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Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm

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Abstract B- and C-hordein gene transcription is severely reduced in the endosperm of the regulatory barley mutant *lys3a*, and this is correlated with persistent hypermethylation of the promoters. In contrast, D-hordein is expressed at normal levels in the mutant. To confirm the connection between methylation and transcriptional activity, a genomic D-hordein clone was isolated and sequenced. The nucleotide composition of the promoter region revealed a CpG island and methylation analysis, using bisulphite treatment of genomic DNA, confirmed that the D-hordein promoter is unmethylated in endosperm and leaf tissue. Immunocytochemical studies localized D-hordein to the reticular component of protein bodies in both the wild-type Bomi and *lys3a*. Transient expression of *GUS* reporter gene constructs in barley endosperm, following transfection by particle bombardment revealed the D-hordein promoter to be 3–5 fold more active than B- or C-hordein promoters. Comparison of transient expression in Bomi and *lys3a* endosperm demonstrated that the activities of the unmethylated D-hordein and the *Hor1-14* C-hordein promoters were equivalent, while the activities in the mutant of the *Hor1-17* C-hordein and the *Hor2-4* B-hordein promoters were reduced two- and tenfold, respectively. Methylation of plasmids in vitro prior to expression severely inhibited B- and D-hordein promoter activities. Based on these observations two categories of promoters for endosperm-specific expression of storage proteins are recognized and a model involving methylation and modulation of chromatin structure in the regulation by the *Lys3* gene is presented.

Key words *lys3a* · CpG island · Transient expression · Particle bombardment · Immunocytochemistry

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Introduction

In plants, DNA methylation is mainly found in the cytosine of the di- and trinucleotides CpG and CpNpG (Gruenbaum et al. 1981), but evidence has in one case been provided for non-symmetrical methylation of a transgene in petunia (Meyer et al. 1994). In mammals the major sites of methylation are CpG dinucleotides (Gruenbaum et al. 1981). Despite this difference, the functional significance of methylation appears to be the same in the two groups of organisms. A clear correlation between transcriptional silencing and hypermethylation of promoter sequences has thus been demonstrated in both plants and mammals (Meehan et al. 1992; Finnegan et al. 1993). Two types of experiments have shown that methylation status is not just a consequence of the transcriptional state, but apparently participates actively in regulation of gene expression. Genomic demethylation induced by treatment of cells with 5-azacytidine (Bouchard and Momparler 1983) can induce activity of otherwise silent genes (Sasaki et al. 1992; Cooper et al. 1993). Methylation in vitro in several cases inhibits transcriptional activity, upon transfection into cells, relative to unmethylated controls. These include human β-globin genes (Yisrali et al. 1988), the promoter and enhancer from human Proα1(I) collagen (Thompson et al. 1991) and the cauliflower mosaic virus 35S promoter (Weber et al. 1990).

Methylation can interfere directly with binding of transcriptional activators (Comb and Goodman 1990; Inamdar et al. 1991) or it can inhibit transcription indirectly via binding of proteins with affinity for methylated DNA. Proteins binding to methylated DNA in a CpG density-controlled manner, but independent of sequence context, have been identified both in mammals (MeCP-1 and -2; Meehan et al. 1989; Lewis et al. 1992) and in plants (DBPm; Ehrlich 1993). There is strong evidence that MeCP-1 participates in transcriptional inactivation (Boyes and Bird 1991), and that the degree of inhibition depends on the density of methylated CpGs in the promoter (Boyes and Bird 1992). Similarly it has been shown that transcription from the cauliflower mosaic virus 35S promoter is inhibited by complete methylation, but not by limited methylation with *Hpa*II and *Hha*I methylases, upon transfection into petunia protoplasts (Hershkovitz et al. 1990).

The 5′ regions of most mammalian housekeeping genes and some tissue-specific genes contain CpG islands. These are unique CpG-rich sequences with a G + C content of around 50%, which remain unmethylated in all tissues (Bird 1986). The presence of CpG islands in the promoter regions of several plant genes has also been demonstrated (Antequera and Bird 1988; Gardiner-Garden and Frommer 1992).

The tissue-specific expression of 23 kDa and 27 kDa zein and B-hordein storage proteins, in the endosperm of maize and barley respectively, is correlated with hypomethylation of the genes (Bianchi and Viotti 1988; Sørensen 1992). For the B-hordein genes, genomic sequencing using ligation mediated PCR (Pfeifer et al. 1989) has demonstrated absence of methylcytosine in their promoters during endosperm development, but complete methylation in DNA from leaves (Sørensen 1992). It has further been shown that B-hordein promoter sequences are hypermethylated in endosperm DNA from barley plants homozygous for a mutation in the *Lys3* gene (Sørensen 1992). This mutant allele (*lys3a*, Risø 1508) is a recessive mutation in a regulatory gene controlling expression of several genes in the barley endosperm and is associated with a high-lysine phenotype in the endosperm (Ingversen et al. 1973). Run-off transcription and analysis of steady-state mRNA levels have demonstrated that *lys3a* causes a drastic reduction (some 50-fold) in transcription levels of B- and C-hordein, protein Z, β-amylase and trypsin inhibitor CMe genes (Sørensen et al. 1989; Kreis et al. 1987; Rodriguez-Palenzuela et al. 1989). Starch synthesis in the *lys3a* endosperm is only slightly affected, and the visual appearance of the mature mutant endosperm is close to the wild type (Ingversen et al. 1973).

