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Cellular localization of the embryo-specific Hybrid PRP from *Zea mays*, and characterization of promoter regulatory elements of its gene

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Abstract The expression, regulation and cellular localization of *ZmHyPRP,* a gene marker of embryo differentiation whose expression declines after ABA induction, was studied. *ZmHyPRP* is a proline-rich protein with a C-terminal domain having eight cysteines in a CM8 pattern. Transient expression in onion epidermal cells, transformed with a *2x35S::ZmHyPRP-GFP* construction, indicated the protein is present in vesicles lining the membrane of the cell. The *ZmHyPRP* gene expression is under the control of classic promoter seed-specific regulatory elements such as Sph/RY and G-boxes, suggesting regulation by B3 and b-ZIP transcription factors. Promoter deletion analysis, by particle-bombardment transient transformation of maize immature embryos with serial deletions of the promoter fused to GUS, showed the presence of two negative regulatory elements, NE1 (−2070 to −1280) and NE2 (−232 to −178), in the *ZmHyPRP* promoter. By selective deletion or mutation of *ZmHyPRP* regulatory promoter elements we conclude that the promoter expression is attenuated by the NE2 element as well as by the G-box2 and the Sph1-2 box together with the G-box2.

Keywords Maize · *ZmHyPRP* · Protein traffic · Promoter analysis · Scutellum tissue specificity

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Introduction

Embryogenesis is an important process in plant development. Three main phases of seed formation can be distinguished: morphogenesis (active cell division and organ differentiation), maturation (accumulation of carbohydrates, storage proteins and lipids) and desiccation (expression of late-embryogenesis abundant genes, *LEA*, giving the seed resistance to water stress). These processes have been the focus of numerous studies in dicotyledonous plants such as Arabidopsis, Brassica and legumes, as well as in monocotyledonous plants including maize, wheat, barley and rice (reviewed by North et al. 2010; Weber et al. 2005; Finkelstein et al. 2008). Many transcriptional factors involved in plant embryogenesis have been identified among plant mutants able to germinate in the presence of ABA, and from leafy cotyledon mutants. Among them are different B3 domain transcription factors such as *VP1* (McCarty et al. 1991), *FUSCA 3* (Luerβen et al. 1998) and *LEC2* (Stone et al. 2001), the basic leucine zipper (b-ZIP) factor *ABI5* (Finkelstein and Lynch 2000), and the HAP3 subunit of the CAAT-box binding factor *LEC1* (Lotan et al. 1998). These transcriptional factors are able to regulate embryo development and maturation processes, metabolic quiescence and adaptation to survive desiccation, but also repress the appearance of leaf traits in cotyledons and germination processes (reviewed by Braybrook and Harada 2008; Santos-Mendoza et al. 2008; and Suzuki and McCarty 2008).

These transcription factors control expression of target genes with the respective consensus binding boxes in their promoters. The targets for B3 domain transcription factors are the Sph/RY boxes (CATGCATGCA/CATGCATG), while b-ZIPs recognize the core ACGT palindromic sequence with different variants in function of the different target genes and studied b-ZIPs (Thomas 1993). Different

combinations and number of these two elements can be found in the promoters of different classes of genes with a function in embryogenesis. One of the most extensively studied B3 domain transcription factors is *VP1/ABI3*, coding for a nuclear protein with an acidic domain in the N-terminal end of the protein, with a transcription activation function, and three basic domains. The B3 domain is involved in DNA interaction, and the B1 and B2 domains are involved in protein–protein interactions with very diverse proteins including 14-3-3-proteins, the homolog of the human helix-loop-helix domain C1 protein involved in cell cycle control, and rice *TRAB1* (b-ZIP factor) among others (Finkelstein et al. 2002; Santos-Mendoza et al. 2008). Different b-ZIPs have been found to be involved in ABA-regulated seed expression. They usually have an N-terminal domain rich in proline and alanine residues, characteristic of transcriptional activation domains, a nuclear localization signal and the leucine zipper domain with a heptad repeat of leucines involved in homo and heterodimerization b-Zip processes previous to DNA binding (Jakoby et al. 2002).

In the present paper we present results on the cellular localization, expression and regulation of *ZmHyPRP*, a gene under the control of Sph and G-box regulatory elements, expressed in the embryo from scutellum differentiation, then declining after ABA induction (Jose-Estanyol et al. 1992; Jose-Estanyol and Puigdomènech 1998). Using maize stable transformation, we have previously shown that 2 Kb of the *ZmHyPRP* promoter fused to GUS contain the necessary information to correctly express the gene in the embryo (Jose-Estanyol et al. 2005). Our results in this paper indicate that *ZmHyPRP* is localized in cellular vesicles lining the membrane of the cell. From deletion and specific mutation studies of the promoter regulatory elements, we propose that the *ZmHyPRP* gene, as a result of its promoter chromatin conformation and regulatory elements distribution, must recruit particular factors before and after ABA induction that define its repressive behavior.

Materials and methods

Biological materials

Unless otherwise stated the plant material used was derived from seeds of a maize (*Zea mays* cv. W64A) pure inbred line grown in a greenhouse in Barcelona, Spain. Kernels were collected at different developmental stages and stored at −80 °C. Homozygous caryopses of *viviparous-1 (vp1)* mutants of *Z. mays* L. were obtained from Dr. R. J. Lambert, Maize Genetic Stock Center, University of Illinois, Urbana, USA.

