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Methylation of B-hordein genes in barley endosperm is inversely correlated with gene activity and affected by the regulatory gene *Lys3*

(genomic sequencing/*Hordeum vulgare*/storage protein/transcriptional regulation)

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ABSTRACT **The methylation status of B-hordein genes in the developing barley endosperm was analyzed by digestion with methylation-sensitive restriction enzymes. Southern blotting revealed specific demethylation of *Hpa* II sites in DNA from wild-type endosperm, whereas leaf DNA and *lys3a* mutant endosperm DNA were highly methylated at these sites. Similar methylation patterns were observed at an *Ava* I site situated at position −260 in the B-hordein promoter. This differential methylation was confirmed by genomic sequencing with ligation-mediated PCR. The analyzed sequence covers most of the B-hordein promoter and includes 10 CpGs from the promoter and 4 CpGs from the adjacent coding region. These sites were all hypomethylated in wild-type endosperm, whereas—except for three partially methylated sites—full methylation was seen in leaf DNA. The four sites in the coding region were partially methylated in *lys3a* endosperm DNA, but the promoter sites remained highly methylated. The possible role of methylation in the regulatory function of the *Lys3* gene product is discussed.**

The barley regulatory gene *Lys3* is involved in the tissue-specific expression of several genes in the developing endosperm. Major storage proteins, such as B- and C-hordeins, are drastically reduced relative to wild-type levels in the homozygous *lys3a* mutant Risø 1508 (1, 2). Synthesis of protein Z (3) and β-amylase (4) is also repressed, whereas the minor D-hordein storage protein is present in normal amounts in *lys3a* endosperm (5). The decreased content of these polypeptides is the consequence of a reduction in steady-state mRNA levels and transcription rates of the affected genes to ≈1% of the wild type (3). This low level of transcription is still specifically induced in the endosperm because hordein transcripts are only detected in this tissue (6).

Inverse correlation between transcriptional activity and cytosine methylation of promoter regions in different tissues and cell lines has been demonstrated for a number of mammalian genes. Transformation of cells with methylated genes revealed them to be transcriptionally inactive (7). Methylation is, therefore, thought to play an active role in transcriptional regulation.

5-Methylcytosine accounts for 20–30% of total cytosine in most plant genomes but accounts for <10% in mammals (8, 9). Furthermore, both CpG and CpNpG di- and trinucleotides are modified in plants compared with the exclusive methylation of CpG in mammals (10). Despite these differences in both levels and patterns, methylation appears to have similar effects in plants and mammals. Thus, correlation between tissue-specific expression and hypomethylation has been shown for a number of plant genes. This correlation includes genes encoding polypeptides involved in photosynthesis (11) and genes for the zein storage proteins in maize (12). Nevertheless, zein genes are hypomethylated in endosperm, regardless of the presence or absence of an active allele of the regulatory opaque-2 gene. Expression of genes under control of the 35S cauliflower mosaic virus promoter in tobacco (13) or petunia (14) protoplasts is inhibited by *in vitro* methylation of the promoter, indicating that methylation also participates actively in plant gene regulation.

The present study shows that expression of hordein genes in the developing barley endosperm is accompanied by hypomethylation and that methylation may be a chief factor in the transcriptional inactivation of genes in the *lys3a* mutant.

MATERIALS AND METHODS

DNA Isolation. Genomic DNA was isolated from the barley variety Bomi and its mutant line Risø 1508 by precipitation with cetyltrimethylammonium bromide as follows. Young leaves or developing endosperms harvested ≈20 days after anthesis were frozen in liquid nitrogen and homogenized in a coffee grinder (Braun KSM2). Subsequent cetyltrimethylammonium bromide extractions and precipitations were as described (15). The DNA was redissolved in 1 M NaCl, incubated with DNase-free RNase A at 10 μg/ml for 1 hr at room temperature, and extracted once with 1 vol of phenol and once with 1 vol of chloroform/isoamylalcohol, 24:1 (vol/vol). DNA was recovered by standard ethanol precipitation and dissolved in 10 mM Tris/1 mM EDTA, pH 7.5.