Interestingly, D-hordein is expressed at a normal level in the *lys3a* endosperm (Sørensen et al. 1989), indicating that transcription of the D-hordein gene is not under control of *Lys3*. D-hordein is encoded by a locus on barley chromosome 5 (Blake et al. 1982), appears as a single polypeptide with an apparent molecular weight of 90–100 kDa in SDS-PAGE of hordein extracts, and is closely related to wheat HMW glutenin (Shewry et al. 1988). A partial cDNA clone encoding the 441 C-terminal amino acids of D-hordein has been isolated and shown to encode a polypeptide with a repeat structure that is different from that of wheat HMW glutenin (Halford et al. 1992). This difference is most pronounced in the C-terminal region where the common Pro-His-Gln-Gly-Gln-Gln hexapeptide is interspersed with 11 copies of a Thr-Thr- Val-Ser tetrapeptide which is not found in HMW glutenin.

The purpose of the present analysis is to investigate the role of DNA methylation in regulation of the expression of the two types of hordein genes. One type is represented by the B-hordein gene *Hor2-4* (Brandt et al. 1985) expression of which is dependent on the activity of the wild-type *Lys3* regulatory gene, and the other is represented by the D-hordein gene *Hor3-1*, which is unaffected by the *lys3a* mutation. Analysis of the D-hordein promoter reveals, in contrast to the B-hordein promoter, that it is unmethylated in both leaves and endosperm. The presence of a CpG island in this promoter is indicated and this provides a possible explanation for its normal expression in the mutant *lys3a*. Transient expression of the glucuronidase (GUS) gene driven by hordein promoters with or without in vitro methylation reveals that methylation inhibits promoter activity in the endosperm. It furthermore demonstrates that expression from unmethylated promoters can be inhibited in the presence of the *lys3a* mutation, which has previously been shown to prevent the demethylation of B-hordein promoters in the endosperm (Sørensen 1992).

Materials and methods

Cloning procedures

DNA was isolated from developing *Hordeum vulgare* (cv. Bomi) endosperms harvested at approximately 20 days after anthesis. The tissue was homogenised in a mortar with liquid nitrogen, and phenol-extracted as described (Hopp and Rasmussen 1983), and DNA was ethanol-precipitated for concentration and further purification. Two 25mer oligonucleotides, starting at positions 265 (5′-GCAGCCGGGACAAGTGCAACAGTTG-3′) and 1378 (5′-GCAAGCACACGTCACGTTTATTGTA-3′) in the D-hordein cDNA clone (Halford et al. 1992), were used to amplify a 1.1 kb fragment from genomic DNA. All PCRs were performed as hot-start PCR using *Taq* polymerase (Perkin-Elmer/Cetus) in a DNA thermocycler (Perkin-Elmer/Cetus) for 30 cycles with denaturation for 1 min at 94° C, annealing for 2 min at 50–65° C depending on primer length and composition, extension for 3 min at 72° C and other conditions as recommended by the manufacturer. Restriction digests were performed as recommended by the enzyme supplier (Promega). Southern blotting was performed using alkaline transfer (Reed and Mann 1985) onto Hybond-N+ (Amersham), using the amplified D-hordein fragment isolated from a 1% agarose gel with Prep-A-Gene

(Bio-Rad) and labelled by random priming (Feinberg and Vogelstein 1983), as a probe. For construction of the lambda ZAPII library, 100 µg of genomic DNA was digested with *Hin*dIII and size-separated on a 1% agarose gel; fragments of around 1.9 kb in size were extracted from 1 mm gel strips by electroelution and the D-hordein containing fraction was identified by PCR. Cloning, screening and recovery of the recombinant insert in pBluescript SK$^-$ were performed as described by the vector manufacturer (Stratagene). Altogether 2×10^5 plaques were screened to yield one positive D-hordein genomic clone. Sequencing was performed using Taq cycle sequencing with fluorescent dye-deoxy terminators and the products were separated and analysed on a Applied Biosystems 373A automated sequencer (Perkin-Elmer/Cetus). The Wisconsin package (Genetics Computer Group Inc.) was used for computer analysis of DNA sequences.

SDS-PAGE and immunoblotting

Hordein was extracted from developing barley endosperm and separated in 12% SDS-polyacrylamide gels and fixed and stained in Coomassie R250 in 10% TCA, as described by Rechinger et al. (1993). Proteins were transferred to PVDF membranes using a semi-dry blotter, and probed with the monoclonal antibody D304/13-1B2 (diluted 20,000:1) and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (DAKO AS, Denmark), diluted 2,000:1.

Immunocytochemistry

Thin sections of endosperm from Bomi and *lys3a* stocks were embedded in London Resin White and incubated with the D hordein monoclonal antibody (diluted 100:1) and gold-labelled goat anti-mouse secondary antibody, as described by Rechinger et al. (1993).

Methylation analysis

Endosperm DNA for methylation analysis was isolated from Bomi and the mutant strain *lys3a* as described above, while leaf DNA was isolated with a cetyltrimethylammonium bromide precipitation procedure as previously described (Sørensen 1992). The bisulphate catalysed conversion of cytosine to uracil was performed essentially as described (Frommer et al. 1992), except that a Prep-A-Gene (Bio-Rad) purification procedure replaced dialysis for the removal of bisulphite from the DNA (Feil et al. 1994). PCR was performed with T4 polynucleotide kinase (Promega)-phosphorylated, plus-strand specific primers starting at positions -424 (5'-T$^C/_T$GT$^C/_T$ GATTTGT-TAGTAATGGTTAATAGATAT-3') and $+107$ (5'-CTAAAACT-C$^G/_A$G$^G/_A$CTCACACTATAACTACCTAA-3') relative to the D-hordein translational start site. PCR fragments of correct size were isolated from 2% NuSieve agarose (FMC) gels using Prep-A-Gene and cloned in *Sma*I-digested and dephosphorylated pBluescript II SK$^-$ (Stratagene).

Transient expression

Vectors containing C-hordein (pE25 and pE26; Entwistle et al. 1991) and constitutive (pEmu; Last et al. 1991) promoters were previously described. The sequences from position -434 to $+7$ of the pHor3-1 D-hordein and from -550 to $+7$ of the λHor2-4 (Brandt et al. 1985) B-hordein gene were inserted in front of the GUS gene by PCR amplification and digestion with primers containing 5'*Eco*RI and 3' *Ecl*XI sites and ligation into a *Eco*RI/*Ecl*XI-digested GUS-NOS vector as described (Müller and Knudsen 1993). The in-frame insertion in the resulting plasmids (D-hordein:pHor3/GUS and B-hordein:pE16) was verified by sequencing.

