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Expression of the promoter of HyPRP, an embryo-specific gene from *Zea mays* in maize and tobacco transgenic plants

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Abstract

zmHyPRP is a gene specifically expressed in maize immature embryos where its transcripts are mainly observed in the scutellum. It has been shown that *zmHyPRP* expression in the embryo is arrested when ABA levels increase at the beginning of the maturation stage. Here we report the ability of 2 Kb *zmHyPRP* promoter to reproduce the *zmHyPRP* gene specific expression pattern in the maize embryo and its repression by ABA at the end of the morphogenetic process. Three different approaches have been used, transient particle bombardment of maize immature excised embryos and stable transformation of maize and tobacco plants with a construct containing 2 Kb of *zmHyPRP* promoter fused to the *GUS* gene. This construct has shown to confer specific expression to maize and tobacco embryos but in tobacco expression in the embryo was very low. The same construct was also negatively regulated by ABA in embryos of both species. This suggests that 2 Kb of the *zmHyPRP* promoter contain all regulatory elements sufficient to confer the developmental expression patterns of the gene characterized to date.
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Keywords: *Zea mays* hybrid proline-rich protein (*zmHyPRP*); *zmHyPRP* promoter; Transgenic plants; Particle bombardment; Abscisic acid, ABA

1. Introduction

Cell wall structural proteins are known to be interesting markers of plant development. The main representative families are arabinogalactan proteins (AGPs), hydroxyproline-rich proteins (HRGPs) and proline-rich proteins (PRPs). They have in common a signal peptide necessary for the export to the cell wall and the presence of repeats of proline or glycine of different length and number. Nevertheless, important differences in the degree of protein glycosylation can be observed when the different families of proteins are compared. The main glycosylated proteins are multidomain AGPs that contain a signal peptide, a central proline-rich repetitive domain and a C-terminal domain which directs the addition of a glycosylphosphatidy-linositol (GPI) anchor (Gaspar et al., 2001; Johnson et al., 2003). They may have a signalling or communication role in embryo and plant development (Pennell et al., 1991, 1992; Acosta-Garcia and Vielle-Calzada, 2004). HRGPs also called extensins in some systems, possessing a signal peptide followed by a domain of proline repeats of different length and composition. Their SPPPP repeat motives are mainly *O*-glycosylated in serines by galactose and in hydroxyprolines by arabinose (Jose-Estanyol and Puigdomènech, 2000). HRGPs are involved in plant cell wall structure and defence (Showalter, 1993), and they have shown to be essential for embryo development (Hall and Cannon, 2002). The third family of proteins are the PRPs that contain the PPVYK repeat motif. Similar

Abbreviations: ABA, abscisic acid; ABRE, aba responsive element; AGPs, arabinogalactan proteins; BAP, benzylamino purine; 8CM-HyPRPs, eight cysteine motif hybrid proline-rich proteins; dap, days after pollination; HRGPs, hydroxyproline-rich glycoproteins; *GUS*, β-glucuronidase gene; MS, murashige and skoog medium; NAA, α-naphthalenacetic acid; *nos*, nopaline synthase gene; *pat*, phosphinothricin acetyltransferase; PRPs, proline-rich protein; *vp2*, viviparous 2; X-Glu, 5-bromo-4-chloro-3-indolyl β-D-glucuronide; *zmHyPRP*, *Zea mays* hybrid proline-rich proteins.

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motives are also present in multidomain or chimeric proteins that includes the maize hybrid proline rich-protein (HyPRPs) (Jose and Puigdomènech, 1994, 2000) where the proline-rich domain precedes a C-terminal hydrophobic domain including a particular pattern containing eight cysteines. The presence of this particular domain in the C-terminal end relates this family of proteins with a big family of mainly seed, defence proteins and proteins involved in cell–cell interactions (Motose et al., 2004) to which we refer as the eight cysteine motif (8CM) family of proteins (Jose-Estanyol et al., 2004).

A particular HyPRP from maize, *zmHyPRP* is specifically expressed in the embryo (Josè-Estanyol et al., 1992). Its expression is associated with early embryo stages, marking the initial steps of scutellum development and it is arrested at the embryo maturation when embryo abscisic acid (ABA) levels increase. The presence of an abscisic acid responsive element (ABRE) (Guiltinan et al., 1990) in the *zmHyPRP* promoter suggests that the hormone may be involved in the regulation of the expression of this gene. Different experimental results appear to indicate a role of ABA in the modulation of *zmHyPRP* expression during embryo development. *zmHyPRP* expression is repressed when maize immature embryos are supplemented with exogenous ABA and in *viviparous 2* (*vp2)* maize ABA deficient mutants, *zmHyPRP* expression is maintained until embryo germination is attained, but it can be repressed by the addition of exogenous ABA to excised immature embryos (Josè-Estanyol et al., 1992; Josè-Estanyol and Puigdomènech, 1998a). Thus, the ABA hormone responsive element in *zmHyPRP* appears until now to be mainly related to embryo development processes. In addition to ABA, *zmHyPRP* expression in the embryo can also be arrested when the embryogenic program is suddenly interrupted by hydration or induction of a dedifferentiation program (Josè-Estanyol and Puigdomènech, 1998b).