Southern Analysis. Ten-microgram samples of barley DNA were incubated with 50 units of restriction enzyme for 3 hr under the conditions recommended by the manufacturer. All restriction enzymes were purchased from Boehringer Mannheim. Restricted DNA was separated on 0.7% agarose gels and transferred by alkaline blotting to positively charged nylon membranes (Hybond-N+, Amersham) as described (16). Restriction fragments used as probes were isolated from agarose gels using the Prep-A-Gene DNA purification kit (Bio-Rad). DNA was labeled by random priming (17) and separated from unincorporated nucleotides by using a Sephadex G-50 column (Nick-columns, Pharmacia). Prehybridization, hybridization, and washing of filters were as described (18).

Ligation-mediated PCR (LMPCR). Base-specific chemical modification and cleavage were as described (19) with 30 μg of genomic barley DNA used in each reaction. The LMPCR principle and protocol described in ref. 20 were modified as follows. First-strand synthesis was initiated by mixing 5 μg of chemically modified genomic DNA with 0.3 pmol of primer 1 in 15 μl of 40 mM Tris/50 mM NaCl, pH 7.7, heating to 95°C for 2 min, followed by annealing at 58°C for 30 min. Primer extension and ligation of linker were as described (20). Twenty-five cycles of PCR were done in a thermocycler

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Abbreviation: LMPCR, ligation-mediated PCR.

(Perkin–Elmer/Cetus) with denaturation at 94°C for 1 min (2 min in the first cycle), annealing at 55°C for 2 min, and extension at 72°C for 3 min. After annealing (2 min, 54°C) and extension of the end-labeled primer 3 the reaction product was purified by passage through a Sephadex G-25 spun column (Quick-Spin, Boehringer Mannheim), dried *in vacuo*, and separated on a standard 8% sequencing gel. The dried gels were autoradiographed at room temperature without intensifying screens for 16–24 hr.

RESULTS

Genomic Southern Blots. Methylation patterns of B-hordein genes were studied by hybridization of a 550-base-pair (bp) *Eco*RI–*Xmn* I fragment from the λhor2-4 B-hordein promoter [(21) and Fig. 1*A*] to blots of genomic barley DNA digested with methylation-sensitive restriction enzymes. The homology between known B-hordein promoter sequences is 90% (21, 22) and thus under the selected hybridization conditions this probe hybridized to all of the 10–15 gene copies in the barley genome.

Digestion with *Hpa* II is inhibited when either one or both of the two cytosines in its CCGG recognition sequence are methylated. As expected from the high frequency of methylation at CpG positions in plant DNA (10), most hybridizing fragments in *Hpa* II-digested DNA were larger than 15 kilobase pairs (kbp) (Fig. 2). In wild-type endosperm DNA two fragments of 2.1 and 1.7 kbp were evident, indicating hypomethylation of specific sites in this tissue relative to both leaf and *lys3a* endosperm. Nevertheless, a high proportion of the genes were still present in high-molecular-weight fragments demonstrating that the *Hpa* II sites either remain partially methylated or are absent due to sequence polymorphisms. Sequence analysis supports the latter explanation because B-hordein clone λhor2-4 (21) contains no *Hpa* II sites, whereas the clone pBHR184 (22) contains only two *Hpa* II sites at positions 1528 and 2425. Both of these sites are downstream from the coding region, where homology is low. Cleavage of the *Hpa* II site at 1528 and a potential site at ≈−600, which is just 5′ to the known promoter sequence, could produce the observed fragment of 2.1 kbp. However, location of the sites giving the 1.7-kbp fragment cannot be determined from the available sequence data.

