ELSEVIER

Plant Science 125 (1997) 189–200

PLANT SCIENCE

Promoter tissue specific activity and ethylene control of the gene coding for the maize hydroxyproline-rich glycoprotein in maize cells transformed by particle bombardment

Marcelo Menossi *, José Antonio Martínez-Izquierdo, Pere Puigdomènech

Departament de Genètica Molecular, CID-CSIC, Jordi Girona, 18, 08034 Barcelona, Spain

Received 28 November 1996; received in revised form 12 March 1997; accepted 17 March 1997

Abstract

The translational construct, containing 719 bp of promoter and 5′-untranslated region and the first 16 bp of coding region, of the maize gene *Hrgp* encoding a hydroxyproline-rich glycoprotein fused to a glucuronidase reporter cassette, has been tested for activity in different maize tissues by microprojectile bombardment. The promoter has been found to be very active in the tissues of the plant, such as meristems or young shoots, with high cell wall formation activity where a high expression has also been shown for the endogenous gene. The promoter was also shown to be very active in cell types with a protection role such as in pericarp or styles and in cell types where the reinforcement of the cell wall is needed, as styles, auricles and cortical cells in the root tip. The promoter activity is developmentally regulated in the endosperm, being highest simultaneously with active cell division at the early-mid stages of development. In the presence of ethylene, the promoter shows an increased activity in accordance with the increment of mRNA accumulation observed in the plant upon ethylene treatment. It is concluded that the promoter fragment starting at -719 bp (numbering related to the ATG) of the *Hrgp* gene keeps the essential *cis*-DNA elements necessary for spatial, temporal and hormonal gene expression in maize. © 1997 Elsevier Science Ireland Ltd.

Keywords: Maize; *Hrgp*; Particle bombardment; Hydroxyproline-rich glycoprotein

1. Introduction

Hydroxyproline-rich glycroproteins (HRGP) are structural components of the plant cell wall. They have been described in both monocotyledonous and dicotyledonous species, although they show distinct features in these two classes of

* Corresponding author. Present address: Centro de Biologia Molecular e Engenharia Genética, Cx P. 6109, Universidade Estadual de Campinas, 13083-970 Campinas, Brazil

PII S0168-9452(97)00066-6

plants. In both classes the proteins are highly repetitive and rich in proline, although the repeated motif varies [1]. Although the proteins are ubiquitous in the plant, the corresponding genes have been shown to be expressed mainly in meristems and in response to mechanical wounding.

The best studied gene coding for an HRGP in monocots is the one isolated from maize [2,3]. It has been cloned from coleoptile tissue where its mRNA accumulates in great proportion, as well as in root tips and coleoptile nodes [4], and in general in tissues rich in dividing cells [6]. At the same time its mRNA level correlates with regions rich in cells where the cell wall is not completely formed [7]. The accumulation of maize mRNA *Hrgp* is taking place transiently at new vascular sites in immature embryos [7], roots and germinating embryos [2]. Additional features of maize *Hrgp* gene expression include its induction by mechanical wounding and ethylene [5] and in the embryo it has been shown to be accumulated mainly in the axis [7]. Finally, the presence of *Hrgp* mRNA has been reported in maize pericarp and silks [8], and comparative studies at protein level showed the highest HRGP accumulation in protecting or supporting organs like maize silks and presumably pericarp [9].

The *Hrgp* gene itself has also distinct features when compared to the ones described in dicotyledoneae. In the graminaceous species studied so far (*Zea diploperennis* teosinte, maize, sorghum and rice), *Hrgp* mRNA appears to be encoded by a single gene showing high sequence similarity between species including the 5′ flanking region of the gene. In fact, the comparison of the genomic sequences flanking the coding region of *Hrgp* gene in maize and related species have revealed that a region of around 500 bp upstream of the coding region is well conserved in different maize varieties, teosinte and sorghum [3] suggesting a possible regulatory function of this region. Preliminary studies indicated that this region is able to direct β-glucuronidase (GUS) expression in maize coleoptiles, leaves and calli [5].

The ubiquity of the HRGP protein and the restricted spatial and developmental control of *Hrgp* gene expression makes the study of the expression of these genes an interesting example of gene regulation. In the present report it is shown that the promoter region conserved among *Zea* and sorghum *Hrgp* genes is able to drive the expression of a GUS reporter construct in different maize tissues and organs in transient expression experiments using microprojectile bombarding and in transgenic Black Mexican Sweet maize (BMS) cells. In addition, it is shown that *Hrgp* promoter activity is under spatial and developmental control.

2. Methods

2.1. Plant material

Seeds and plantlets from maize W64A pure inbred line were used throughout the transient expression studies employing microprojectile bombardment. Transgenic maize cells were obtained from cell suspension cultures of the BMS line of maize. BMS cells were maintained in MSE medium (MS salts and vitamins (Duchefa, Holland), 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.02 mg/l BAP and 30 g/l sucrose, pH 5.8).

2.2. DNA constructs

A *BamHI/FokI* 734 bp fragment spanning the -719 to +16 region (numbering related to ATG) of the *Hrgp* gene from an AC1503 genomic clone [2] was fused in-frame with a cassette containing the GUS gene and the NOS terminator producing the construct p719. p35SI was obtained by fusing a GUS/NOS cassette to a vector containing a 450 bp 35S promoter and the ADH1-S intron, obtained from pCAMVI1NEO [10]. p35S was obtained by inserting a 800 bp 35S promoter/GUS/NOS cassette from pBi121.1 into pUC18 and it corresponds to pBi221 [11]. pAHC18 (kindly provided by Dr P.H. Quail) is the construct used as internal control (see Section 3), and it contains the maize ubiquitin promoter and its first intron fused to the luciferase coding region [12].