In the present study, *zmHyPRP* 2 Kb promoter activity was investigated by transient particle bombardment in maize immature excised embryos and by stable transformation of maize and tobacco plants. The results indicate that 2 Kb of *zmHyPRP* promoter contain the information needed to confer its characterized hitherto embryo-specific expression.

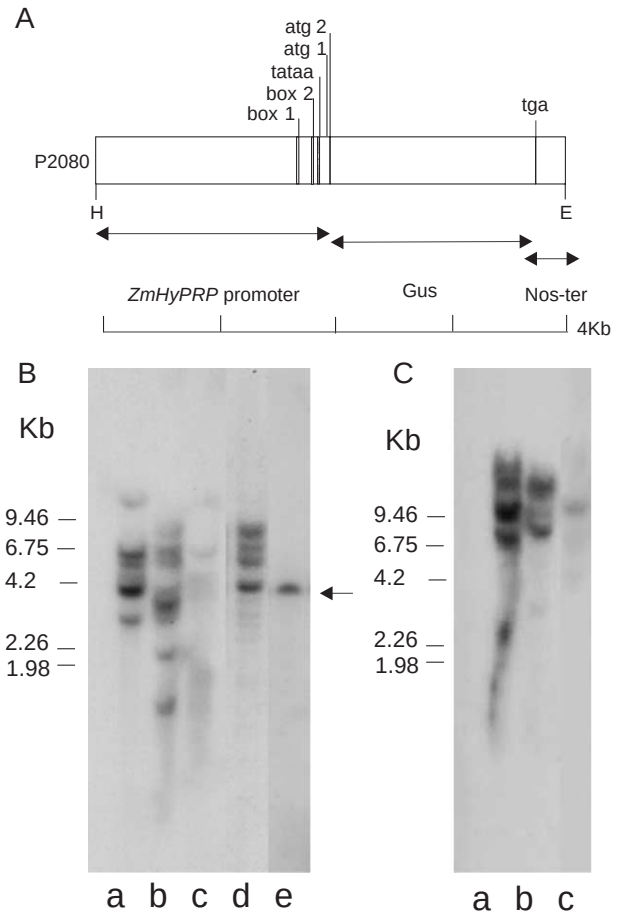

Fig. 1. *Zea mays* plant transformation with *zmHyPRP*: :*GUS* construct. (A) Schematic representation of the construction used in plant stable transformation studies. This construct contains 2080 bp of *zmHyPRP* promoter fusioned to *GUS* fused to the nos-ter. box1, abre like element (Guiltinan et al., 1990); box2, motif1 like element (Mundy et al., 1990); atg1, atg from *zmHyPRP* gene; atg2, atg from *GUS* gene in frame with atg1. A scale in Kb of the casette can be observed at the bottom of (A). (B and C) Southern analysis of *Z. mays* different lines transformed with *zmHyPRP*: :*GUS* construct. Genomic DNA from different transgenic lines were digested with HindIII+Eco RI (B) and with HindIII (C) restriction enzymes. (a) line 357.1S; (b) line 357.1R; (c) line 357.1F; (d) line 332.1D; (e) line 312.2E. The size of λ DNA marker fragments after digestion by Hind III restriction enzyme are showed at the left of each panel. The arrow signals the correct DNA size insertion in transgenics plants.

2. Materials and methods

2.1. Maize particle bombardment

Maize transient transformation was performed by particle bombardment with a 2 Kb fragment (SacI–DdeI) of *zmHyPRP* promoter in the construct *zmHyPRP*: :*GUS*. The promoter was fusioned to the *β-glucuronidase (GUS)* gene and the *nopaline synthase (nos)* gene terminator, as it is shown in Fig. 1A. The *zmHyPRP* promoter was cloned in the plasmid pBI201.3 in the site Sma of the polylinker. This plasmid was derived from pBI101.3 (Jefferson et al., 1987) by cloning the HindIII–EcoRI fragment in pUC19. Gold particles coated with this construction were used for bombardment of maize 16 dap immature embryos (with or without 50 μM ABA), and 26 dap mature embryos. After excision embryos were placed on MSO medium (murashige and skoog-MS-medium supplemented with 30 g/l sucrose). Biolistic assays were carried out 24 h after excision with a PDS1000/He (Dupont, Biorad) using a helium pressure of 900 psi, as described by Menossi et al. (1997).