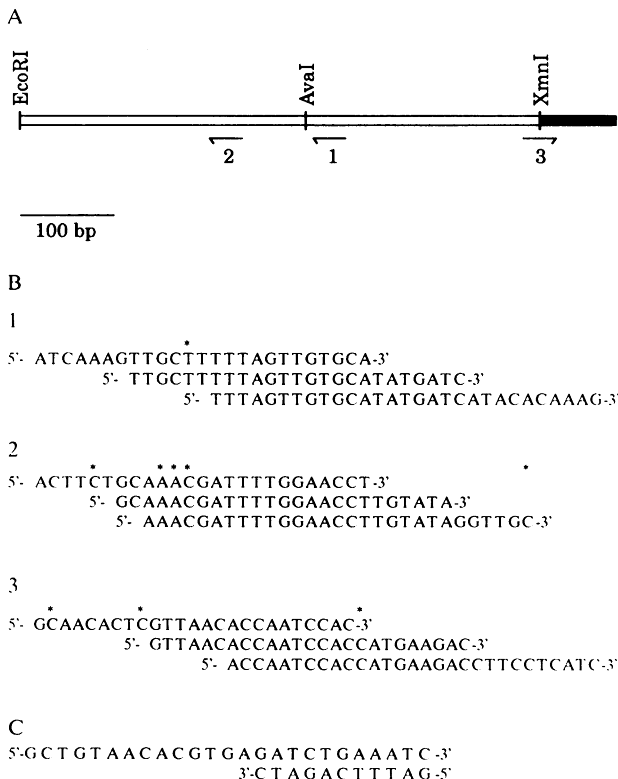

FIG. 1. Probes and oligonucleotides used in the analysis of B-hordein promoter methylation. (*A*) Restriction map of the hor2-4 promoter region. Open bar indicates the fragment used as probe in the hybridizations; closed bar represents the beginning of the coding region. Positions of the LMPCR primer sets are shown below the map. (*B*) Oligonucleotide sequences of the three primer sets used in LMPCR. Primer sets 1 and 2 represent lower-strand sequences, and sequencing will be toward the 5′ end of the gene. Primer set 3 represents upper-strand sequences, and sequencing will, therefore, be toward the 3′ end. Asterisks above sequences denote sequence differences between the two genomic B-hordein clones pBHR184 (22) and hor2-4 (21). (*C*) Sequence of the common linker used in LMPCR.

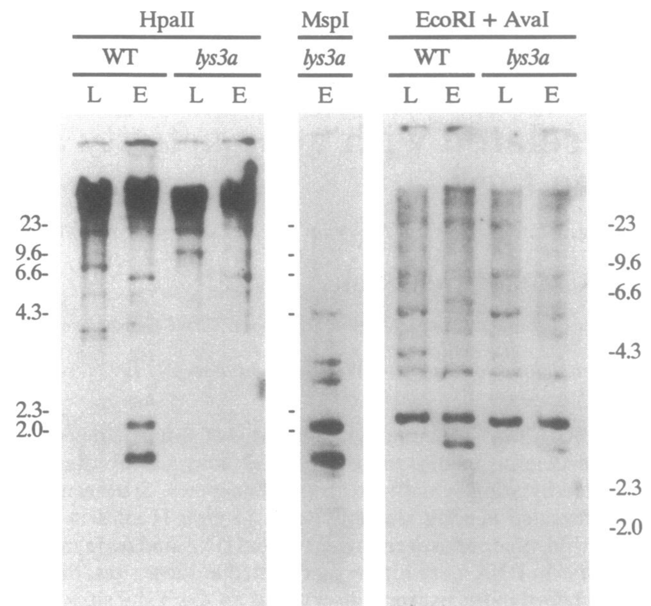

FIG. 2. Hybridization of the B-hordein promoter probe to genomic barley DNA. Leaf (L) or endosperm (E) DNA from Bomi [wild type (WT)] or its mutant Risø 1508 (*lys3a*) was digested with restriction enzymes *Hpa* II, *Msp* I, or *Ava* I plus *Eco*RI and hybridized to the 550-bp *Eco*RI–*Xmn* I B-hordein promoter fragment from λhor2-4. The size marker was λ DNA-digested with *Hin*dIII.

When digested with *Msp* I (an isoschizomer of *Hpa* II that is sensitive to methylation at the first but not the second cytosine) leaf and endosperm DNA from wild type and *lys3a* all gave identical patterns of hybridization (Fig. 2 and data not shown). Thus, the methylation pattern of CpCpG trinucleotides is maintained irrespective of B-hordein gene activity.