Endosperm isolation, particle bombardment and GUS assays were performed as previously described (Müller and Knudsen 1993), with the exception that, in some experiments, a pressure of 1100 psi helium replaced gun-powder as the motive force.

Results

Cloning and sequencing of a D-hordein genomic clone

Based on the published partial D-hordein cDNA sequence which was isolated from the barley variety Hiproly (Halford et al. 1992) specific primers were constructed and PCR amplification of Bomi barley DNA allowed the isolation of a 1.1 kb fragment from the 3' end of the D-hordein gene (Fig. 1A). The PCR fragment was used as probe in a Southern blot of Bomi DNA digested with *Nco*I, *Bam*HI and *Hin*dIII (Fig. 2). This confirmed that D-hordein is encoded by a single-copy gene, although the presence of weakly hybridising bands in the *Bam*HI digest indicates that additional sequences with some homology to the 3' end of D-hordein are present in the barley genome. The 0.9 kb hybridising *Hin*dIII band is slightly larger than the 825 bp fragment expected from the presence of *Hin*dIII sites at positions 624 and 1450 in the cDNA clone (Fig. 1A). Subsequent PCR analysis and sequencing of this region in genomic DNA revealed the difference to be caused by the presence of additional copies, in Bomi relative to Hiproly, of the 30-bp repeat that constitutes this part of the gene. The hybridising 1.9 kb *Hin*dIII genomic fragment represents sequences extending in the 5' direction from the cDNA. This fragment was deduced to contain the entire 5' end of the coding sequence and part of the promoter sequence, based on the general absence of introns in cereal storage protein genes and assuming D-hordein to be of a size similar to HMW glutenin. A genomic library in Lambda ZAP II was constructed from size-selected *Hin*dIII digested Bomi DNA as described in Materials and methods. Screening of 2×10^5 plaques with the D-hordein PCR probe yielded one positively hybridising lambda clone.

Sequencing of the derived pBluescript clone (pHor3-1) revealed a 1855 bp insert, overlapping the cDNA clone by the expected 624 bp (Fig. 1A). Three single nucleotide differences were found between the cDNA and the genomic clone; none of these were silent, leading to three amino acid differences between the two clones (Fig. 1B). Some sequence differences were expected since the two clones were isolated from different barley varieties (Hiproly and Bomi). In addition to the entire 5 end of the D-hordein coding region, pHor3-1 also contained 434 bp of sequence upstream of the ATG start codon (Fig. 1C).

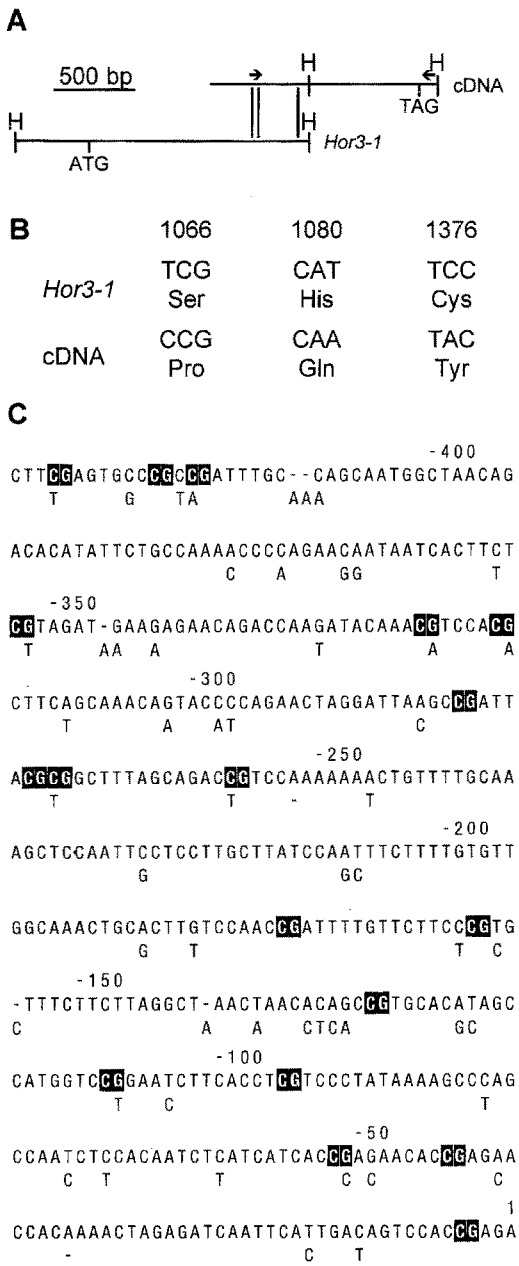


Fig. 1 Cloning and analysis of D-hordein. **A** Diagram showing the overlap between a previously analysed cDNA and the *Hor3-1* genomic clone containing the promoter. *Arrows* indicate the positions of primers used to amplify the 1.1 kb D-hordein probe. H, *Hin*dIII sites; translational start and stop sites are indicated as ATG and TAG, respectively, and polymorphisms are shown by vertical lines between the two sequences. **B** Polymorphisms between a cDNA clone from the variety Hiproly and the *Hor3-1* genomic clone from Bomi. Numbers indicate nucleotide positions relative to the translational start. **C** Nucleotide sequence of the promoters for D-hordein (*upper line*) and the high molecular weight glutenin gene Glu-1D-1b (Anderson et al. 1989; X12928) on the *lower line*. The positions of the CpG dinucleotides are *highlighted*. The *Hor3-1* sequence has been deposited in the EMBL database under the accession number X84368