2.2. Maize transgenic plants

Embryogenic type II calli were initiated by culture of immature embryos (10 dap) of maize hybrid Hi-II and

maintained by regular subculturing for 3–6 months (Armstrong, 1994). Callus were co-bombarded with the plasmid containing the *zmHyPRP*::*GUS* construct above described and plasmid pDM302 carrying a phosphinothricin acetyltransferase *(pat)* gene under the control of a rice *actin* gene promoter as indicated by Bonello et al. (2000). Then the calli were subcultured under selective pressure every 2 weeks for 3 months and then placed on regeneration medium to regenerate plants (Vain et al., 1993).

2.3. Tobacco transgenic plants

For tobacco stable transformation, the fragment HindIII–EcoRI from 2 Kb pBI201.3 plasmid containing the *GUS* coding region was subcloned in pBIN19 and used to transform *Agrobacterium tumefaciens* A.T.GV2260 and transferred to *Nicotiana tabacum* L. cv. Petit Havana SR1 leaves. Transformed tobacco shootlets were selected in a shoot-inducing medium containing 1 μg/ml BAP (benzylamino purine), 0.2 μg/ml NAA (α-naphthalenacetic acid), 500 μg/ml cefotaxime sodium salt and 50 μg/ml kanamycin. Regenerated shootlets were rooted in a root-inducing medium containing 500 μg/ml cefotaxime sodium salt and 50 μg/ml kanamycin. Plants were grown in a greenhouse and T_1 and T_2 seeds were collected and germinated in a 2 MS medium with 50 μg/ml kanamycin, as described by Rieping et al. (1994). Positive plants were selected by PCR, Southern analysis and *GUS* expression assay.

2.4. Molecular analysis

Genomic DNA from transgenic plants was isolated by the method described by Dellaporta et al. (1983). Enzymatic amplification by PCR was performed using Taq DNA polymerase. The primers used for amplification were Pro5 (GAA TGG AAT TGG GAG GAC GC) from *zmHyPRP* proximal promoter and *GUS* (GATTTCACGGGTTGGGGTTTCT) from initial *GUS* coding region. The plasmid 2 Kb pBI201.3 was used as positive template DNA. The conditions for the PCR were as described by Montoliu et al. (1992). The number of incorporated copies of the *GUS* gene and possible recombinations was analyzed by Southern blot (Southern, 1975). This was carried out by digesting genomic DNA by HindIII alone or with HindIII and EcoRI, gel electrophoresis running, transfer to nylon paper N-BIODINE and paper hybridization with a fragment of *GUS* coding region (HincII–HincII) as probe.

2.5. Histochemical and fluorometric GUS assay

The histochemical localization of *GUS* in transformed maize plants was performed essentially as described by Jefferson (1987). Maize embryos, sectioned kernels, and small pieces of several tissues from stable maize transformed plants were immersed in a histochemical reaction mixture containing 3 mg/ml X-Glu (5-bromo-4-chloro-3-indolyl β-D-glucuronide) in 100 mM sodium phosphate buffer pH 7.0, 0.06% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide. The histochemical reaction was performed in the dark at 37 °C until a blue indigo dye colour as precipitate appeared at the site of enzymatic cleavage after 2–15 h. Tissues were rinsed several times in 50 mM phosphate buffer to stop the reaction, rinsed in 70% v/v ethanol, mounted in 50% glycerol and examined with a Zeiss (Stemi SV6) stereomicroscopy or an AxioPhot microscope. Histochemical analysis of maize embryos after particle bombardment was similar to that described for transgenic plants but with the particularity that prior to immersion in the reaction mixture, tissues were reposed in bombardment MSO medium dishes for 24 h. After reaction blue spots were counted.

Histochemical analysis of tobacco transgenic plants was performed by immersion of plant tobacco tissues and seed embryos manually dissected in a reaction mixture containing 0.5 mg/ml X-Glu in 50 mM sodium phosphate buffer pH 7.0, 0.1% Triton X-100. Fluorometric *GUS* assay of maize and tobacco transgenic plants was performed following the protocol described by Jefferson (1987), modified by adding methanol to the assay buffer as suggested by Kosugi et al. (1990). Protein concentration in the extracts was measured using the Bradford assay (Bradford, 1976). Measurements were carried out with a TKO 100 Mini-Fluorimeter (Hoefer Scientific Instruments, San Francisco, CA).