Protein fusion expression

The coding region of *ZmHyPRP* was fused to GFP by cloning in the NcoI site of the S65C/T vector (gift from Dr. Robert Blanvillainn) from $pZmHyPRP_{3500}$. Previously, three mutations were introduced in the coding region of *ZmHyPRP* using the QuickChange Site-directed Mutagenesis Kit (Stratagene). The first was to eliminate an internal NcoI site in the *ZmHyPRP* coding region: **5′** 5′ CAA GCA CCA CCA **C**GG CAA GCC GCC C 3′ and **3′** 5′ GGG CGG CTT GCC **G**TG GTG GTG CTT G 3′ with no change in protein sequence. The second was to introduce a NcoI restriction enzyme site in the ATG of the first protein codon: **5′** 5′ TTG AGC CAA GGC GC**C** ATG GCA ACC TCC ACC 3′ and **3′** 5′ GGT GGA GGT TGC CAT **G**GC GCC TTG GCT CAA 3′. And the third mutation was to eliminate the stop codon, and to introduce a nucleotide to allow phasing with the GFP as well as a Nco restriction enzyme site: **5′** 5′ GCC GCT CTA CGA C**AC C**AT G**G**A CAC CAC CAC CAG 3′ and **3′** 5′ CTG GTG GTG GTG T**C**C AT**G G**T**G** TCG TAG AGC GGC 3′ (mutated nucleotides are in each case underlined and in bold). The new NcoI–NcoI fragment in the mutated $pZmHyPRP_{3500}$, was cloned, after digestion with NcoI, in the S65C/T NcoI cloning site under the control of a 2x35S promoter before the GFP coding region. Microbombardment with gold tungsten particles, coated as described in Jose-Estanyol et al. (2005), was used for transient expression of the GFP fusion protein in onion bulb epidermis cells. These cells were incubated in PIPES solid medium (Scott et al. 1999) for two hours before bombardment and then for 16, 24 or 48 h in the same medium before mounting on slides for imaging with a laser scanning confocal microscope, Fv1000-ASW (Olympus, Tokyo, Japan). Excitation was at 488 nm laser lines and green emission detected at 515 nm.

RNA preparation and gel-blot analysis

RNA preparation, RNA gel blot transfer to nylon membranes, hybridizations and probes were as indicated in Jose-Estanyol and Puigdomènech (1998) except for the following modifications. The hybridization signal was quantified from a storage phosphor screen with the Storm 820 PhosphorImager (GE Healthcare Bio-Sciences) and normalized to gel rRNA levels after gel staining with ethidium bromide (EtBr). Each membrane was hybridized with the probes of interest, with prior dehybridization following the manufacturer's instructions.

Genomic cloning and sequencing

Genomic cloning and sequencing was as described in Jose-Estanyol et al. (1992).

Particle bombardment

Maize embryo transient transformation was by particle bombardment of 16 dap immature embryos with constructions containing *ZmHyPRP* promoter deletions or mutated consensus boxes. The *ZmHyPRP*$_{-2070}$*::GUS* construct in pBI201.3 was as described in Jose-Estanyol et al. (2005). Successive promoter deletions and mutations of the *ZmHyPRP* promoter were obtained as described in supplementary material 1. Gold particles coated with the different constructions were used for bombardment. After ear excision, embryos were placed on MSO medium (Murashige and Skoog medium supplemented with 30 g/l sucrose) with or without 50 μM ABA. Biolistic assays were carried out 24 h after excision with a PDS1000/He (Dupont, Biorad) using a helium pressure of 900 psi, as described in Jose-Estanyol et al. (2005). After bombardment, embryos were left in the MSO medium for 24 h before fluorometric or quantitative histochemical analysis as described in Jose-Estanyol et al. (2005), with one modification in the histochemical quantitative analysis. Blue spots/basic units were classified as a function of their diameter and then quantified as follows: twelve basic units for spots of 80 μm ϕ, six basic units for spots of 40 μm ϕ, 3 basic units for spots of 20 μm ϕ and one basic unit for spots of less than 20 μm ϕ. This procedure allowed a more precise histochemical quantification. In each experiment, three dishes with nine embryos each were bombarded for each construction studied. The results are the average value of one to four different experiments. The standard deviation was calculated from the means of the different experiments when more than two experiments were considered; when only one or two experiments were considered it was calculated from the means of each replica. For fluorometric quantification, internal controls of bombardment efficiency were by co-bombardment of each construct with one containing a constitutive promoter, maize ubiquitin, fused to luciferase (*pUBI::LUC-nos-ter*). For quantitative histochemical analyses, a different set of embryos were bombarded with a constitutive promoter for monocots, such as *OsActine::GUS-nos-ter* (McElroy et al. 1990), to establish visual differences in the expression level between experiments. Samples from the same extracts were used to measure sample luminescence, with a Luciferase Assay System (Promega) and fluorescence (Jose-Estanyol et al. 2005). Measurements were taken using a Spectra max M3 apparatus (bioNova cientifica S.L.). The *p value*, to determine statistically significant differences between constructs and between the values for −/+ ABA, was calculated using the average values from the different replicas or from the different experiments in a Student's *t* test (http://www.physics.csbsju.edu).

Results

Cellular localization of *ZmHyPRP*

ZmHyPRP is a gene mainly expressed in the scutellum of maize immature embryos. The coding region contains a signal peptide for export out of the cell, followed by a domain of proline-rich repeats, similar to those described for cell wall proteins, and a hydrophobic C-terminal domain, not proline-rich and with eight cysteines in a CM8 pattern (Jose-Estanyol et al. 1992, 2004). We studied the *ZmHyPRP* cellular localization by transient expression after particle bombardment of onion bulb epidermal cells with the *2x35S::ZmHyPRP-GFP* construct. The fluorescence was observed in a cell pattern similar to vesicle-like structures in the cell cytoplasm (Fig. 1a, b) and lining the plasma membrane in sections where the volume of the cell is mainly occupied by the cell tonoplast-vacuole (Fig. 1c).

ZmHyPRP gene expression levels in maize embryos related to different developmental markers

ZmHyPRP expression is mainly associated to the period of cell division, but also to the elongation phase. As previous studies indicated that the expression of *ZmHyPRP* and that of the *H4*, a marker of cell division, is repressed after treatment of excised maize immature embryos with exogenous ABA hormone (Jose-Estanyol and Puigdomènech 1998) we used RNA analysis to compare their expression patterns in maize embryos at different development stages. Results show (supplementary material 2a) that after ABA induction at 18 dap, when ABA levels in maize embryos are around 200 ng/gr in DWT (dry weight tissue) (Jones and Brenner 1987), the disappearance of *H4* gene expression is faster than for *ZmHyPRP*. *ZmHyPRP* expression slowly declines to a basal level after 30 dap. In contrast, the *LEA* gene *RAB28* is expressed during the maturation and desiccation phases, mainly after maximum ABA levels are attained in the embryo (1,500 ng/gr DWT), around 25 dap (Jones and Brenner 1987).

