 25, 41]. Motif AATGCAAAA, which is related to the canonical endosperm box [36], is present in the non-coding strand between positions -228 and -236. Several potential binding sites for transcription factors of the bZIP class [15] are present up to position -343 (see Fig. 1).

There are two long repeats (88% identical) of approximately 660 bp each, separated by 93 bp, upstream of position -343. Each repeat includes two copies of a 24 bp element (wavy lines in Fig. 1) that is present also in the 3'-untranslated region of the *Ac* transposable element of maize [21, 29]. About 40 copies of the repeat are dispersed throughout the barley genome (data not shown). Additionally, just upstream of the two direct repeats, there is a 605 bp region that is homologous (ca. 90% identical nucleotides) to the 5' terminus of the long terminal repeat (LTR) from barley retrotransposon BARE-1 [26].

Table 1. Average *GUS* specific activity on barley protoplasts derived from developing endosperms, aleurone, leaves and roots of cv. Bomi, transiently transfected with chimeric constructs derived from the *ltrJ* promoter fused to the *β*-glucuronidase reporter gene¹.

Constructs	Endosperm ²	Aleurone ²	Leaves ³	Roots ³
-2369	316 ± 61	1.3 ± 0.3	80 ± 1.3	6.5 ± 0.9
-83	55.6 ± 9.7	7.3 ± 2.4	15.1 ± 4.0	3.8 ± 1.1
35S	776 ± 125	185 ± 66	827 ± 172	409 ± 46

¹Data are the average values of three different experiments (with their standard deviations) expressed as pmol 4-MU per minute per mg protein.

²Transfected by the PEG method.

³Transfected by electroporation.

Fig. 2. Transcription initiation of the *ltrI* gene by primer extension analysis. Oligonucleotide 5'-AGAATGGCGAGCATGAC-3' (positions 2446 to 2430 in Fig. 1) was used for priming the reverse transcriptase reaction using as templates either total RNA (t) or poly(A)+ RNA (+) from developing barley endosperm. The 5' end of this reaction (horizontal arrows) was determined by electrophoresis of the primer extension products, next to the sequencing reactions (G, A, T, C) of the genomic clone primed with the same oligonucleotide.

Transcription initiation

The mRNA transcription start site of the *ltrI* gene has been determined by primer extension analysis using poly(A)+ RNA from barley developing endosperms from both cv. Bomi and its mutant Ris01508. Previously, the proximal promoter regions of the *ltrI* gene were isolated by PCR amplification and confirmed by sequencing to be identical to that contained in the genomic clone *Δ*gCMe described here. The major putative transcription start sites for cv. Bomi were adenosines at positions -22, -23, -25 and -26 (Fig. 2). The same transcription start points were detected in the Ris01508 mutant, although the gel bands were fainter (data not shown), corroborating that transcription in the mutant is correct but occurs at a lower rate [31]. It can be assumed that position

-26 is the *in vivo* transcription start, and that the others are likely to be premature terminations of reverse transcription *in vitro*.

Functional analysis of the promoter

In an initial experiment, fusions of the *GUS* reporter gene to the complete *ltrI* promoter (-2369) and to its minimal version (-83) were subcloned into the polylinker of the pUC 19 plasmid and introduced into protoplasts from leaves, roots, aleurone and developing endosperm from barley cv. Bomi, using a *GUS* fusion to the CaMV 35S promoter as control. As summarized in Table 1, both *ltrI* constructions gave endosperm-specific expression, although the enzyme activity was much higher with the complete promoter. Additional fusions were obtained with promoter fragments whose 5' ends respectively extended to positions -1969, -816, -343, -289, -241, -211, -179, and -113, and transiently expressed, together with those previously obtained, in endosperm protoplasts from cv Bomi and from its mutant Ris01508 (Fig. 3). Deletions of the promoter up to position -343 did not affect expression of the reporter. Expression directed by the truncated promoters -289, -241, -211, -179 and -113 was about 50% of that of the complete promoter (-2369), indicating the presence of a cis-acting sequences between positions -343 and -289 affecting quantitatively the expression of the *ltrI* gene. The expression driven by the minimal promoter fragment assayed (-83), which contained the TATA box, was 20% that of the full promoter, again indicating the existence of positive cis-elements between positions -113 and -83 and downstream from the latter. All the gene constructions were only marginally active (<5% of maximal expression in the wild type) in the mutant protoplasts (Fig. 3).