3. Results

3.1. zmHyPRP promoter activity in transgenic maize plants

zmHyPRP is highly expressed in the scutellum of maize immature embryos (Josè-Estanyol et al., 1992). We have studied by maize stable transformation the ability of *zmHyPRP* 5′ non-coding region to mimic the expression pattern defined by in situ hybridization (Josè-Estanyol et al., 1992). Maize callus derived from 10 dap immature embryos were transformed by microparticle bombardment with *zmHyPRP*::*GUS* construct in Blue Script as described in Materials and methods. The promoter region analyzed ranges from position −2080 to the +18, a DNA fragment that has been fused to the *GUS* coding region and joined to the *nos* terminator (Fig. 1A). Forty-five embriogenic callus plates were bombarded. Callus were growth in a selective medium and then plants were regenerated. Twelve lines of T2 regenerated plants from three different transformation events (312, 332 and 357) were examined. Exogenous DNA insertion was studied by PCR (not shown) and Southern analysis. Genomic DNA digestion from transgenic plants by Hind III plus EcoRI restriction enzymes allowed to control the intactness insertion of the promoter-GUS fusion in the plant by the appearance of a 4 Kb band using a GUS probe, as the studied fusion is flanked by unique copies of these

Table 1
Maize transgenic plants

line	$e_{15\ dap}$	$e_{25\ dap}$	$e_{30\ dap}$	$s_{2\ cm}$
357.1R	37	61	26	n.d.
357.1F	47	106±10	27±0	n.d.
357.1S	755±55	9694±525	372±107	40

zmHyPRP promoter expression in different lines of maize transgenic plants. Analysis correspond to T_2 transgenic plants harbouring the *GUS* gene controlled by 2 Kb of *zmHyPRP* promoter. *GUS* expression of plant tissues where the promoter is expressed is presented as pmol/h/mg. e, embryo; s, spikelet; dap, days after pollination; (±) indicate standard deviation for at least three different plants; n.d. not determined.

enzymes (Fig. 1A). Southern analysis of five different transgenic lines is shown in Fig. 1B. Three of them present the expected 4 Kb band (Fig. 1Ba,d,e) but in the other two cases the band is not so clear (Fig. 1Bb,c). Some lines present extra hybridization bands of varying sizes that may represent rearranged extra copies of the construct. Studies of GUS expression in maize immature embryos from lines of the three transformation events were done. Results showed higher values for some lines from transformant 357. These 357 lines (357.1S, 357.1R, 357.1F) that have the strongest level of *GUS* expression were studied in detail by Southern analysis, digesting with Hind III restriction enzyme alone in order to obtain information about the number of different copies inserted (Fig. 1C). Results indicated that these transgenic plants contained between one to three copies of the inserted sequence (Fig. 1C). Their quantitative fluorometric analysis results are shown in Table 1. In immature maize embryos at different days after anthesis a maximum of expression was observed around 25 DAP but expression analysis in other tissues was negative (not shown) for the three analyzed lines. Expression levels in immature embryos of lines F and R was nearly 100 times reduced in relation to line S. This important difference might be explained from the Southern blot studies as only in line 357.1S, the 4 Kb band expected for an intact insertion was observed. Histochemical analysis of *GUS* expression in kernels and embryos from maize transgenic plants allowed to define which embryo tissues were involved in *zmHyPRP*::*GUS* expression. Results for 357.1S maize transgenic line were similar to the ones of lines 357.1R and 357.1F and are shown in Fig. 2. *zmHyPRP*::*GUS* expression was first observed in 10 dap embryos as blue scutellar spots in both embryo sides, before homogeneous distribution was attained, and as a band in the scutellar nodular region of the adaxial side (Fig. 2A). We understand that these spots are not the result of wounding as embryos were not cut before treatment with the substrate and in embryo sections (see next Fig. 2D–F) the spotted pattern was not observed. Since stage 1 to stage 3 of maize embryo development, expression was observed in a uniform way in the scutellar abaxial side and loosely in the adaxial side as well as in the nodular scutellar region of the adaxial side (Fig. 2B and C). Longitudinal and transversal sections of maize transgenic kernels at stage 1 and 2 of development allowed to distinguish the embryo specific expression pattern (Fig. 2E) and the scutellar gradient from the abaxial side toward the inner zones (Fig. 2D,F). Thus, we can conclude that *zmHyPRP* in situ hybridization expression pattern (Josè-Estanyol et al., 1992) has been reproduced by the *zmHyPRP*::*GUS* construct in stable transformed maize plants.