Methylation changes correlating with transcriptional activity are most often found in the promoter or the beginning of the coding region. Because positions of the *Hpa* II sites relative to the B-hordein promoter could not be determined, this was examined for the presence of other methylation-sensitive restriction sites. This analysis revealed a conserved *Ava* I site at position −260 (Fig. 1*A*). To analyze the methylation status of this particular site *Eco*RI/*Ava* I double digests were hybridized to the B-hordein promoter probe (Fig. 2). The prominent 3.0-kbp fragment seen in all lanes is the result of cleavage at two *Eco*RI sites at positions −550 and 2450. These sites are present in pBHR184 and are apparently conserved in most B-hordein genes. When unmethylated, the *Ava* I site will be digested and reduce the size of the hybridizing fragment to 2.7 kbp. There is no evidence of a 2.7-kbp fragment in digests of leaf DNA, whereas weakly and strongly hybridizing fragments are seen in *lys3a* and wild-type endosperm DNA, respectively. The 2.7- and 3.0-

kbp fragments in Bomi endosperm are equally intense and, in parallel to the *Hpa* II digests, this result can be caused by partial methylation or sequence polymorphism abolishing the *Ava* I site in one or more of the B-hordein genes.

Visual inspection of the *Hpa* II digests in ethidium bromide-stained agarose gels showed that no gross differences in general methylation levels exist between leaf and endosperm DNA or between the wild type and *lys3a* (data not shown). Correlation of the observed methylation differences with tissue-specific and phenotypic expression of genes was further confirmed by rehybridization of the blots with the α-amylase cDNA clone pM/C (23). Digestion with *Hpa* II, *Msp* I, or *Eco*RI/*Ava* I gave identical hybridization patterns for the four DNA samples (data not shown), which was expected because α-amylase genes are not expressed in endosperm or leaf tissue.

LMPCR. The methylation pattern of cytosines not positioned in sites for methylation-sensitive restriction enzymes can be determined by genomic sequencing. This technique uses the chemical sequencing reactions described in ref. 19. The distinction between 5-methylcytosine and cytosine is caused by the lack of reactivity of the cytosine-specific reagent hydrazine toward 5-methylcytosine (24).

To obtain information on methylation of most CpGs in the B-hordein promoters, three sets of overlapping primers for LMPCR were synthesized (Fig. 1*A*). The primers were positioned in highly conserved sequences to facilitate simultaneous sequencing of all or most of the B-hordein promoters in the barley genome. This strategy was used to avoid sequencing of a silent or otherwise abnormal single gene. The two known promoters differ at 1-, 5-, and 3-nucleotide positions in the sequences covered by primer set 1, 2, and 3, respectively (Fig. 1*B*). Where polymorphism occurred, the primer sequences were identical to genomic clone pBHR184 (22) because this clone was isolated from a barley cultivar (Sundance) with a B-hordein polypeptide pattern identical to Bomi. The sequence of the common linker was modified from the original LMPCR protocol (20) to give an oligonucleotide with a melting temperature similar to that of the other primers (Fig. 1*C*).

Cytosine-specific reactions were done on leaf and endosperm DNA from both wild-type and the *lys3a* mutant. The guanine, adenine plus guanine, and thymine plus cytosine reactions were included for a single DNA sample to facilitate alignment of sequences and confirm that methylation only occurs at the expected positions (CpGs and CpNpGs). The genomic sequencing gels are shown in Fig. 3.

Intense bands are present at most CpG positions (indicated by arrowheads) in the Bomi endosperm lane, whereas the corresponding bands are weak or absent in the cytosine-specific reactions of wild-type leaf, *lys3a* leaf, and *lys3a* endosperm DNA. Variations in band intensity within a lane and the relatively strong background ladder hindered a fully quantitative analysis of methylation. Instead, cytosines are described as fully methylated when the corresponding band is absent or has an intensity similar to the neighboring interbands. The promoter sequence of Bomi endosperm DNA appears unmethylated because the band intensity at potential methylation sites is comparable to the other cytosine bands in the sequence, except at sites of probable polymorphism (see below). Bands with intensities between these two states are described as partially methylated.