Combination of the genomic and cDNA sequences gives an open reading frame of 2121 bp, suggesting a size for D-hordein of 707 amino acids and a deduced

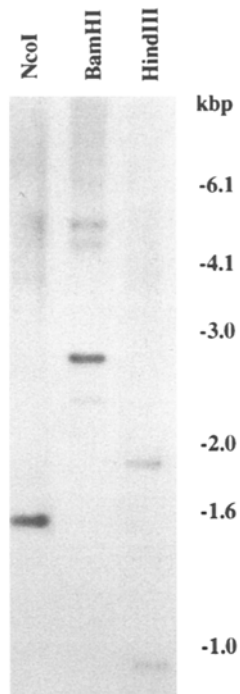


Fig. 2 Southern blot analysis of genomic DNA with the D-hordein probe. A 1.1 kb PCR fragment from the 3′ end of the D-hordein gene was amplified from genomic Bomi DNA, labelled and used as probe in a Southern blot analysis of Bomi DNA digested with *Nco*I, *Bam*HI or *Hin*dIII as indicated. The size marker is a 1 kb ladder (Gibco-BRL)

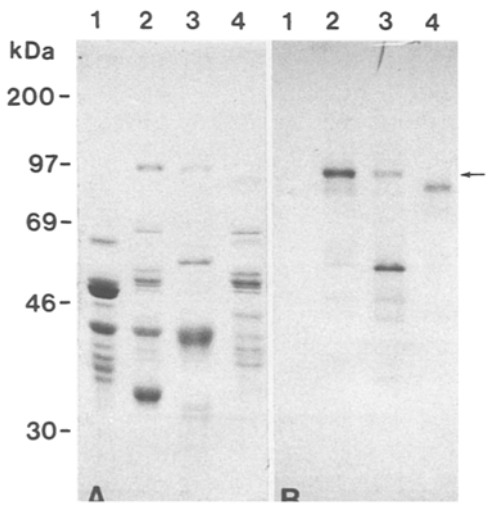


Fig. 3 SDS-PAGE and immunoblotting analysis of D-hordein. SDS-PAGE of hordeins of the D-3203 mutant lacking D-hordein (lane 1), Bomi (2), *lys3a* (3) and Hiproly (4), followed by Coomassie staining (**A**) or immunoblotting (**B**), with the monoclonal antibody D304/13-1B2. The *arrow* indicates the position of the mature D-hordein polypeptide in *lys3a* and Bomi, whereas a smaller polypeptide is produced from the *Hor3* allele of Hiproly. Immuno positive bands of lower molecular weight are breakdown products of D-hordein, and are missing from the mutant lacking D-hordein

molecular weight of 75.1 kDa. In SDS-PAGE, D-hordein migrates at an apparent molecular weight of 90–95 kDa (Fig. 3A). A similar discrepancy is seen for the homologous HMW glutenins and might be due to differences in SDS-binding relative to the reference proteins (Greene et al. 1988). As mentioned above, the

repeat number differs between barley varieties and the product size will therefore also be variety dependent. Mainly due to the difference in composition of the repeats, the nucleic acid sequence homology between wheat HMW glutenin genes and barley D-hordein is only 71–79% in the coding region, while it is 85–89% in the promoter region (Fig. 1C), confirming the close relation between these genes and indicating that the tissue-specific and temporal expression pattens in barley and wheat endosperm are very similar.

Intracellular localisation of D-hordein

The monoclonal antibody D304/13-1B2 was used to determine the intracellular localisation of D-hordein. Immunoblotting of total hordein separated by SDS-PAGE demonstrated that this antibody specifically recognises D-hordein polypeptides (Fig. 3B). The immunoreactive bands of lower molecular weight, which, together with the mature D-hordein band, are not found in the mutant lacking D-hordein, do not correspond to Coomassie-staining bands, and are thus presumed to be breakdown products of D-hordein. Sections from wild-type and *lys3a* mutant endosperm were prepared and immunocytochemistry was performed. The antibody specifically labelled the reticular component of the protein bodies located in the vacuoles of both Bomi and *lys3a* (Fig. 4). The higher density of labelling in *lys3a* (Fig. 4B) is probably a reflection of the higher relative concentration of D-hordein in this mutant in comparison with Bomi. This indication that the reticular component is the site of D-hordein deposition is in contrast to findings for B-, and C-hordein, which in similar experiments have been localised to the translucent globules (Rechinger et al. 1993).

Analysis of D-hordein promoter methylation

Analysis of nucleotide composition revealed that the D-hordein promoter and the 5′ part of the coding region have some of the characteristics of a CpG island. Thus, there is no apparent deficiency in the CpG dinucleotide frequency, the ratio of observed to expected occurrences being similar to that of GpC dinucleotides. The C + G content is, however, somewhat lower than 50% (Fig. 5A), but still conforms to the criteria that have previously been used to analyse plant sequences for the presence of CpG islands (Gardiner-Garden and Frommer 1992). In addition, the CpG frequency in the D-hordein promoter is quite distinct when compared with the major part of the D-hordein coding region (Fig. 5A) and the *Hor2-4* B-hordein gene (Fig. 5B). The first 500 bp of the D-hordein clone, containing the putative D-hordein CpG island sequence, contains 25 CpG dinucleotides compared with 5 in 550 bp of the *Hor2-4* promoter. The CpG frequency in B- and D-hordein coding regions is higher, 17/500 bp on average, but this is due to a generally high C + G content caused by the unusual amino acid composition of the polypeptides, though the frequency is still lower than expected for a random distribution (Fig. 5).