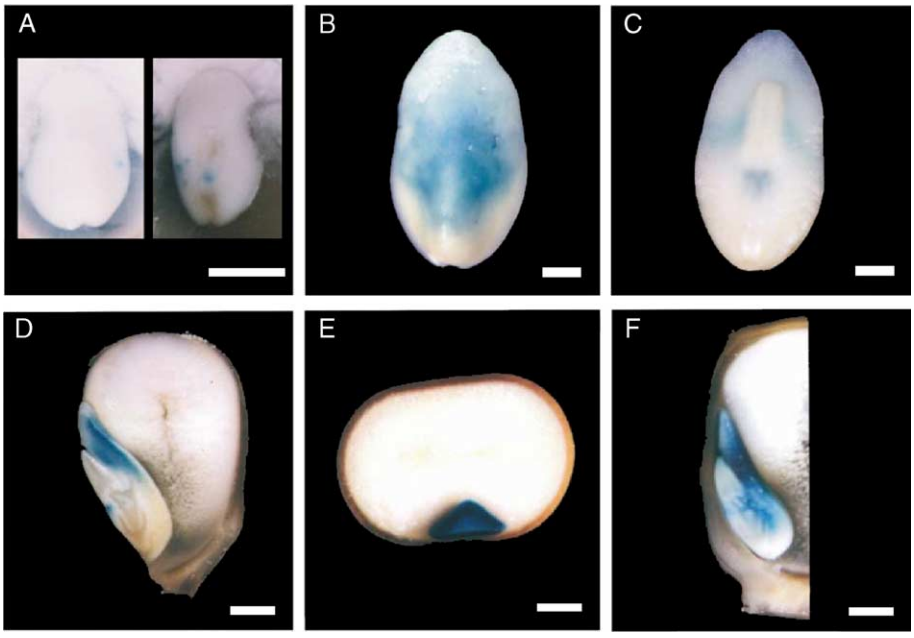

Fig. 2. Histochemical localization of *GUS* activity in transgenic maize kernels. Detection of *GUS* in maize transgenic plants transformed with the *zmHyPRP*::*GUS* construct (line 357.1S-T2-). (A) 10 dap embryo abaxial and adaxial sides. (B) 25 dap embryo abaxial side. (C) 25 dap embryo adaxial side. (D, F) Longitudinal sections of maize 20 dap kernels. (E) Transversal section of maize 20 dap kernel. Bar=1 mm.

Table 2
Tobacco transgenic plants

line	$s_{15\ dap}$	$s_{20\ dap}$	$s_{30\ dap}$	$s_{40\ dap}$	l_{2cm}	r_{2cm}	l_a	r_a
8	542±363	704±128	293±179	238±133	<	200	<	240±126
27	85±29	283±80	101±4	126±2	<	86	<	261±5
66	236±178	428±131	199±36	142±48	<	290	<	724±160

zmHyPRP promoter expression in different lines of tobacco transgenic plants. Analysis correspond to T_2 transgenic plants harbouring the *GUS* gene controlled by 2 Kb of *zmHyPRP* promoter. *GUS* expression is presented as pmols/h/mg. Expression in different plant parts is indicated. dap, days after pollination; s, seeds; l, leaf; r, root; 2 cm, plant size of 40 days germinated seeds; a, adult flowering plants; (±) indicate standard deviation for at least three different plants; <, values under the detection limit of the technique.

3.2. zmHyPRP promoter activity in tobacco transgenic plants

Tobacco was transformed with the *zmHyPRP*::*GUS* construct (Fig. 1A) in order to study whether the expression pattern observed in maize transgenic plants could be also obtained in a dicot plant. Tobacco was chosen in this study for two reasons. First, most of the promoter studies of dicotyledoneous seed expressed genes have been made in this easily transformable plant. Secondly, the size of the seed allows studies at different days after anthesis as in maize. Tobacco leaves were transformed with *zmHyPRP*::*GUS* construct in pBIN19 using *Agrobaterium tumefaciens*. DNA insertion in T2 transformed plants was routinary confirmed by PCR and Southern analysis (not shown). Transgenic plants usually contained one to three copies of the gene. 4 Kb *GUS* hybridizing bands confirmed a correct insertion of the *zmHyPRP*::*GUS* gene cassette in the tobacco genome of the different transformants. *GUS* expression by quantitative fluorometric analysis was observed in tobacco seeds between 15 and 40 days after anthesis. The maximum expression was observed around 20 days. Expression analysis in other plant tissues as roots and leaves of 2 cm plants was negative or very low, as well as for roots and leaves of adult plants (Table 2). Histochemical analysis of the seeds only allowed sometimes to observe expression mainly in embryo cotyledons as a faint blue. We conclude that the *zmHyPRP*::*GUS* construction can be specifically expressed in tobacco embryos but this expression is very diminished in relation to maize.

3.3. zmHyPRP promoter activity in response to ABA

zmHyPRP is expressed during the morphogenetic stage in maize immature embryos. After this stage ABA is induced and no *zmHyPRP* mRNA accumulation is observed. The presence of two ACGT-elements at −260 pb (Guiltinan et al., 1990) and −93 (Mundy et al., 1990) of *ZmHyPRP* promoter suggests that in this particular gene ABA could be involved in the repression of the gene by the hormone. The ability of *zmHyPRP* promoter to be inhibited by ABA has been studied in three different systems. First by microprojectile bombardment of maize wild type excised embryos with *zmHyPRP*::*GUS* construct in Blue Script vector. Second in excised embryos from maize T2, 357.1 S transgenic plants transformed with the same construct used in particle bombardment studies above described and third in excised seeds from tobacco T2, 8 transgenic plants transformed with the *zmHyPRP*::*GUS* construct inserted in pBin19 vector. Results of the particle bombardment experi-