A total of 14 CpGs was analyzed by LMPCR. Of these, 10 CpGs were in the promoter region, and 4 CpGs were within the first 56 nucleotides of the coding region. The two CpGs found at positions −281 and −324 are a consequence of sequence polymorphisms within the gene family because they are not present in pBHR184, which has TpGs at both these positions. The CpG located approximately at position −550 is also missing in pBHR184, but this position is such that the sequence from the LMPCR cannot be aligned with the cloned sequence.

The methylation patterns in wild-type and *lys3a* leaf DNA are identical, except at position +56. This difference might be

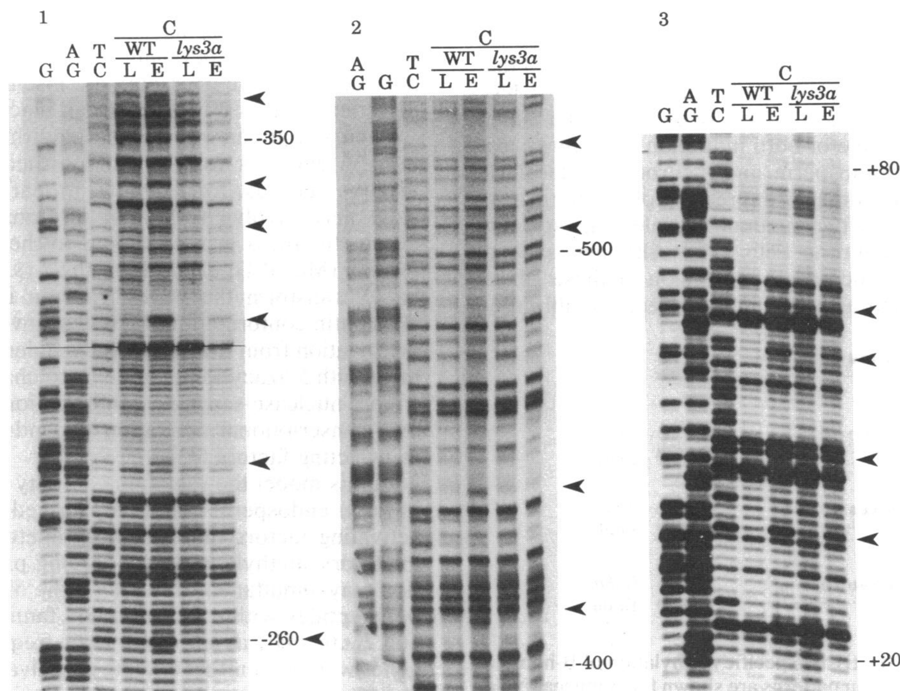

FIG. 3. Genomic sequences of B-hordein promoters. LMPCR was done with the three primer sets (sets 1, 2, and 3) shown in Fig. 1. With primer sets 1 and 3 the guanine (G), adenine plus guanine (A+G), and thymine plus cytosine (T+C) control reactions contained DNA from Risø mutant 1508 endosperm, whereas Bomi endosperm DNA was used with primer set 2. Cytosine (C)-specific reactions were made on DNA from Bomi [wild type (WT)] and Risø mutant 1508 (*lys3a*) isolated from leaf (L) or endosperm (E). CpG dinucleotides are indicated by arrowheads, and nucleotide positions are given relative to the translation start site in the genomic clone pBHR184 (22).

an artifact because the background ladder in the lane with *lys3a* endosperm DNA is very strong. Leaf DNA escapes full methylation at three positions—the first position at −336 probably represents a polymorphism because λhor2-4 differs from pBHR184 at this position. The other two sites (−260 and +39) might exemplify additional sequence requirements of the methylation mechanism, as they are both part of CpGpG trinucleotides and are surrounded by additional cytosines and guanines. A similar bias against full methylation of CpGs in (G+C)-rich surroundings has been observed in the human phosphoglycerate kinase 1 gene on the inactive X chromosome of females (25).