We also compared the time of expression of the described genes, with respect to the time of maximum enlargement of the seed, under our greenhouse conditions. To do this we excised and weighed maize kernels at different days after anthesis, then excised and weighed

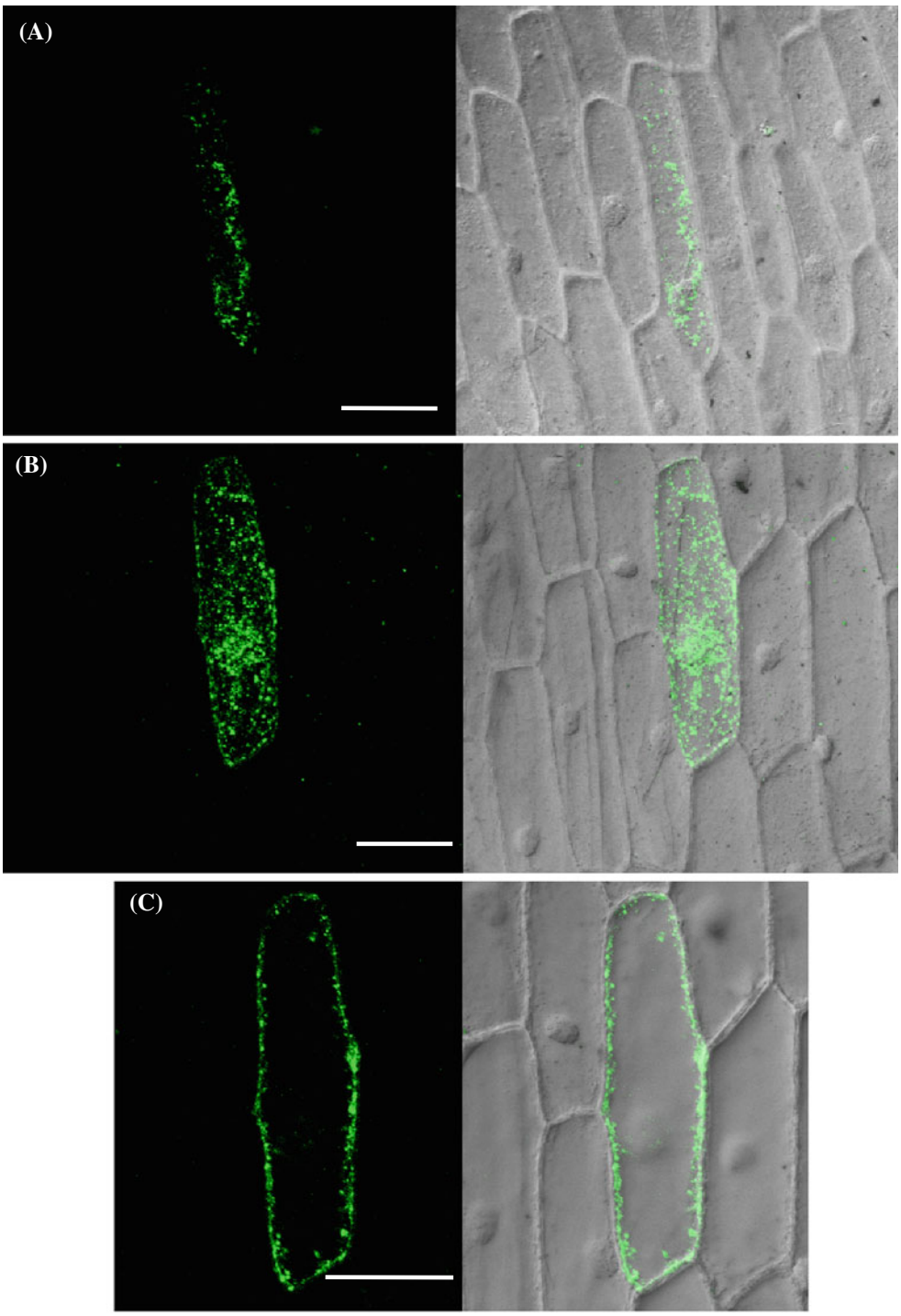

Fig. 1 *ZmHyPRP* cellular localization. Onion epidermal bulb cells bombarded with the *2x35S::ZmHyPRP-GFP* construct were observed through a confocal microscope. Images of different cells in pipes medium: **a**, **b** Fluorescent pattern images revealed presence of the protein in vesicle-like structures. **c** An optical section image of the fluorescent pattern in **b** corresponding to the vacuole region, showing vesicles lining the membrane of the bombarded onion epidermis bulb cells. *Left* dark field. *Right* superimposed dark and light fields. *Bars* 100 μm

the embryos (supplementary material 2b). We found that the increase in weight of the kernel and embryo evolved in parallel. The maximum weight increase was at 30 dap, at the time of maximum RAB28 expression, much later than the period of H4 expression, coinciding with the disappearance of *ZmHyPRP* mRNA expression.

ZmHyPRP promoter sequence and delineation of cis-acting elements by 5′ deletion analysis of the *ZmHyPRP* promoter

Our previous results have allowed us to define *ZmHyPRP* as a gene mainly expressed in the scutellum of maize embryos. After ABA induction its expression is reduced, and disappears before desiccation of the kernel. Different consensus boxes have been identified in the *ZmHyPRP* promoter sequence (http://www.genomatix.de/). The location of the transcription start site has been proposed at −36 bp from the ATG, as a result of analysis of maize ESTs in the maize databank (http://www.maizegdb.org) and of ATG established consensus sequences (Cavener and Ray 1991). A classical TATAAA box is located at −74 bp from the ATG. Other regulatory elements related to seed expression have been identified (Fig. 2). The described *ZmHyPRP* promoter sequence of *Zea mays* cv. W64A has been found to have 94.67 % similarity with *Zea mays* cv. B73, from −1 to −1,038 bp, and this also includes all the described regulatory elements (http://www.maizegdb.org).