Fig. 3. Functional analysis of the *ltr1* promoter by transient expression in barley endosperm protoplasts. **a.** Schematic representation of the genomic clone for *ltr1*. Restriction endonuclease sites for *Sall* (S), *KpnI* (K), *HaeIII* (H), *SphI* (Sp), *PvuII* (P) are indicated. The coding region is hatched. Chimeric constructs were obtained by in-phase fusion to the *GUSINOS* reporter gene, of several fragments of the promoter spanning in its 5' end to the indicated restriction sites (1=S; 2=K; 3=H; 4=Sp; 10=P) or to different *ExoIII* digestion stop points (5, 6, 7, 8, 9). **b.** *GUS* specific activity (pmol MU per minute per mg protein) after transfection of endosperm protoplasts derived from barley cv. Bomi (white bars) or from its mutant Ris01508 (hatched bars) with the chimeric constructs described in **a.** The CaMV 35S constitutive promoter was used as a positive control. Background values obtained with the promoter-less *GUS* plasmid was subtracted from all data. The average of four different experiments with their standard errors are represented. Insert: developing endosperm protoplasts from barley cv. Bomi.

To ascertain that the Bomi and Ris01508 endosperms were at the same developmental stage, a construct driven by the promoter of the sucrose synthase *SsI* gene that is not under *Lys3a* control was used to transfect endosperm protoplasts of both types and the same *Gus* specific activity was obtained both in

wild-type and mutant protoplasts ($\bar{x} = 900 \pm 130$ pmol MU per mg protein per minute).

Binding of nuclear proteins to promoter fragments

Gel retardation assays were carried out with nuclear proteins isolated from developing barley endosperms

a

SphI	SspI	PvuII	HaeIII
-343	-211	-83	+28

b

Fig. 4. Gel retardation analysis of the *ltrI* proximal promoter. **a** Restriction fragment *SphI/HaeIII*, spanning from nt -343 (upstream of the ATG codon) to position +28 of the coding region, was separated into the three indicated fragments after digestion with restriction endonucleases *SspI* and *PvuII*. **b** Each ³²P-labelled DNA fragment was incubated with crude protein preparations derived from endosperm nuclei of cvs. Bomi (B) or Ris1508 (R) and the mixture was analysed by gel electrophoresis and autoradiography. Lane (-) contains the appropriate ³²P-labelled DNA-fragment without protein. Arrowheads indicate differentially retarded bands in the wild type and in the mutant.

from wild type cv. Bomi and mutant Ris1508. Based on the functional analysis, the 350 bp *SphI-HaeIII* region reaching up to position -343 (Fig. 1) was digested with *SspI* and *PvuII* restriction endonucleases, producing fragments *SphI-SspI* (127 bp), *SspI-PvuII* (133 bp) and *PvuII-HaeIII* (90 bp), corresponding to the three regions of the promoter associated with changes in its activity. The three fragments were ³²P-labelled and incubated with endosperm nuclear extracts. Differential retardation bands (present in cv. Bomi and absent in Ris1508) were detected for each of the three fragments (Fig. 4), although for the proximal fragment a weak trace of the differential band was barely detected in the mutant.

The binding specificity was demonstrated in all cases by competition titrations with the respective unlabelled DNA fragment at 20 x concentrations (data not shown).