The mutant endosperm DNA retains the methylation pattern of leaf on eight of the CpGs in the promoter, whereas the remaining two sites are partially methylated. This contrasts with the complete absence of methylation in Bomi endosperm. Nevertheless, the CpGs in the coding region of *lys3a* endosperm are hypomethylated, which is similar to the wild-type endosperm methylation pattern.

Results of the LMPCR experiments are summarized in a schematic form in Fig. 4. Interestingly no methylation was seen in any of the CpNpGs included in the sequence analyzed by LMPCR (Fig. 4), except in one CpGpG position described above. This result lends support to the hypothesis that CpNpG methylation in plants has a function distinct from the CpG methylation, which is based on experiments showing inhibited expression of DNA methylated *in vitro* on CpG positions and introduced into petunia protoplasts (14).

DISCUSSION

This study has shown that hypomethylation of B-hordein promoters is correlated not only with the specific expression of the corresponding genes in the endosperm, as for the zeins in maize (12), but also with the presence of a wild-type allele of the regulatory *Lys3* gene.

The phenotypic effect of the *lys3a* mutation on expression of storage proteins is somewhat similar to that of the maize opaque-2 mutation. The opaque-2 gene was isolated by transposon tagging (26, 27) and encodes a transcriptional activator of the leucine zipper type (28, 29), which will bind to sequences in the zein promoter region (30, 31). It has recently been suggested that *Lys3* encodes an opaque-2-like trans-acting factor capable of both induction and repression and present in both endosperm and aleurone tissue. This suggestion is based on increased expression of chymotrypsin inhibitor 1 and 2 and of two unidentified aleurone-specific transcripts in *lys3a* aleurone cells (32). Although such a model is possible, the increased expression of these genes can easily be explained as a secondary effect possibly mediated by the increased level of free amino acids in *lys3a* endosperm (33). This hypothesis is supported by the equally increased expression of chymotrypsin inhibitor 1 in the *lys3a* and *hor2ca* mutants (34), as the latter is a deletion of the structural genes encoding B-hordein (35). The genomic sequencing shows that *lys3a* is more probably involved in early events of barley endosperm differentiation because the absence or inhibition of a trans-acting factor that interacts at specific sites in the promoters of the affected genes could not cause the differential methylation observed throughout the B-hordein promoters.

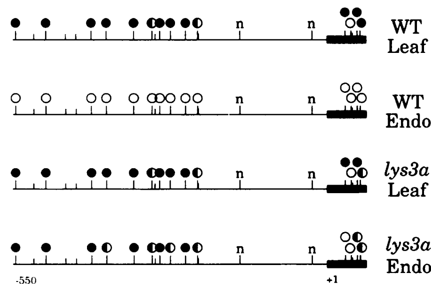

FIG. 4. Summary of the tissue-specific methylation of B-hordein promoters. DNA methylation patterns are shown for Bomi leaf [wild type (WT) Leaf], Bomi endosperm (WT Endo), Risø mutant 1508 leaf (*lys3a* Leaf), and Risø mutant 1508 endosperm (*lys3a* Endo). Short and long vertical lines indicate CpNpG and CpG tri- and dinucleotides, respectively. Circles represent the methylation status of CpGs: ●, full methylation; ◐, partial methylation; ○, no methylation; and n, site not analyzed.

The suggestion that the *Lys3* gene product is involved in events before transcriptional activation is supported by the equal reduction in protein Z and hordein mRNA levels in the *lys3a* mutant (3), as the sequences of their promoters share little homology (36). Further support comes from the observation that demethylation of zein genes apparently precedes the activation of these genes (12).