The regulatory elements in the *ZmHyPRP* promoter agree with those for seed specific genes except that *ZmHyPRP* promoter expression does not increase after ABA induction and it is not expressed in the aleurone endosperm cell layer as are seed specific genes, usually (Kroj et al. 2003; Panitz et al. 1995; Hong et al. 1992). This particular behavior led us to study the contribution of *ZmHyPRP* promoter regulatory elements to its expression. Successive promoter deletions (Fig. 3) of the *ZmHyPRP* promoter (−2070) fused to GUS in the pBI201.3 vector were studied by transient expression, using particle bombardment of excised 16–18 dap maize immature embryos maintained in MSO in the absence or presence of 50 μM ABA. Deletions were initiated from −2070 bp as, in a previous study, *ZmHyPRP*$_{-2070}$*::GUS* maize transgenic plants (Jose-Estanyol et al. 2005) were able to reproduce the *ZmHyPRP* embryo specific cellular expression pattern defined by in situ hybridization (Jose-Estanyol et al. 1992).

We first used RNA analysis to confirm that the experimental conditions did not alter the embryogenesis program, as the expression of different embryo markers is unchanged (supplementary material 3). The GUS expression level of the deletion constructions determined by quantitative fluorometric analysis is shown in Fig. 3a. Two negative regulatory elements were identified, one in the region between −2070 bp and −1280 bp and a second between −232 bp and −178 bp. Apart from these two exceptions, serial deletions resulted in a successive diminution of promoter activity as a result of the loss of positive regulatory elements. In all cases, expression was lower in the presence of ABA. As *ZmHyPRP* expression is mainly localized in the embryo abaxial scutellar side and extracts were from full embryos, the expression level of the deletions was considerably diluted as the cells involved in *ZmHyPRP* expression represent only a small fraction of the pool of embryo cells. For this reason, histochemical quantitative analysis was done in parallel (Fig. 3b), corresponding with the results shown in Fig. 3a, but better defined. The difference in expression between −232 and −178 constructions was found to double and −/+ ABA treatments were statistically significant for all constructs.

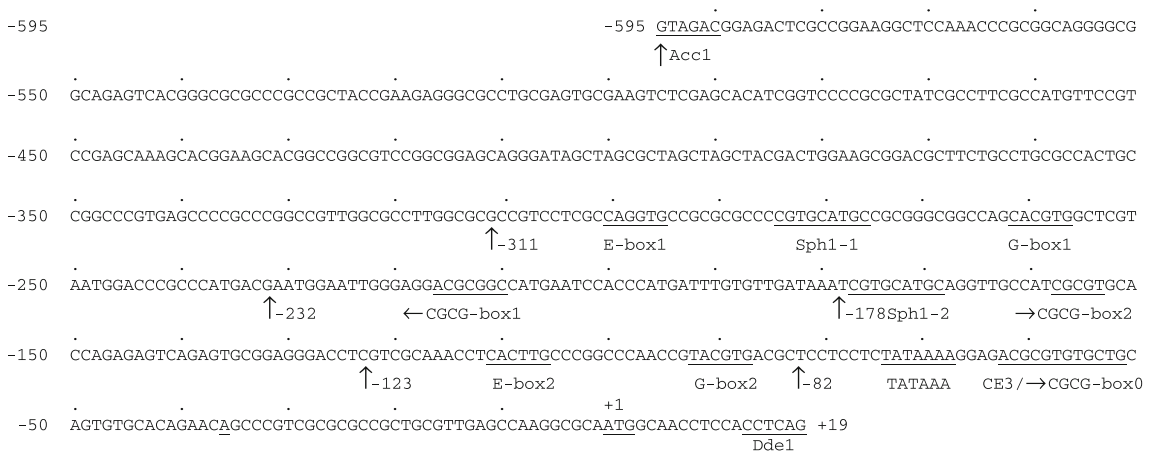

Fig. 2 Nucleotide sequence of the *ZmHyPRP* promoter. Nucleotides are numbered from the start codon for translation to −595 bp. Restriction enzymes used to subclone the promoter fragment and consensus identified promoter boxes are underlined. Regulatory elements: −74 putative TATAAA box; −262 and −92, G-boxes, targets of b-Zips transcription factors (Jakoby et al. 2002; Marcotte et al.1989; Mundy et al. 1990); −300 and −111, E-boxes, targets for b-helix-loop-helix transcription factors (Thomas 1993); −284 and −177, Sph/RY boxes, targets for B3 transcription factors (supplementary material 6) (Thomas 1993); −211 and −158, CGCG boxes, targets for calmodulin binding/CGCG box binding proteins (Yang and Poovaiah 2002); and −63 CE3 coupling element/CGCG box (Shen et al. 1996). *Arrows* (↑) indicate the location and distance from the ATG, for the different promoter serial deletions analyzed in the study of the *ZmHyPRP* promoter