The methylation pattern of the D-hordein promoter was analysed using selective conversion of cytosine but not 5-methylcytosine to uracil by reaction with bisulphite (Frommer et al. 1992), as described in Materials and methods. Following bisulphite treatment, PCR-amplified fragments were cloned into pBluescript SK^- and sequenced. When this approach is used, each clone represents the methylation pattern in a single DNA molecule from the original tissue, and it is therefore necessary to analyse several clones in order to obtain a reliable estimate for the methylation pattern. Examples of sequencing data covering the partially

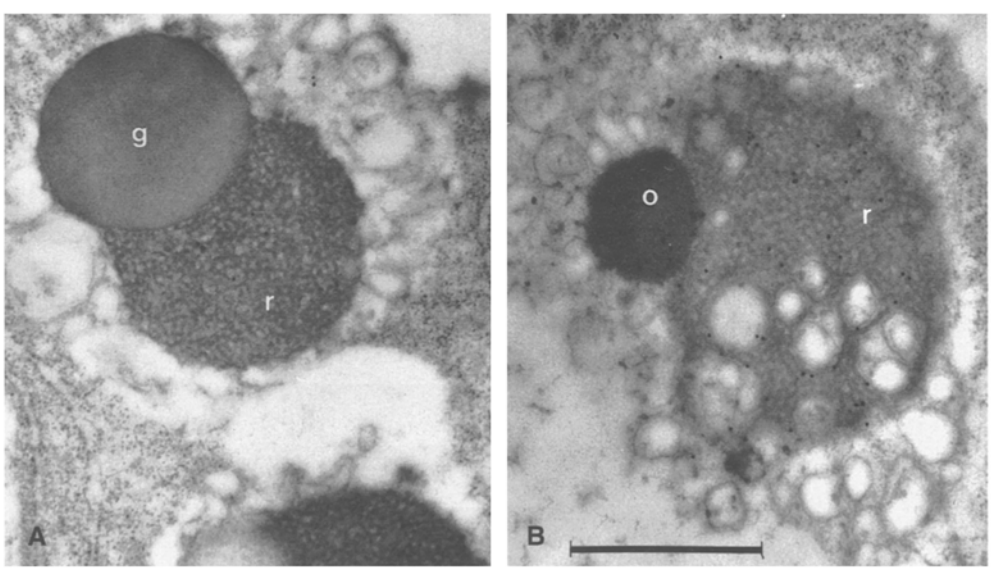


Fig. 4A, B Intracellular localization of D-hordein. Immunocytochemistry of Bomi (**A**) and *lys3a* (**B**) using the monoclonal antibody D304/13-1B2 to D-hordein, followed by a gold-labelled second antibody. Labelling by 10 nm gold particles is confined to the reticular component (r) of the protein bodies, and is not found over the globules (g) containing B- and C-hordeins, or the osmiophilic globule (o) of unknown composition. The dark spots in the osmiophilic globule (o) are not gold particles as they are of irregular size and shape. There is a higher density of labelling in mutant *lys3a* than in its parent Bomi. Bar represents 1.0 μm

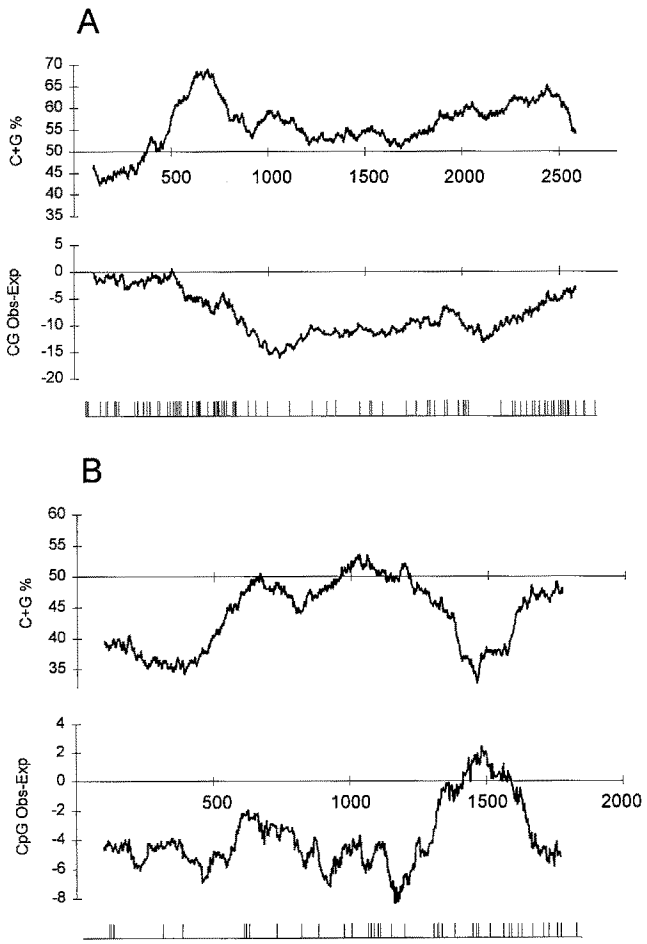
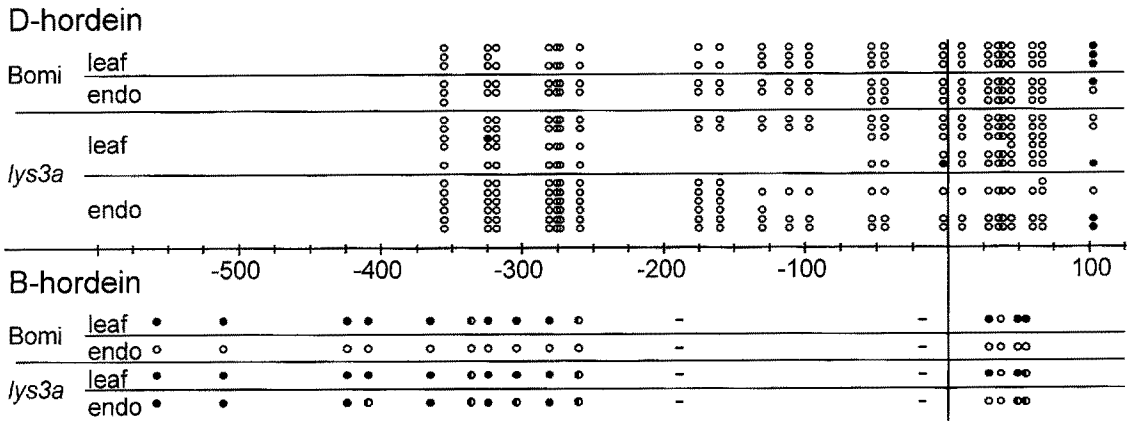