Discussion

The 343 bp upstream of the ATG initiation codon of the gene *ltrI* contain all the information required for full activity and for endosperm specificity, whereas the region further upstream (up to -2369) seems to be of no relevance for promoter function. The most significant feature of this 2 kb dispensable region is the presence of a BARE-1 LTR fragment. Complete LTRs are 1.8 kb long, but this one is interrupted by two tandem, unrelated repeats of 660 bp, which may explain why it does not have any enhancing effect, in contrast to what has been reported in other cases [26]. Partial BARE-1 LTRs have been reported previously in the promoter regions of other barley genes, such as those encoding acyl carrier protein ACP III [12] and nitrate reductase [37]. The gene *ltrI* is part of a very divergent multi-gene family that is dispersed over several chromosomes and encodes both protease and α -amylase inhibitors [3]. The presence of a BARE-1 fragment near the gene suggests that this element might have been involved in the alluded dispersal.

Promoter activity gradually decreases with the extent of the 5' deletions and the -83 minimal promoter still retains 20% of the maximum activity. This is in agreement with the evidence of binding of nuclear factors to the distal, central and proximal promoter segments in gel retardation experiments. All constructions tested gave similar low level of activity in mutant protoplasts, under conditions in which the CaMV 35S promoter showed normal function, and all of them, including that with the minimal -83 promoter, gave differential expression in wild-type protoplasts. This suggests that the proximal -83 region is involved in the interaction affected by the *lys3a* mutation, but does not exclude the involvement of other promoter regions in the effects of the mutation. Indeed, differential retardation bands have been observed for each of the three promoter segments, which would point to the latter possibility. We have previously shown that a single dose of the wild-type *Lys3a* allele, in the triploid endosperm, completely restores the expression of the *ltrI* gene [31], which indicates the lack of a functional positive factor in the mutant genotype. This factor could play one of the following roles: (1) activate the expression of genes

encoding the transcription factors that would bind to the different promoter regions; (2) mediate binding of different transcription factors to these regions; (3) directly bind to multiple sites along the promoter, either alone or as a heterodimer. These alternatives have not been yet investigated, but there are some suggestions concerning the third one. The *opaque2* mutation in maize has very similar functional effects to those of the *lys3a* in barley, and it has been shown that the *Opaque2* gene encodes a transcriptional activator of the bZIP class [13, 34]. Multiple putative binding sites for this class of factors are distributed along the *ltr1* promoter. In the distal, *SphI-SspI* fragment, there are three such sites, *TAGTA*, *AACTC*, and *CACTG*, between positions -343 and -289, which would be congruent with the decrease of activity observed when the segment between these positions is deleted (Fig. 3). In the central, *SspI-PvuII* fragment, sites *CACCT* and *TACTT* are present between positions -113 and -83, and an additional *CAGCTG* site coincides with the *PvuII* site (Fig. 1). Again, an activity decrease is associated with the deletion of the segment between positions -113 and -83 (Fig. 3). Another three potential bZIP-binding sites, *CACTA*, *CACCT*, and *ACATGT*, are located in the *minimal -83* promoter (Figs. 1 and 3).

Further analysis of the cis-regulatory elements of the *ltr1* promoter, and of the corresponding protein factors that interact with them, will be required to discriminate between the above alternative hypothesis.

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1. Brandl A, Svendsen I, Hejgaard J: A plant serpin gene. Structure, organization and expression of the gene encoding barley protein Z₄. *Eur J Biochem* 194: 499-505 (1990).
2. Castillo AM, Vasil V, Vasil IK: Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). *Bio/technology* 12: 1366-1371 (1994).
3. Carbonero P, Salcedo G, Sanchez-Monge R, García-Maroto F, Royo J, Gomez L, Mena M, Medina J, Diaz I: A multigene family from cereals which encodes inhibitors of trypsin and heterologous α -amylases. In: Aviles FX (ed) *Innovations on*