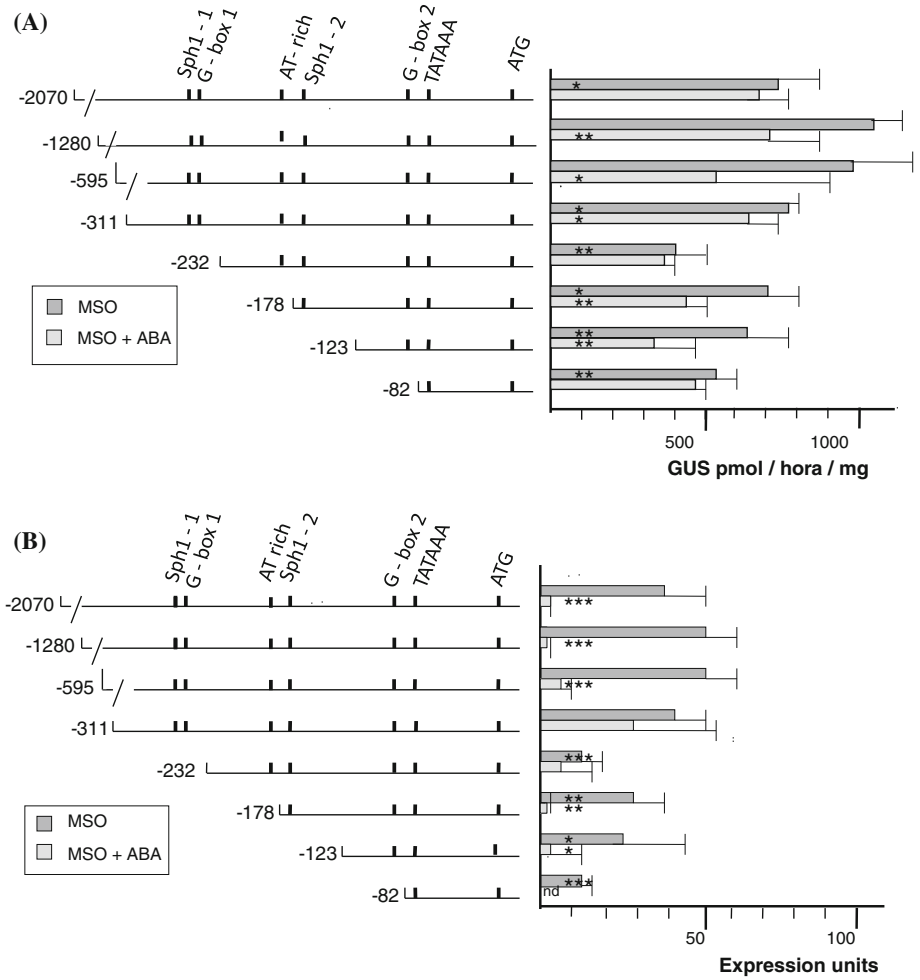

Fig. 3 Deletions of *ZmHyPRP*$_{-2070}$*::Gus* fusion promoter construct analyzed by transient expression after particle bombardment of maize excised immature 16–18 dap embryos. *Left* **a**, **b** The constructions and the location of the regulatory elements studied. *Right* **a** Quantitative fluorometric analysis, **b** Quantitative histochemical analysis. The *p* value or probability that differences are not a result of the serial deletions or of the (−/+) ABA treatments is indicated by asterisks: from 25 to 5 % (*); 5 to 1 % (**) and less than 1 % (***)

The exception of the −232 deletion was the consequence of large variation in values obtained from the replicas studied, due to partial inhibition in one of them.

Construction of new promoters by internal deletions of the *ZmHyPRP* promoter

The *ZmHyPRP* promoter has at least two negative regulatory elements in its promoter and lacks a positive response to the ABA hormone, possibly as a result of the absence of any functional ABA coupling element or a consequence of the presence of negative regulatory elements in the promoter.

For further insight, we first deleted the NE2 (−232 to −178 bp) negative regulatory element (Δ −225 to −179 bp), from the promoter deletion of −595 bp, as reference, to confirm its functionality in the presence of preceding sequences.

Expression levels of bombarded immature embryos were quantified by histochemical analysis, as for the aforementioned promoter serial deletions. The NE2 deletion resulted in a nearly two-fold increase in expression with respect to the control, confirming its silencing function either in the presence or in the absence of distal promoter elements (Fig. 4).

Secondly, we created new promoters based on 50 bp deletions of each regulatory element (30 bp for G-box2 deletion), from the −595 bp *ZmHyPRP* promoter deletion, to define their positive or negative contribution to the *ZmHyPRP* promoter expression (Fig. 4, left). The results indicated that there was a significant reduction of expression with deletion of Sph1-1/G-box1 elements (Δ −294 to −233 bp), while there was a significant increase on deletion of the G-box2 (Δ −112 to −83). Minor differences were observed after deletion of the Sph1-2 element (Δ 179 to 124) (Fig. 4, right). We consider that these results

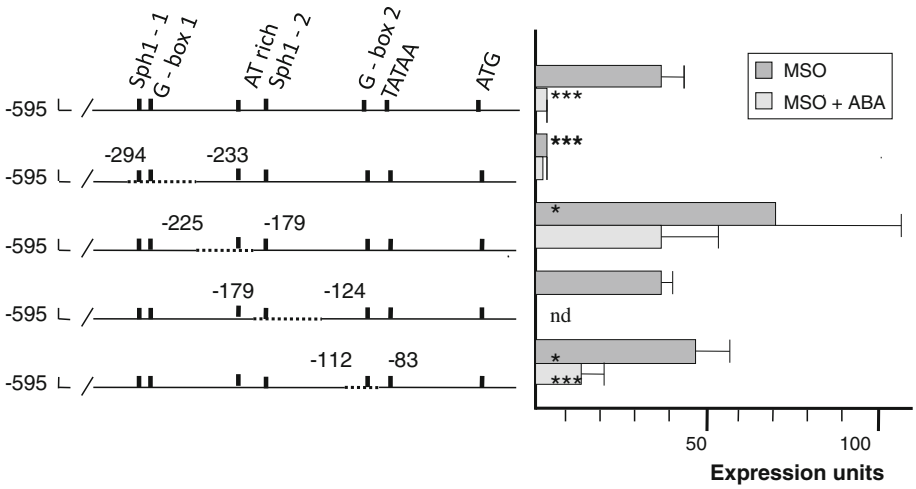

Fig. 4 Internal deletions of *ZmHyPRP*$_{-595}$*::Gus* fusion promoter analyzed by transient expression after particle bombardment of maize excised immature 16–18 dap embryos. *Left* The constructions with internal deletions and the location of the regulatory elements studied. Deletion location is indicated by (….) and the nucleotide distance from the ATG to the deletion limits is given. **Right**: Quantitative histochemical analysis. The *p* value or probability that differences are not a result of the internal deletions or of the (−/+) ABA treatments is indicated by asterisks: from 25 to 5 % (*); 5 to 1 % (**) and less than 1 % (***)

confirm the presence of sequences in the *ZmHyPRP* promoter that result in attenuation of its expression in the maize embryo, independent of (Δ NE2) or dependent on (Δ G-box2) the preceding promoter sequences, as expression is enhanced in the absence of these sequences. In contrast, Sph1-1/G-box1 elements appear to be necessary for promoter expression as it is reduced in their absence. None of the deletions allowed clear induction of expression of the *ZmHyPRP* promoter by ABA, but a statistically significant difference in the −/+ ABA results, similar to that described for the −595 control construction, was observed for the G-box2 deletion.