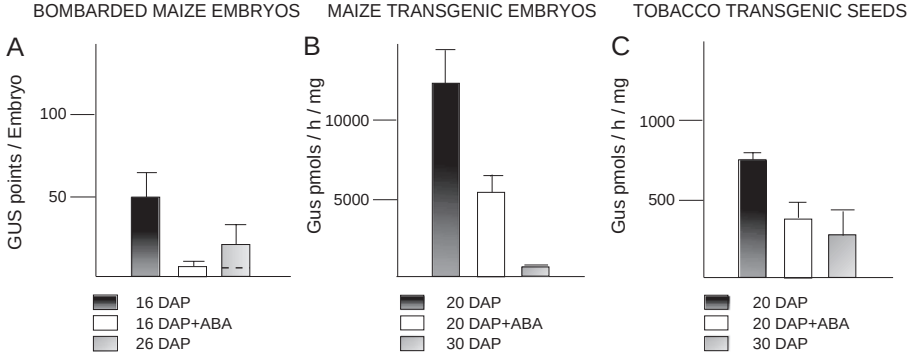

Fig. 3. Modulation of *zmHyPRP* promoter expression in presence and absence of ABA. (A) Modulation of *zmHyPRP* promoter expression in presence and absence of ABA by particle bombardment of maize excised embryos. Bar chart of the results of the *GUS* blue spots/embryo counted on the scutellar abaxial side of maize embryos after being bombarded with the *zmHyPRP*::*GUS* construct described in Fig. 1A. Black bar: 16 dap excised immature embryos; White bar: 16 dap excised immature embryos after 24 h in 50 μM ABA; Gray bar: 26 dap excised immature embryos. Error bars indicate standard deviation (results are the average of three different experiments, three samples of nine embryos/experiment). (B) Modulation of *zmHyPRP* promoter expression in presence and absence of ABA in maize transgenic embryos from plants transformed with the construction described in Fig. 1A. Bar chart of the results of the quantitative fluorometric *GUS* analysis of maize transgenic excised embryos in pmol/h/mg. Black bar: *GUS* expression in 20 dap immature embryos; White bar: *GUS* expression in 20 dap immature embryos after being treated with 50 μM exogenous ABA for 24 h. Gray bar: *GUS* expression in 30 dap maturating excised embryos. Error bars indicate standard deviation for at least three different plants of T2 357.1S transgenic plants. (C) Modulation of *zmHyPRP* promoter expression in presence and absence of ABA in tobacco transgenic seeds from plants transformed with the construction described in Fig. 1A in pBin19. Bar chart of the results of the quantitative fluorometric *GUS* analysis of tobacco transgenic seeds in pmol/h/mg. Black bar: *GUS* expression in 20 dap seeds; White bar: *GUS* expression in 20 dap seeds treated with 50 μM exogenous ABA for 24 h. Gray bar: *GUS* expression in 30 dap maturating seeds. Error bars indicate standard deviation for at least three different plants of T2, 8 transgenic plants.

ments are presented in Fig. 3A. The activity of the *zmHyPRP* promoter was analyzed in maize immature embryos (16 dap) in the presence and in the absence of 50 μM ABA and in maturating embryos (26 dap). To measure the transient activity of the promoter, blue spots on the abaxial side of the bombarded embryo were counted. The 2 Kb region of *zmHyPRP* promoter showed to be able to induce *GUS* expression in maize immature embryos in the absence of ABA after particle bombardment. In presence of the hormone, 90% reduction of the expression was observed. In maturating embryos (26 dap) the reduction in *GUS* expression was of a 50% in relation to the control, 16 dap immature embryos. This reduction was in fact 87% as the bombarded surface in 26 dap embryos was four times larger than the one of 16 dap embryos. This detail is indicated by a transversal dotted line in the 26 dap bombarded embryos bar in Fig. 3A.

Negative regulation of *zmHyPRP* promoter by ABA was also observed when embryos from maize and tobacco *zmHyPRP*::*GUS* transgenic plants were studied. Quantitative fluorometric *GUS* expression analysis of maize 20 dap transgenic embryos excised and incubated in presence of 50 μM ABA was reduced to 40% with respect the expression determined for non-treated embryos (Fig. 3B). Similarly in 20 dap seeds of tobacco transgenic plants *GUS* expression in presence of 50 μM ABA was reduced to 50% with respect the control (Fig. 3C). A reduction of the expression of a 96% for maize 30 dap maturating embryos (Fig. 3B) and of a 60% for tobacco 30 dap maturating seeds (Fig. 3C) in relation to their respective controls could also be observed. Similar results were obtained for the other maize and tobacco transgenic lines described in this paper (not shown). The *zmHyPRP* promoter activity showed by this way to be reduced after ABA treatment in all the systems analyzed.