- Proteases and their inhibitors*, pp. 333-348. Walter de Gruyter, Berlin/New York (1993).
4. Christou P, Ford TL, Kofrar M: Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties by electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/technology* 9: 958-962 (1991).
 5. Diaz I: Optimization of conditions for DNA uptake and transient GUS expression in protoplasts from different tissues of wheat and barley. *Plant Sci* 96: 179-187 (1994).
 6. Diaz I, Carbonero P: Isolation of protoplasts from developing barley endosperm: a tool for transient expression studies. *Plant Cell Rep J O*: 595-598 (1992).
 7. Diaz I, Royo J, O'Connor A, Carbonero P: The promoter of the gene *ltr1* from barley confers different tissue specificity in transgenic tobacco. *Mol Gen Genet* 248: 592-598 (1995).
 8. Entwistle JE: Primary structure of a C hordein gene from barley. *Carlsberg Res Commun* 53: 247-258 (1988).
 9. Forde BG, Heyworth A, Pywell J, Kreis M: Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. *Nucl Acids Res* 13: 7327-7339 (1985).
 10. Gallusci P, Salamini F, Thompson RD: Differences in cell type-specific expression of the gene *Opaque-2* in maize and transgenic tobacco. *Mol Gen Genet* 224: 391-400 (1994).
 11. Grasser KD, Maier U-G, Haass MM, Feix G: Maize high mobility group proteins bind to CCAAT and TATA boxes of a zein gene promoter. *J Biol Chem* 265: 4185-4188 (1990).
 12. Hansen L, von Wettstein-Knowles P: The barley genes *Acll* and *Ac3* encoding acylcarrier proteins I and III are located on different chromosomes. *Mol Gen Genet* 229: 467-478 (1991).
 13. Hartings H, Maddaloni M, Lazzaroni N, Di Fonzo N, Motto M, Salamini F, Thompson R: The *O2* gene, which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. *EMBO J* 8: 2795-2801 (1989).
 14. Hopp HE, Rasmussen SK, Brandl A: Organization and transcription of B1 hordein genes in high lysine mutants of barley. *Carlsberg Res Commun* 48: 201-210 (1983).
 15. Izawa T, Foster R, Chua NH: Plant bZIP protein DNA binding specificity. *J Mol Biol* 230: 1131-1144 (1993).
 16. Jefferson RA: Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387-405 (1987).
 17. Jensen ES, Marcker KA, Schell J, Bruijn FJ: Interaction of a nodule specific trans-acting factor with distinct DNA elements in the soybean leghaemoglobin *lbc3* 5' upstream region. *EMBO J* 7: 1265-1271 (1988).
 18. Karlsson KE: Linkage studies on the *lys* gene in relation to some marker genes and translocations. In: Gaul H (ed) *Barley Genetics III*, pp. 536-541. Verlag Karl Thiemig, Munich (1976).
 19. Kreis M, Shewry PR, Forde BG, Rahman S, Mifflin BJ: Molecular analysis of the effects of the mutant *lys 3a* gene on the expression of *Hor* loci in developing endosperms of barley (*Hordeum vulgare*). *Cell* 34: 161-167 (1984).
 20. Kreis M, Williamson M, Buxton B, Pywell J, Hejgaard J, Svendsen I: Primary structure and differential expression of β -amylase in normal and mutant barleys. *Eur J Biochem* 169: 517-525 (1987).
 21. Kunze R, Stochaj V, Laufs J, Starlinger P: Transcription of transposable element *Activator (Ac)* of *Zea mays* L. *EMBO J* 6: 1555-1563 (1987).