Expression analysis of the *ZmHyPRP* promoter mutated in consensus regulatory boxes

Deletion studies have described the presence of positive and negative elements in the *ZmHyPRP* promoter. Some ambiguity arises as to whether they are a consequence of the regulatory boxes present in the different deletions, of the sequences surrounding them, or of both.

To distinguish between these different possibilities we mutated the different boxes and combinations of them (Fig. 5a, b, left). Expression levels of bombarded immature embryos were measured by quantitative histochemical analysis. Only a small decrease in the level of expression was observed for *Sph1-2*$_m$, and *G-box1*$_m$*/Gbox2*$_m$ when compared to the *ZmHyPRP*$_{-595}$*::GUS* unmodified promoter, but there was a threefold increase for *G-box2*$_m$ and a 4.5-fold increase for the *Sph1-2*$_m$*/Gbox2*$_m$ double mutation. A smaller increase was observed for G-*box1* $_m$ (Fig. 5b, right). In these experiments, there was a moderate level of expression with the control construction (−595 deletion), so it is difficult to establish whether the presence of both G-boxes is important for promoter expression, but it is clear that suppression of only one of them is enough for promoter derepression. These results could support the hypothesis that, as a consequence of *ZmHyPRP* promoter chromatin conformation and regulatory elements distribution, the expression driven by this promoter must be the result of an established repressive complex where interactions between factors targeting the regulatory elements is necessary to define the observed behavior.

ZmHyPRP expression in *vp1* and *vp2* mutants in agreement with cell division and desiccation markers

The results from specific mutations and deletions show that the *ZmHyPRP* promoter is under the control of negative regulatory elements that result in attenuation of its level of expression. Some of them are under the control of transcription factor families that can positively or negatively modulate gene expression. One of the candidates is *Vp1/ABI3*, a B3 transcription factor that can control gene expression through Sph/RY elements (Hattori et al. 1992) and through G-boxes by interaction with b-Zip factors (Vasil et al. 1995). To see if *Vp1* could be involved in *ZmHyPRP* regulation we decided to study how the gene was expressed in the maize *vp1* mutant. For this, *vp1* homozygous embryos were excised at different times of embryo development and frozen prior to RNA extraction and gene expression analysis. *ZmHyPRP* gene expression was observed in immature embryos (16 dap), but was very low at 25 dap and in germinated embryos at 32 dap. For

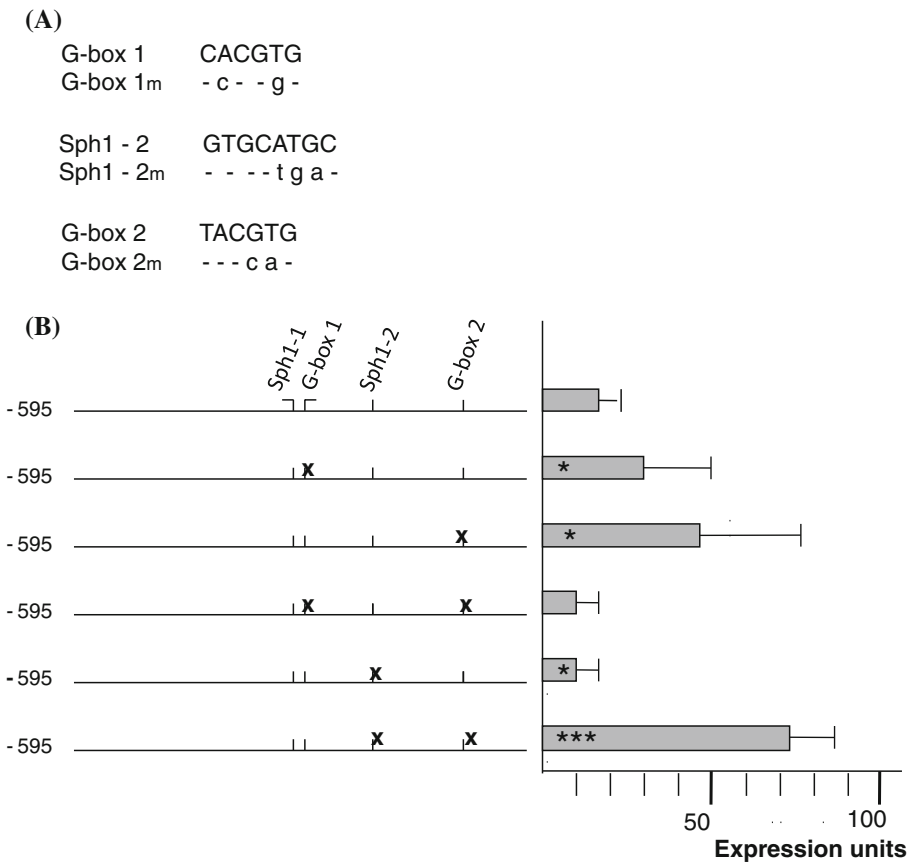

Fig. 5 Mutations in *ZmHyPRP*$_{-595}$*::Gus* fusion promoter analyzed by transient expression after particle bombardment of maize excised immature 16–18 dap embryos. **a** Mutation changes introduced in the sequence of the regulatory elements. **b** *Left* the constructions with the mutated regulatory elements marked with (**x**); *Right* quantitative histochemical analysis. The *p* value or probability that differences are not a result of the mutations is indicated by asterisks: from 25 to 5 % (*), 5 to 1 % (**) and less than 1 % (***)

ZmHRGP, as a control of cell division activity, there was a progressive increase in expression after 25 dap (Fig. 6a). We conclude that *Vp1* function is not relevant for *ZmHyPRP* expression, but in its absence, arrest of *ZmHyPRP* expression is accelerated, possibly by the initiation of the germination program, as indicated by the increase in *ZmHRGP* expression (Fig. 6a) in the absence of RAB28 expression (not shown). In contrast, in *vp2* mutants, *ZmHyPRP* (Jose-Estanyol et al. 1992 and Fig. 6b) and H4 (Fig. 6b) gene expression arrest was avoided in the absence of ABA in spite of the presence of *Vp1*. Nevertheless, in both mutants, in precocious germination *ZmHyPRP* expression finally declines.