4. Discussion

The present work has been carried out in the context of the study of gene promoters that are regulated in specific stages of plant development. The *zmHyPRP* gene is of interest because it is mainly expressed in the embryo from the onset of coleoptil embryo development stage (Josè-Estanyol and Puigdomènech, 1998a), and because its expression is arrested at the end of the embryo morphogenetic process as result of its negative regulation by ABA (Josè-Estanyol et al., 1992).

Our results show that 2 Kb of *zmHyPRP* promoter are able to specifically express *GUS* in the scutellum of maize transgenic plants since the end of the coleoptilar stage. Scutellar expression showed the same gradient from the abaxial side toward the embryo inner zones as was previously reported by *in situ* hybridization studies (Josè-Estanyol et al., 1992). *GUS* expression was also observed in the embryo adaxial side mainly in the nodular region, but neither in the endosperm nor in the pericarp. Histochemical and quantitative fluorometric analysis detected no expression in other tissues.

Different results were obtained when *zmHyPRP*::*GUS* expression was analyzed in the heterologous system, tobacco. Expression in seeds of tobacco transgenic plants by quantitative fluorometric analysis of *GUS* expression was very low and it could be observed sometimes in embryo cotyledons by histochemical analysis. In conclusion our results indicate that regulatory elements present in *zmHyPRP* full promoter can drive expression to tobacco embryos but with a very low efficiency in relation to the once observed in maize transgenic plants.

Results on ABA regulation studies during embryogenesis showed that *zmHyPRP*::*GUS* construct contained signals sufficient for the repression by the ABA hormone as *GUS* expression was consistently reduced or repressed, when maize excised immature embryos that had been supplied with exogenous hormone or maturating embryos with endogenous ABA were bombarded with the studied construct. Similar results were obtained when excised embryos from maize and tobacco transgenic plants were analyzed.

Genes expressed in the seed have been shown until now to be under the control of different combinations of cis-control elements (Jofuku and Goldberg, 1989) that appear to be particular for each gene (Nunberg et al., 1994). Different seed/embryo specific expression elements have been described until now (Thomas, 1993; Schwechheimer et al., 1998; Finkelstein et al., 2002). Usually promoter proximal elements (around 100 bp) define seed expression while more distal regions enhance expression. Sequence analysis of regulatory elements present in *zmHyPRP* promoter indicate that this gene may behave similarly. Two different G-box-like ABRE elements (G/ABREs) are present in *zmHyPRP* promoter. The first one at −260 pb (CACGTG) is similar to the ABRE of the wheat *Em* ABA inducible promoter (Guiltinan et al., 1990) and the second one at −93 (TACGTG) is similar to the motif1 present in rice *RAB16A* promoter (Mundy et al., 1990). The core of these boxes are ACGT-elements known to be present in the promoter of genes differently regulated (Izawa et al., 1993; Menkens et al., 1995; Busk et al., 1997). It would appear that in *zmHyPRP* promoter these elements together with the sequences by which they are flanked and a specific combination of transcriptional factor levels result in specific embryo expression mainly restricted to the scutellum and in ABA repression instead of induction when the maturation and desiccation processes are attained during embryo development.

Authors conclude from the transformation studies here described that the tissue specific regulatory elements of *zmHyPRP* gene expression in the scutellum are contained in the 2 Kb fragment of its promoter. The same region contains as well the sequences needed for the repression of its activity by ABA at the end of the embryo morphogenetic development stage. Deletion studies of *zmHyPRP* promoter by transient and stable transformation may allow in a future

to clarify the real function of the different regulatory elements and factors responsible of the *zmHyPRP* particular expression pattern here described.

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References

Acosta-Garcia, G., Vielle-Calzada, J.-P., 2004. A classical arabinogalactan protein is essential for the initiation of female gametogenesis in Arabidopsis. Plant Cell 16, 2614–2628.

Armstrong, C.L., 1994. Regeneration of plants from somatic cell cultures: applications for in vitro genetic manipulation. In: Freeling, M., Walbot, V. (Eds.), The Maize Handbook. Springer, New York, pp. 663–671.

Bonello, J.F., Opsahl-Ferstad, H.G., Perez, P., Dumas, Ch., Rogowsky, P.M., 2000. Esr genes show different levels of expression in the same region of maize endosperm. Gene 246, 219–227.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 248–254.

Busk, P.K., Jensen, A.B., Pagés, M., 1997. Regulatory elements in vivo in the promoter of the abscisic acid responsive gene rab17 from maize. Plant J. 11, 1285–1295.

Dellaporta, J.L., Wood, J., Licks, J.B., 1983. A plant DNA minipreparation. Plant. Mol. Biol. Report. 1, 19–21.

Finkelstein, R.R., Gampala, S.S.L., Rock, Ch.D., 2002. Abscisic acid signalling in seeds and seedlings. Plant Cell, S15–S45.