Methylation has been reported to participate in transcriptional regulation by different mechanisms. Methylation at specific CpGs can directly inhibit the binding of activating factor, as for the cAMP-responsive element present in a number of mammalian genes (37). Alternatively, binding of activating factors can be excluded indirectly by methylated DNA-binding proteins. These might be site-specific—e.g., binding of the methylated DNA-binding protein (MDBP-2) to the promoter of the avian vitellogenin gene (38) or bind to any sequence containing enough methylated CpGs. Such a protein is methyl-CpG-binding protein (MeCP-1), which inhibits transcription from several promoters when these promoters are methylated (39). Methylation has also been correlated with chromatin conformation (40). When a methylated herpes simplex virus thymidine kinase gene was introduced into mouse or rat cell cultures by microinjection, transcriptional inhibition only occurred after assembly of the introduced DNA into chromatin. In contrast, transcription of *in vitro* reconstituted chromatin made from methylated DNA and isolated histones was inhibited immediately after microinjection (41). It has been proposed that the methyl-CpG-binding protein (MeCP-1) participates in the assembly of methylated DNA into transcriptionally inactive chromatin because it provides the same characteristic insensitivity toward *Msp* I digestion as does chromatin in an inactive form (42).

Therefore it can be hypothesized that methylation of the hordein genes is responsible for their maintenance in an inactive state as, for example, in leaf tissue. This inactive state may involve formation of transcriptionally inactive chromatin and a protein similar to the methyl-CpG-binding protein (MeCP-1). The role of the *Lys3* product would then be to transform the genes from an inactive to an active chromatin conformation, a process involving the removal of methylation from the genes. Indeed demethylation, by treatment with 5-azacytidine, appears to change the chromatin to a more nuclease-sensitive conformation, whereas simultaneous transcriptional activation depends on the presence of trans-acting factors (43).

In this model the remaining activity of the affected genes in *lys3a* endosperm could be ascribed to the abundance of activating factors that would compete with binding of the inhibitory methylated DNA-binding protein. This competition may simultaneously explain the different prevalence of polypeptides within each hordein family between wild type and *lys3a* (44), as different CpG frequencies in individual genes would not affect binding of activating factors but could change affinity for the inhibitory protein. A similar model for reactivation by strong trans-activators of methylated promoters containing relatively few CpGs has been suggested (39). In any case, the low but significant transcription of hordein genes in the mutant endosperm might interfere with the maintenance of methylation to cause the partial demethyl-

ation seen in the B-hordein-coding region of the mutant endosperm.

The validity of this hypothesis could be further tested by transient expression studies of hordein promoters in wild-type and *lys3a* mutant endosperm and by analysis of chromatin conformation by nuclease treatment of intact isolated nuclei.