After bombardment studies, we repeated the *vp1* mutant study in more detail, analyzing additional points during embryogenesis and comparing *ZmHyPRP* expression with that of a different cell division marker, *H4*. In this case, due to the greenhouse conditions or *vp1* phenotype variability, there was abortion of precocious initiated germination and the seed dried on the plant (semi-dormant embryos). Under these conditions, expression of *ZmHyPRP* increased at 23 dap and then declined and ceased at 28 dap after abortion of germination, when desiccation was initiated, as indicated by *RAB28* expression induction (supplementary material 4). As the ABA content in *vp1* seeds has been described as similar to that in wild type plants (Neil et al. 1986), one interpretation of the results could be that germination abortion in the absence of *Vp1* and presence of ABA, allows *ZmHyPRP* expression in semi-dormant embryos before desiccation initiation. We consider that if a *Vp1* repressive function were involved in the *ZmHyPRP* response to ABA it would act in concert with the hormone, as in the absence of *Vp1* there is not a decrease but an increase of *ZmHyPRP* expression in semi-dormant *vp1* embryos, while in the absence of ABA (*vp2* embryos) *ZmHyPRP* expression is unaltered.

Discussion

We studied the regulation and cellular localization of an embryogenesis expressed gene, *ZmHyPRP*, whose pattern of expression has been shown to be associated with embryo morphogenesis processes from the scutellum differentiation stage (Jose-Estanyol and Puigdomènech 1998). Its expression increases up to 18 dap, followed by a decrease after ABA induction. The *ZmHyPRP* coding region has a signal peptide followed by proline repeats characteristic of cell wall *HRGPs/PRPs* and a non-repetitive C-terminal hydrophobic domain, with eight cysteines in the CM-8

pattern (Jose-Estanyol et al. 2004). The presence of proline repeats in the protein suggests that *ZmHyPRP* could be a cell wall structural protein, as its expression is mainly associated with the cell division phase, although it is also related to embryo elongation processes during the maturation phase of embryo development.

Defining its cellular localization by transient transformation experiments in onion bulb epidermal cells, *ZmHyPRP* appears to be associated to vesicle-like structures in the cytoplasm and lining the plasma membrane. This localization could be the result of transit of *ZmHyPRP* through the Golgi apparatus to be glycosylated, and its presence in vesicles lining the cell membrane could suggest retention before delivery to the cell wall, where GFP observation may be difficult. From the code established for Hyp-*O*-glycosylation of plant *HRGPs* (Kieliszewvski 2001), *ZmHyPRP* could be glycosylated in the $S[Z]_2PVPPTPRP$ unique repeat due to its homology to the SOKPOTPKPT well-studied decamer repeat of *ZmTHRGP* (Kieliszewvski and Lamport 1994), where [Z] is a duplicated repeat of the PPYV motif within the maize extensin repeat motif. In the *ZmHyPRP* repeat, the *ZmTHRGP* Lys residue three is substituted by Val (K3V), the Lys in position eight by Arg (K8R), a basic amino-acid usually present in *GRPs* but not in *HRGPs* (Jose and Puigdomènech 1993), and the Thr in position ten is lost. The $S[Z]_2PVPPTPRP$ repeat in *ZmHyPRP* confers extensin characteristics to the protein as a consequence of the Thr and Ser amino-acids, absent in *PRP* repeats. The main divergence between *ZmTHRGPs* and *ZmHyPRP* repeats results, as mentioned above, from the two insertions of a highly degenerated duplicated repeat [Z] in *ZmHyPRP*, between the first and second amino-acids of the *ZmTHRGP* decamer repeat. The inserted repeat is similar to the classical PPVYK (PPXYK) repeat described for soybean *PRPs*, also present in the tomato P2 extensin repeat. Although purification and aminoacid composition is needed for the precise glycosylation pattern of *ZmHyPRP*, we can predict an intermediate glycosylation level between that described for *THRGPs* and *PRPs*.

We went on to study the regulatory mechanisms that control the main expression of *ZmHyPRP* during the embryogenesis proliferation cellular phase and its decrease in expression after ABA induction in vivo (Jose-Estanyol et al. 1992) or in vitro (Jose-Estanyol and Puigdomènech 1998). Analyses of the regulatory elements present in the *ZmHyPRP* promoter suggest a duplication of the four main regulatory elements described (Sph/RY, G-box, E-box and CGCG box). This duplication defines the limits of the proximal and distal promoter regions connected by an AT-rich region close to the Sph1-2 box (Fig. 2), in agreement with the previously established concept that proximal promoter regions are involved in seed-specific expression

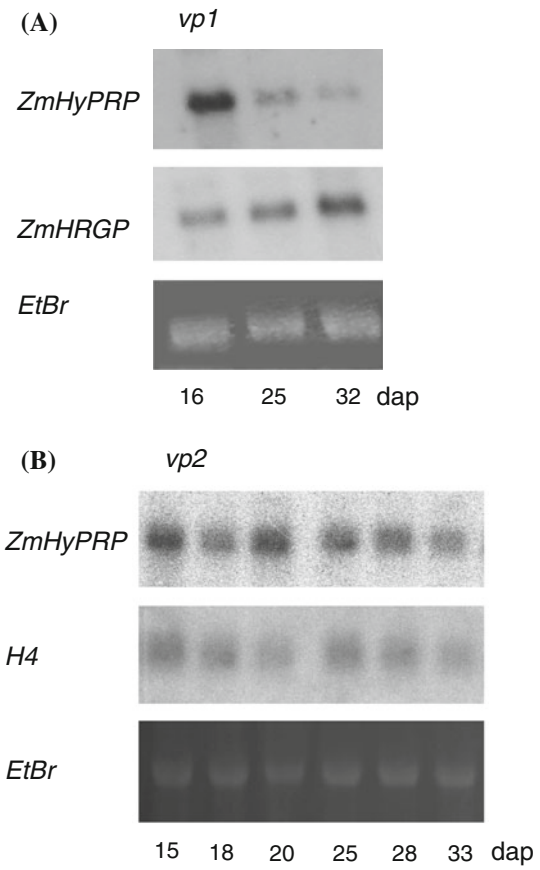

Fig. 6 Comparative gel blot analysis of the mRNA expression pattern of genes during maize *vp1* and *vp2* embryogenesis. Each of the indicated cDNA probes was hybridized to total RNA isolated from **a** *vp1* or **b** *vp2* non-dormant embryos harvested at different days after anthesis. Equal input was verified by EtBr staining. The same blot was successively hybridized with all the probes. 10 μg RNA/slot