Gaspar, Y., Johnson, K.L., McKenna, J.A., Bacic, A., Schultz, C.J., 2001. The complex structures of arabinogalactan-proteins and the journey towards understanding function. Plant Mol. Biol. 47, 161–176.

Guiltinan, M.J., Marcotte, W.R., Quatrano, R.S., 1990. A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250, 267–271.

Hall, Q., Cannon, M.C., 2002. The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in *Arabidopsis*. Plant Cell 14, 1161–1172.

Izawa, T., Foster, R., Chua, N.-H., 1993. Plant bZIP protein DNA binding specificity. J. Mol. Biol. 230, 1131–1144.

Jefferson, R.A., 1987. Assaying chimeric genes in plants: the *GUS* gene fusion system. Plant Mol. Biol. Rep. 5, 387–405.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Jofuku, K.D., Goldberg, R.B., 1989. Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. Plant Cell 1, 1079–1093.

Johnson, K.L., Jones, B.J., Schultz, C.J., Bacic, A., 2003. Non-enzymic cell wall (glyco) proteins. In: Rose, J.K.C. (Ed.), In The Plant Cell Wall. Blackwell Publishing. CRC Press, pp. 111–153.

Jose, M., Puigdomènech, P., 1994. Hybrid-proline rich and related proteins. Curr. Top. Mol. Genet. (Life Sci. Adv.) 2, 109–126.

Josè-Estanyol, M., Puigdomènech, P., 1998a. Developmental and hormonal regulation of genes coding for proline-rich proteins in female inflorescences and kernels of maize. Plant Physiol. 116, 485–494.

Josè-Estanyol, M., Puigdomènech, P., 1998b. Rapid changes induced in developmental programmes of the maize embryo detected by analysis of the expression of genes encoding proline-rich proteins. FEBS Lett. 422, 400–402.

Jose-Estanyol, M., Puigdomènech, P., 2000. Plant cell wall glycoproteins and their genes. Plant Physiol. Biochem. 38, 97–108.

Josè-Estanyol, M., Ruiz-Avila, L., Puigdomènech, P., 1992. A maize embryo-specific gene encodes a proline-rich and hydrophobic protein. Plant Cell 4, 413–423.

Jose-Estanyol, M., Gomis-Rüth, F.X., Puigdomènech, P., 2004. The eight-cysteine motif, a versatile structure in plant proteins. Plant Physiol. Biochem. 42, 355–365.

Kosugi, S., Ohashi, Y., Nakajima, K., Arai, Y., 1990. An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. 70, 133–140.

Menkens, AE., Schindler, U., Cashmore, A.R., 1995. The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends Biochem. Sci. 20, 506–510.

Menossi, M., Martinez-Izquierdo, J.A., Puigdomènech, P., 1997. Promoter tissue specific activity and hormone control of the gene coding for the maize hydroxyproline-rich glycoprotein in maize cells transformed by particle bombardment. Plant Sci. 125, 189–200.

Montoliu, L., Rigau, J., Puigdomènech, P., 1992. Analysis by PCR of the number of homologous genomic sequences to α-tubulin in maize. Plant Sci. 84, 179–185.

Motose, H., Sugiyama, M., Fukuda, H., 2004. A proteoglycan mediates inductive interaction during plant vascular development. Nature 429, 873–878.

Mundy, J., Yamaguchi-Shinozaki, K., Chua, N.-H., 1990. Nuclear proteins bind conserved elements in the abscisic-acid-responsive promoter of a rice Rab gene. Proc. Natl. Acad. Sci. U. S. A. 87, 1406–1410.

Nunberg, A.N., Li, Z., Bogue, M.A., Vivekananda, J., Reddy, A.S., Thomas, T.L., 1994. Developmental and hormonal regulation of sunflower helianthinin genes: proximal promoter sequences confer regionalized seed expression. Plant Cell 6, 473–486.

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M., Roberts, K., 1991. Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3, 1317–1326.

Pennell, R., Janniche, L., Scofield, G.N., Booij, H., De Vries, S.C., Roberts, K., 1992. Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J. Cell Biol. 119, 1371–1380.

Rieping, M., Fritz, M., Prat, S., Gatz, Ch., 1994. A dominant negative mutant of PG13 suppresses transcription from a cauliflower mosaic virus 35 S truncated promoter in transgenic tobacco plants. Plant Cell 6, 1087–1098.

Schwechheimer, C., Zourelidou, M., Bevan, M.W., 1998. Plant transcription factor studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 127–150.

Showalter, A.M., 1993. Structure and function of cell wall proteins. Plant Cell 5, 9–23.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Thomas, T.L., 1993. Gene expression during plant embryogenesis and germination: an overview. Plant Cell 5, 1401–1410.

Vain, P., Keen, N., Murillo, J., Rathus, C., Nemes, C., Finer, J.J., 1993. Development of the particle inflow gun. Plant Cell, Tissue Organ Cult. 33, 237–246.