Fig. 5 Sequence composition of D- and B-hordein genes. C + G content, CpG dinucleotide frequency (number observed minus the number of expected random occurrences) and CpG distribution is shown in **A** for the combined nucleotide sequences of the *Hor3-1* and D-hordein cDNA clones and in **B** for *Hor2-4*. The window size for the C + G content and CpG frequency plots is 200 bp. The CpG island in *Hor3-1* is indicated by the high density of CpG dinucleotides in the first 800 bp

methylated CpG dinucleotide at position +103 (relative to the translation start) are shown in Fig. 6. The specificity of the reaction is demonstrated by the complete conversion of the nearest neighbour non-CpG cytosines.

In the experiment reported here, 18 individual clones were sequenced. Occasional stretches of DNA where no conversion of any cytosines had occurred were observed in some clones. These stretches were randomly distributed, and never interrupted by modified cytosines as would be expected if they represented non-symmetrically methylated DNA (Meyer et al. 1994). They arise rather as a consequence of reannealing of DNA during the bisulphite reaction, as has previously been reported (Feil et al. 1994), and therefore these sequences have been excluded from data represented in Fig. 7. All CpG dinucleotides between positions −356 and +103 relative to the translation start site of the D-hordein gene were, however, analysed in 10–17 individual clones, representing at least 2 from each tissue and genotype. The results of the analysis are summarised and compared to the previously published tissue-specific methylation pattern of B-hordein promoters in Fig. 7. The B-hordein promoter methylation was analysed by ligation-mediated PCR with primers recognising multiple genes (Sørensen 1992). Due to polymorphism, it is therefore likely that each individual B-hordein promoter contains fewer CpG dinucleotides than shown in Fig. 7 (e.g. the *Hor2-4* promoter contains only five CpGs).

Fig. 6 Methylation of D- and B-hordein promoters. Genomic DNA of Bomi was bisulphite treated, PCR amplified and cloned. Each row above the scale represents methylation analysis of one clone. *Open circles*, unmethylated CpG; *filled circles*, methylated CpG. Significant methylation of D-hordein is only observed at the most 5′ position analysed. The incomplete information from some clones is caused by stretches of unconverted DNA. The translation start is indicated by a *vertical line* and numbering is relative to this site. Each DNA was analysed in at least two separately bisulphite treated samples. B-hordein promoter methylation data are from Sørensen (1992). Methylation was analysed by ligation-mediated PCR on hydrazine-treated genomic DNA, and each row represents the methylation pattern in the indicated tissue. *Half-filled circles*, partial methylation; *dash*, CpG site not analysed; other symbols as for D-hordein



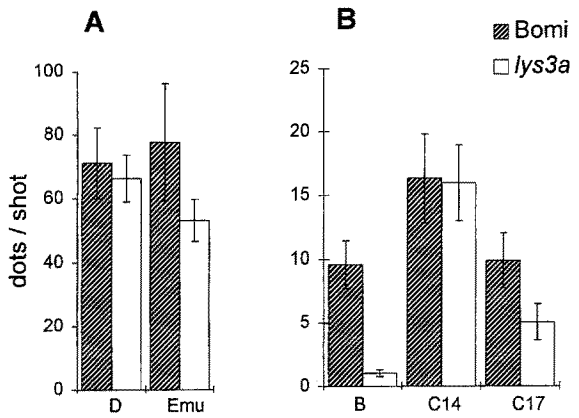


Fig. 7A, B Activities of unmethylated hordein promoters in wild-type (Bomi) and mutant (*lys3a*) endosperm. Glucuronidase activity driven by hordein promoters was measured by the number of blue dots. Each bar represents the average of between 14 and 32 shots each at 10 isolated endosperms. No significant differences between *lys3a* and Bomi were found for the D-hordein (D) promoter, the C14-hordein (C14) promoter or the Emu promoter (Emu). B-hordein promoter (B) activity was reduced almost tenfold, while the C17-hordein promoter (C17) was 50% active in *lys3a* relative to Bomi. These differences are significant at the 1% and 5% levels, respectively. Note the difference in scale between **A** and **B**. Bars indicate standard errors. Details of experiments are listed in Table 1

The D-hordein promoter methylation pattern was analysed in leaves and developing endosperm from both Bomi and *lys3a* barley plants. No methylation was detected in any tissue in the 22 CpGs between positions −355 and +67, except for two independent clones showing methylation at positions −325 and −3, respectively. These are likely to represent instances of incomplete conversion of unmodified cytosines, since 1–4 unconverted cytosines in non-CpG positions were detected in most clones, in addition to the aforementioned stretches of unconverted cytosines. The absence of methylation in leaf DNA (Fig. 7) provides strong evidence for the CpG island nature of this endosperm tissue-specific gene. Interestingly 3/5 endosperm and 4/6 leaf DNA clones analysed were methylated at the most 3′ position analysed (+103). This position coincides with the 3′ end of the region in which CpG frequency is not reduced and might mark the end of the CpG island.

None of the 33 CpNpG trinucleotides in the region of the D-hordein gene analysed were methylated. This could be taken as an indication that both CpNpG and CpG dinucleotides are unmethylated in plant CpG islands. CpNpG methylation is however also absent from B-hordein promoters in leaf DNA where the CpG sites are completely methylated (Sørensen 1992). It is therefore possible that the extensive CpNpG methylation found in plants (Gruenbaum et al. 1981) has a different sequence specificity and is not involved in the transcriptional regulation exerted by CpG methylation.