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1. Ingversen, J., Køie, B. & Doll, H. (1973) *Experientia* **29**, 1151–1152.
2. Shewry, P. R., Faulks, A. J. & Miflin, B. J. (1980) *Biochem. Genet.* **18**, 133–151.
3. Sørensen, M. B., Cameron-Mills, V. & Brandt, A. (1989) *Mol. Gen. Genet.* **217**, 195–201.
4. Kreis, M., Williamson, M., Buxton, B., Pywell, J., Hejgaard, J. & Svendsen, I. (1987) *Eur. J. Biochem.* **169**, 517–525.
5. Kreis, M., Shewry, P. R., Forde, B. G., Rahman, S., Bahramian, M. B. & Miflin, B. J. (1984) *Biochem. Genet.* **22**, 231–255.
6. Forde, B. G., Kreis, M., Bahramian, M. B., Matthews, J. A. & Miflin, B. J. (1981) *Nucleic Acids Res.* **9**, 6689–6707.
7. Razin, A. & Cedar, H. (1991) *Microbiol. Rev.* **55**, 451–458.
8. Messeguer, R., Ganal, M. W., Steffens, J. C. & Tanksley, S. D. (1991) *Plant Mol. Biol.* **16**, 753–770.
9. Palmgren, G., Mattsson, O. & Okkels, F. T. (1990) *Biochim. Biophys. Acta* **1049**, 293–297.
10. Gruenbaum, Y., Naveh-Many, T., Cedar, H. & Razin, A. (1981) *Nature (London)* **292**, 860–862.
11. Ngernprasirtsiri, J., Kobayashi, H. & Akazawa, T. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7919–7923.
12. Bianchi, M. W. & Viotti, A. (1988) *Plant Mol. Biol.* **11**, 203–214.
13. Weber, H., Ziechmann, C. & Graessmann, A. (1990) *EMBO J.* **9**, 4409–4415.
14. Hershkovitz, M., Gruenbaum, Y., Renbaum, P., Razin, A. & Loyter, A. (1990) *Gene* **94**, 189–193.
15. Murray, M. G. & Thompson, W. F. (1980) *Nucleic Acids Res.* **19**, 4321–4325.
16. Reed, K. C. & Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221.
17. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
18. Sørensen, M. B. (1989) *Carlsberg Res. Commun.* **54**, 109–120.
19. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
20. Mueller, P. R. & Wold, B. (1989) *Science* **246**, 780–786.
21. Brandt, A., Montembault, A., Cameron-Mills, V. & Rasmussen, S. K. (1985) *Carlsberg Res. Commun.* **50**, 333–345.
22. Forde, B. G., Heyworth, A., Pywell, J. & Kreis, M. (1985) *Nucleic Acids Res.* **13**, 7327–7339.
23. Rogers, J. C. (1985) *J. Biol. Chem.* **260**, 3731–3738.
24. Ohmori, H., Tomizawa, J.-I. & Maxam, A. M. (1978) *Nucleic Acids Res.* **5**, 1479–1485.
25. Pfeifer, G. P., Tanguay, R. L., Steigerwald, S. D. & Riggs, A. D. (1990) *Genes Dev.* **4**, 1277–1287.
26. Motto, M., Maddaloni, M., Ponziani, G., Brembilla, M., Marotta, R., Fonzo, N. D., Soave, C., Thompson, R. & Salamini, F. (1988) *Mol. Gen. Genet.* **212**, 488–494.
27. Schmidt, R. J., Burr, F. A. & Burr, B. (1987) *Science* **238**, 960–963.
28. Schmidt, R. J., Burr, F. A., Aukerman, M. J. & Burr, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 46–50.
29. Hartings, H., Maddaloni, M., Lazzaroni, N., Fonzo, N. D., Motto, M., Salamini, F. & Thompson, R. (1989) *EMBO J.* **8**, 2795–2801.
30. Aukerman, M. J., Schmidt, R. J., Burr, B. & Burr, F. A. (1991) *Genes Dev.* **5**, 310–320.
31. Lohmer, S., Maddaloni, M., Motto, M., Fonzo, N. Di., Hartings, H., Salamini, F. & Thompson, R. D. (1991) *EMBO J.* **10**, 617–624.
32. Jacobsen, K. S., Kalvenes, C. & Olsen, O.-A. (1991) *Physiol. Plant.* **83**, 201–208.
33. Brandt, A. (1976) *Cereal Chem.* **53**, 890–901.
34. Williamson, M. S., Forde, J. & Kreis, M. (1988) *Plant Mol. Biol.* **10**, 521–535.
35. Kreis, M., Shewry, P. R., Forde, B. G., Rahman, S. & Miflin, B. J. (1983) *Cell* **34**, 161–167.
36. Brandt, A., Svendsen, I. & Hejgaard, J. (1990) *Eur. J. Biochem.* **194**, 499–505.
37. Iguchi-Ariga, S. M. M. & Schaffner, W. (1989) *Genes Dev.* **3**, 612–619.
38. Jost, J.-P., Saluz, H.-P. & Pawlak, A. (1991) *Nucleic Acids Res.* **19**, 5771–5775.
39. Boyes, J. & Bird, A. (1991) *Cell* **64**, 1123–1134.
40. Keshet, I., Lieman-Hurwitz, J. & Cedar, H. (1986) *Cell* **44**, 535–543.
41. Buschhausen, G., Wittig, B., Graessmann, M. & Graessmann, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1177–1181.
42. Lewis, J. & Bird, A. (1991) *FEBS Lett.* **285**, 155–159.
43. Michalowsky, L. A. & Jones, P. A. (1989) *Mol. Cell. Biol.* **9**, 885–892.
44. Klemsdal, S. S., Olsen, O.-A. & Rørvik, K. A. (1987) *Hereditas* **107**, 107–114.