while more distal regions function to enhance the basic expression patterns conferred by the proximal regions (Thomas 1993). Comparison of the distribution of the G-box and Sph/RY boxes, mainly involved in the regulation of seed-specific expression in the promoters of different genes, has shown that their distribution is usually similar for genes belonging to families related by a similar function and time of expression in the embryo, as is the case for storage (Dickinson et al. 1988) and lea (Busk and Pagés 1998) protein genes (supplementary material 5). The main difference between the *ZmHyPRP* promoter and that of storage proteins, which have a major increase in expression after ABA induction, results from the absence, in the *ZmHyPRP* promoter, of an Sph box close to the G-box which is 5′ of the TATAAA box. In the *ZmHyPRP* proximal promoter, the nearest TATAAA box Sph element is located at 103 bp from it, at the limit of the proximal promoter and equidistant, 85 bp, between G-box1 and 2. In our opinion, this particular distribution of regulatory elements in the *ZmHyPRP* promoter, in a nucleosome

conformation (160pb) with two turns of DNA (80 bp/turn), could favor interactions between the factors that target the G-box1, Sph1-2 and G-box2 boxes. These interactions could result in the recruitment of particular factors not only in the initial chromatin/nucleosome architecture modification by *VP1* (Li et al. 1999), but also in the establishment of the transcriptional complex during embryogenesis, before and after ABA induction.

The functional study of the regulatory elements described in the *ZmHyPRP* promoter was initiated in a previous study by stable transformation of maize plants with the *ZmHyPRP*$_{-2070}$ promoter fused to a reporter gene (Jose-Estanyol et al. 2005). Results indicated that this construction was able to reproduce the *ZmHyPRP* expression pattern defined by in situ hybridization (Jose-Estanyol et al. 1992). In the present paper, 5′ serial deletions of the *ZmHyPRP*$_{-2070}$*::GUS* construct were studied by transient expression in bombarded, excised immature maize embryos. From the results we identified two negative regulatory elements, NE1 (−2070 to 1280 bp) and NE2 (−232 to −178 bp). Outside these two regions, successive promoter deletions result in a successive decrease in the expression level as a result of progressive lost of enhancer elements. Nevertheless, these deletions never allow an ABA positive response. Further studies on promoter expression, by deletion of different regulatory elements together with their surrounding elements or by their specific mutation, indicated that both the proximal and distal promoter of *ZmHyPRP* are involved in the major attenuation of *ZmHyPRP* expression. While a single mutation of G-box2 and, to a lesser extent of G-box1, was enough to allow promoter derepression, when both G-boxes were mutated *ZmHyPRP* promoter expression decreased slightly, which indicates that at least one of them must be present for derepression. Derepression could also be by dual Sph1-2/G-box2 mutation with a major synergistic effect. In contrast, Sph1-1/G-box1 deletion results in a decrease of expression. Corroborating our previous hypothesis, our results indicate that the particular chromatin conformation and distribution of regulatory boxes in the *ZmHyPRP* promoter are, to a different degree, involved in the recruitment of targeted factors that result in a complex with a repressive function, as, when one of the elements (NE2, G-box2, G-box1 and Sph1-2 with G-box2) is deleted, expression is increased. A major attenuation of *ZmHyPRP* promoter expression has previously been described for *ZmHyPRP*$_{-2070}$*::GUS* (Jose-Estanyol et al. 2005) and *ZmHyPRP*$_{-595}$*::GUS* (unpublished results) constructions after stable transformation of tobacco plants, as only low levels of GUS expression were reported in embryo cotyledons. These results can now be explained as a consequence of greater negative modulation of *ZmHyPRP* promoter expression in dicotyledonous than in monocotyledonous plants. As B3 transcription factors have been shown to be able to negatively regulate gene expression (Rohde et al. 2000; Braybrook and Harada, 2008) as well as b-Zips transcription factors (Chern et al. 1996a, b; Bensmihen et al. 2002; Nantel and Quatrano 1996) we can not discard their involvement in this process.

Our studies on *ZmHyPRP* promoter elements involved in the control of the decrease of gene expression by endogenous (in vivo) (Jose-Estanyol et al. 1992) as well as exogenous (in vitro) ABA (Jose-Estanyol and Puigdomènech 1998) have shown that the G-box2, at least, does not appear to be involved in this process since after its deletion, expression is reduced in a similar way as the −595 control construction after bombardment of excised embryos treated with exogenous ABA. The lack of involvement of other *ZmHyPRP* promoter elements is not so clear. Among the factors that could be involved in this negative ABA response by the *ZmHyPRP* promoter, *Vp1* is a good candidate. It has been associated with cell cycle arrest to allow maturation and desiccation processes while *ZmHyPRP* expression decreases (Rohde et al. 2000). *Vp1* together with ABA protect seeds from water deficit and also, from our results, both ABA and *Vp1* are needed to decrease *ZmHyPRP* expression during embryogenesis. More studies are required to analyze the involvement of B3 transcription factors, well characterized in *Arabidopsis* but not in maize, and of basic/leucine-zipper proteins with a repressive function. Other processes could involve DNA methylation, and previous or parallel post-translational modification of histone N-terminal tails during embryogenesis that could generate a code for the recruitment of some of the proteins or protein complexes, mentioned above, affecting chromatin structure and finally gene expression.

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