DNA from *lys3a* plants was included in the analysis to test whether the differences in methylation observed between wild-type and mutant endosperm (Sørensen 1992) is restricted to genes that are affected by the mutation. The absence of methylation in the D-hordein promoter from both Bomi and *lys3a* endosperm DNA confirms this hypothesis, since D-hordein is expressed at similar levels in the mutant and wild type (Sørensen et al. 1989). Thus, hypermethylation in *lys3a* endosperm is not a random phenomenon but is gene specific, and actively participates in the transcriptional inhibition of the affected genes.

Hordein promoter activity in mutant and wild-type endosperm

For heterologous expression of a reporter gene driven by the wheat HMW glutenin promoter in transgenic tobacco, between 338 and 433 bp of 5′ upstream sequence has been shown to be sufficient to give seed-specific expression in two different studies (Halford et al. 1989; Robert et al. 1989). Based on the high degree of homology between D-hordein and HMW glutenin promoters, it was concluded that the 434 bp of D-hordein promoter sequence present in pHor3-1 should contain all cis-elements necessary for endosperm specific expression. The D-hordein promoter was fused to the coding sequence of the GUS gene and the resulting plasmid (pHor3/GUS) was introduced into isolated developing endosperms by particle bombardment. After 48 h of culture, the endosperms were stained for β-glucuronidase activity and promoter activity was measured by counting the number of blue dots, as quantitative β-glucuronidase assays are not feasible in this system. This approach relies on the probability that an individual transformation event leading to detectable amounts of β-glucuronidase activity increases with promoter strength, as a result of a reduced copy number requirement. It has previously been used with success (Müller and Knudsen 1993).

As expected, the D-hordein promoter driven GUS activity was not significantly different in Bomi and *lys3a* endosperms. In both tissues the D-hordein promoter showed approximately the same activity as the strong constitutive EMU promoter (Fig. 8A).

To test the possibility that hypermethylation of endogenous B- and C-hordein promoters is the basis for the reduced transcriptional activity of these genes in *lys3a* endosperm, constructs containing B- (*Hor2-4*; Brandt et al. 1985) and C-hordein promoters (*Hor1-14*, Entwistle 1988; *Hor1-17*, Entwistle et al. 1991) were transiently expressed in wild-type and mutant endosperm. If all trans-acting factors necessary for B- and C-hordein expression are present in the mutant endosperm, then expression from the introduced unmethylated promoters should be equivalent to wild-type activity. As can be seen in Fig. 8B this is indeed the case

Table 1 Effect of in vitro methylation on transient hordein promoter activity in endosperm of wild-type and mutant

Genotype	Promoter[a]	Unmethylated			Methylated			Unmeth./meth.
		dots/shot		n (shots)[c]	dots/shot		n (shots)[c]	
Bomi	D-hordein	130.7	± 25.5[b]	6	5.8	± 0.8	6	22[d]
lys3a	D-hordein	44.7	± 9.5	6	2.8	± 0.8	6	16[d]
Bomi	B-hordein	4.2	± 2.6	6	0.0	–	6	–[d]

[a] Constructs used were: D-hordein: pHor3-1/GUS and B-hordein: pE16
[b] Standard error
[c] Ten isolated endosperms were used for each shot
[d] Significant difference between methylated and unmethylated at 1% level

for one C-hordein promoter (*Hor1-14*), while activity of the other C-hordein promoter (*Hor1-17*) is approximately half of that found in Bomi. Although this difference is just significant at the 5% level, it is not as large as the 50-fold reduction in transcription rate found by run-off transcription analysis (Sørensen et al. 1989).

On the other hand, the B-hordein promoter activity was reduced tenfold in the *lys3a* endosperm (Fig. 7B). This correlates well with the reduction in mRNA levels and run-off transcription rates (Sørensen et al. 1989). The activity of an introduced unmethylated B-hordein promoter thus closely mimics that of the endogenous methylated genes in the endosperm of the mutant. This provides strong evidence that the mutant endosperm lacks or has only limiting levels of trans-acting factors needed for normal expression of B-hordein genes, and that persistent promoter methylation is not the only reason for reduced hordein transcription in the *lys3a* mutant. The results of transient expression in Bomi and *lys3a* endosperms are summarised in Table 1.

Methylation inhibits hordein promoter activity

To confirm that methylation has the capability to inhibit transcriptional activity in the developing barley endosperm, the transient in vivo activity of B- and D-hordein promoters methylated in vitro was examined. Plasmids were methylated at all CpG positions with *Sss*I methylase before introduction into the endosperm. Both B- and D-hordein promoter activities in Bomi and D-hordein promoter activity in *lys3a* are significantly reduced when the promoters are methylated. D-hordein promoter activity was reduced to approximately 5% of the unmethylated activity. The difference seen between unmethylated D-hordein promoter activity in this experiment and those reported in Table 1 is probably due to the small sample size of the former and did not score as significantly different when analysed statistically. The methylated B-hordein promoter showed no detectable activity, but the activity of the unmethylated control is also low and the inhibition is likely to be of the same relative size as that seen for the methylated D-hordein promoter. Thus methylation is capable of inhibiting activity of both the CpG-dense D-hordein promoter and the less CpG-dense B-hordein promoter in barley endosperm.

Discussion

The finding that the 5′ end of the D-hordein gene contains a CpG island was unexpected, as D-hordein expression is highly endosperm tissue-specific. Nevertheless, methylation analysis of leaf DNA confirmed the permanently unmethylated status of the promoter. This finding is not without precedent since a number of mammalian genes showing tissue-specific expression contain CpG islands (Bird 1986). The promoter of the maize *Adh-1* gene, which is expressed in root and scutellum tissues, is also unmethylated in non-expressing tissues (Nick et al. 1986), indicating that this gene might also contain a CpG island.