22. Lazara A, Barber D, Salcedo G, Mendez E, García-Olmedo F: Differential effects of high lysine mutations on the accumulation of individual members of a group of proteins encoded by a disperse multigene family in the endosperm of barley (*Hordeum vulgare* L.). *Eur J Biochem* 149: 617-623 (1985).
23. Lee BT, Murdoch K, Topping J, Jones MGK, Kreis M: Transient expression of foreign genes introduced into barley endosperm protoplasts by PEG-mediated transfer or into intact endosperm tissue by microprojectile bombardment. *Plant Sci* 78: 237-246 (1991).
24. Luthe DS, Quatrano RS: Transcription of isolated wheat nuclei. Isolation of nuclei and elimination of endogenous ribonuclease activity. *Plant Physiol* 65: 305-308 (1980).
25. Maier U-G, Grasser KD, Haass MM, Feix G: Multiple proteins bind to the P2 promoter region of the zein gene pMS1 of maize. *Mol Gen Genet* 221: 164-170 (1990).
26. Manninen L, Schulman AH: BARE-1 a copia-like retroelement in barley (*Hordeum vulgare* L.). *Plant Mol Biol* 22: 829-846 (1993).
27. Marris C, Gallois P, Copley J, Kreis M: The 5' flanking region of a barley B hordein gene controls tissue and developmental specific CAT expression in tobacco plants. *Plant Mol Biol* 10: 359-366 (1989).
28. Matzke AJM, Stoger EM, Matzke MA: A zein gene promoter fragment drives GUS expression in a cell layer that is interposed between the endosperm and the seed coat. *Plant Mol Biol* 22: 553-554 (1993).
29. Pohlman RF, Federoff NV, Messing J: The nucleotide sequence of the maize controlling element *Activator*. *Cell* 37: 635-643 (1984).
30. Roberts LS, Thompson RD, Flavell RB: Tissue-specific expression of a wheat high molecular weight glutenin gene in transgenic tobacco. *Plant Cell* 1: 569-578 (1989).
31. Rodríguez-Palenzuela P, Royo J, Gómez L, Sánchez-Monge R, Salcedo G, Molina-Cano JL, García-Olmedo F, Carbonero P: The gene for trypsin inhibitor CMe is regulated in *trans* by the *l vs 3a* locus in the endosperm of barley (*Hordeum italicum* L.). *Mol Gen Genet* 219: 474-479 (1989).
32. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
33. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-5467 (1977).
34. Schmidt RJ, Burr FA, Aukerman MJ, Burr B: Maize regulatory gene Opaque-2 encodes a protein with a 'leucine-zipper' motif that binds to zein DNA. *Proc Natl Acad Sci USA* 87: 46-50 (1990).
35. Schubert R, Panitz R, Mantuffel R, Nagy L, Wobus U, Baumlein H: Tissue-specific expression of an oat 12S seed globulin gene in developing tobacco seeds: differential mRNA and protein accumulation. *Plant Mol Biol* 26: 203-210 (1994).
36. Shewry PR, Tatham AS, Field JM, Forde BG, Clark J, Gallois P, Marris C, Halford N, Forde J, Kreis M: The structures of barley and wheat prolamins and their genes. *Biochem Physiol Pfl* 183: 117-127 (1988).
37. Shnorrr KM, Juricek M, Culley D, Kleinholz A: Analysis of barley nitrate reductase cDNA and genomic clones. *Mol Gen Genet* 227: 411-416 (1991).
38. Singh H, Sen R, Baltimore D, Sharp PA: A nuclear factor binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* 319: 145-157 (1986).
39. Suzuki H, Fowler TJ, Tierney ML: Deletion analysis and localization of SbPRP1, a soybean cell wall protein gene in roots of transgenic tobacco and cowpea. *Plant Mol Biol* 21: 109-119 (1993).
40. Taylor B, Powell A: Isolation of plant DNA and RNA. *Focus* 4: 4-6 (1982).
41. Thompson GA, Boston RS, Lyznik LA, Hodges TK, Larkins BA: Analysis of promoter activity from an m-zein gene 5' flanking sequence in transient expression assays. *Plant Mol Biol* 15: 755-764 (1990).
42. Vasil V, Srivastava V, Castillo AM, Fromm ME, Vasil IK: Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. *Bio/technology* 11: 1553-1558 (1993).
43. Wan Y, Lemaux PG: Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol* 104: 17-48 (1994).