Several observations indicate that the expression of D-hordein is controlled independently of the other hordeins, although its temporal and tissue-specific expression patterns are comparable. D-hordein transcription is unaffected by the *lys3a* mutation (Sørensen et al. 1989), and there are no apparent homologies between D- and B-hordein promoters. The CpG island in the D-hordein promoter further emphasises this difference.

The absence of methylated CpNpGs in the plant promoter sequences analysed for methylation so far (Sørensen 1992; Nick et al. 1986) indicates that both the specificity and role of CpNpG methylation is different from that of CpG methylation. It has previously been noted that CpNpG methylation is not needed for methylation-mediated inhibition of the CaMV 35S promoter activity (Hershkovitz et al. 1990), confirming this ifference. Unfortunately, methylases with CpNpG specificity are not available and it is therefore not easy to determine how this type of modification is maintained and whether it plays a similar role in transcriptional regulation to CpG methylation.

Transient expression from the reporter gene construct pHor3-1/GUS demonstrated that the D-hordein

promoter is very active in developing endosperm: its activity in the wild type exceeds that of B- and C-hordein promoters by 3 to 5-fold. Considering that the steady-state level of D-hordein mRNA is almost 50 times lower than that for B- or C-hordein during endosperm development (Sørensen et al. 1989), this result is somewhat surprising. D-hordein is, however encoded by a single gene and transcriptional activity of this one gene might exceed that of individual B- and C-hordein genes, considering that B- and C-hordeins are encoded multigene families comprising 10–20 members (Brandt et al. 1985; Rasmussen and Brandt 1986).

The different responses of the B- and C-hordein promoters in transient expression in the *lys3a* endosperms are unexpected for several reasons. The conserved −300 element, which is important for tissue-specific expression of storage protein genes in endosperm (Müller and Knudsen 1993) is present in both promoters and the overall homology between them is approximately 50%. Furthermore, transcriptional activities of B- and C-hordein genes are highly co-ordinated temporally in the wild type barley endosperm and, more importantly, the responses of the endogenous promoters to the *lys3a* mutation are very similar (Sørensen et al. 1989). One possible explanation for the difference in activity of B- and C-hordeins in transient expression assays is that the promoters respond differently to the high nitrogen levels that are available to the endosperm after shooting. Increased nitrogen levels have previously been shown to lead to a substantial increase in C-hordein mRNA levels, while the B-hordein response is much weaker (Giese and Hopp 1984). It is thus possible that, under the conditions in the endosperm during the transient expression experiment, the response to high nitrogen overrides the effect of the *lys3a* mutation in the case of C-hordein promoters.

A possible explanation for the reduced B-hordein promoter activity in *lys3a* is that the DNA becomes methylated upon introduction into the endosperm. This is considered unlikely since de novo methylation in plants is normally a rare event (Nelsen-Salz and Döring 1990). Nevertheless, it has recently been reported that the presence of specific sequence elements can direct de novo methylation in specific cell types (Hasse and Schulz 1994; Szyf et al. 1990), and it cannot be excluded that the B-hordein sequence contains such an element.

The in vitro methylation and transient expression experiments presented here clearly demonstrate that methylation alone has the capacity to inhibit transcriptional activity even in the *lys3a* mutant. Although plasmids were methylated over their entire lengths, the inhibitory effect is likely to be mediated by promoter methylation, since methylation of 40 non-promoter CpGs in a CaMV 35S construct with M. *Hpa*II and M. *Hha*I did not inhibit promoter activity in plant protoplasts (Hershkovitz et al. 1990). It has also been demonstrated that specific methylation of 5′ sequences is sufficient to inhibit activity of the γ-globin gene promoter (Murray and Grosveld 1987).

Based on the observations that DNA methylation seems to be involved in regulation of hordein gene expression and that *lys3a* endosperm is limited in factors necessary for hordein promoter activity, a model for the mechanism by which the *Lys3* participates in endosperm development can be proposed. According to this model, *Lys3* exerts its influence early in the endosperm differentiation process, converting genes from an inactive to an active or transcriptionally competent state. The structural genes affected are those for B- and C-hordein, protein Z, chymotrypsin inhibitor and possibly other endosperm-specific genes, as well as tissue-specific trans-acting factors needed for the expression of these genes. The conversion is accompanied, and possibly mediated, by demethylation of promoter sequences, and D-hordein escapes the effect of the *lys3a* mutant because of its permanently unmethylated CpG island promoter sequence.

Several other observations support this model. It has been demonstrated that the 23 and 27 kDa zein genes in the maize endosperm are demethylated prior to the onset of their expression (Bianchi and Viotti 1988), indicating that methylation could play an active role in their regulation. Also the fact that *Lys3* affects the expression of genes whose promoters share no homology, suggests that the LYS3 product is not a trans-acting factor that directly contacts these promoters, but rather influences events in endosperm differentiation that precedes hordein expression. A similar model involving the methylated DNA binding protein MeCP-1, in a mechanism determining chromatin conformation and thus transcriptional activity, has previously been proposed to explain the regulatory effect of DNA methylation (Lewis and Bird 1991).

In conclusion, methylation of the two types of promoters for endosperm-specific expression severely inhibits transcription. The promoter category represented by the *Hor3* gene encoding the D-hordein storage protein carries a CpG island, is unmethylated in all tissues and its transcriptional activity is not influenced by the *Lys3* regulatory gene. The other promoter category (*Hor1*, *Hor2*) lacks a CpG island, is hypermethylated in leaves and demethylated in the endosperm. Mutation of the *Lys3* gene prevents the establishment of the active endosperm-specific demethylated state. Introduction of unmethylated promoters into endosperm cells carrying this mutation can lead, in some but not all cases, to efficient transcription.

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