2.3. Manipulation of maize tissues

Mature maize grains were sterilized by rinsing 1 min in absolute ethanol and 12 min in 3% commercial bleach with 0.01% Triton X-100, followed by washing four times for 5 min with water. Grains were placed over three sheets of filter paper saturated with sterile water and allowed to germinate in the dark at 28°C. After 2 days, 6–10 grains were laid in the center of a 5.5 cm diameter Petri dish containing water solidified with 0.6% agar. Seedlings were collected after 7 days and placed carefully in the center of a 15 cm diameter Petri dish containing water/agar. Leaves from 14 days after germination (DAG) seedlings were cut in 2 cm long sections and transferred to MS medium (MS salts and vitamins, 30 mg sucrose, 2.4 mg Gelrite, pH 5.8).

Roots from five DAG seedlings were cut longitudinally with a razor blade and maintained in MS medium. Young silks were analyzed still connected to ears, and older silks were cut in 4 cm long sections, and in both cases placed in MS medium. Immature maize grains of 14, 21 and 30 days after pollination (DAP) were taken from maize ears sterilized as the maize mature grains (except that the wash with bleach solution was done during 20 min), sectioned or not in halves (see text) and placed in Petri dishes with MS medium. Immature embryos from surface sterilized 14 DAP grains were hand-dissected and transferred to MS medium.

2.4. Particle bombardment of maize tissues

All tissues were bombarded immediately after transferring to the Petri dishes, except the immature embryos, which were kept in the medium for 24 h before bombarding. For particle preparation, 60 mg of gold particles (1 μm diameter, Biorad) were transferred to an 1.5 ml polypropylene tube (Treff Lab, Swiss), vortexed 5 min in ethanol (HPLC grade) and pelleted in a microcentrifuge at top speed for 4 min. The pellet was washed three times with sterile water. Gold particles were resuspended in 1 ml of 50% glycerol and stored at 4°C until use.

The procedure for DNA coating used here is a modification of two previous reports [13–15]. The particle stock was vortexed 5 min and aliquots of 30 μl were transferred to 2 ml polypropylene tubes (Sarstedt, Germany). The coating were carried out by adding under continuous vortex (top speed) 8 μl DNA (0.5 μg/μl in TE pH 8.0), 112 μl H_2O, 150 μl of precipitation mix (2.1 M $CaCl_2$ and 0.02 M spermidine free base). The mix was vortexed 3 min and the tubes placed in ice 15 min to sediment the particles. The aqueous solution was discarded completely and the pellet was washed twice with 500 μl ethanol by vortexing. The pellet was resuspended in 48 μl ethanol by vortexing 1 min and sonicated in a water bath three times of 3 s each.

Bombardment was carried out in a PDS1000/He device from DuPont (commercially available from BioRad), with rupture disks of 900 p.s.i., 8 mm between rupture disk and macrocarrier, 6 mm between macrocarrier and stopping screen, 10 cm between tissue sample and stopping screen, under partial vacuum (0.1 atm). A total 8 μl of the gold particles coated with DNA were applied in the macrocarrier and left to dry in the flow hood over 5 min before bombarding. Unless otherwise stated, one shot was carried out per tissue sample.

After bombardment, tissues were kept in MS0 medium and incubated in the dark at 28°C during 24 h. Histochemical detection of GUS activity in tissue was carried out by incubating samples 24 h in the dark at 28°C in 10 ml tubes containing 5 ml X-Gluc solution (0.1 M sodium phosphate buffer pH 8.0, 0.1% Triton X-100, 10 mM EDTA, 5 mM potassium ferrocianyde, 5 mM potassium ferricianyde and 0.5 mg/ml X-Gluc). Fluorometric detection of GUS activity was carried out essentially as described by Jefferson [11], except for the use of 25% (v/v) of methanol in the reaction buffer [16]. Luciferase activity was quantified in a luminometer by mixing 20 μl of the same protein extract that for fluorometric detection of GUS activity with 100 μl of luciferin-containing reaction buffer [17]. Luciferase activity values were considered as the integral of millivolts produced in 10 s. The GUS/LUC ratio for each sample was calculated dividing the fluorescence in units/min due to GUS activity by the mV in 10s observed in the luciferase assay.

2.5. Production of transgenic maize cells

BMS suspension cells were collected 4 days after subculture in new medium. BMS suspension cells (0.5 ml PCV) previously filtered through a 500 μm mesh were transferred to filter paper discs (7.5 cm diameter), covering a circle of 5.5 cm diameter. Cells were kept for 4 days on Petri dishes with solid MSE medium at 28°C in the dark. Each Petri dish was bombarded twice with particles carrying 6.25 μg of pROB5 [18] and 6.25 μg of the DNA carrying the gene of choice. Selection of transgenic clones was carried out using the procedure described by Walters et al. [19]. Briefly, after bombarding, cells were immediately transferred to MSE medium containing 15 mg/l hygromycin. After 1 week, paper discs with cells were transferred to solid MSE medium containing 60 mg/l hygromycin. Clones showing normal growth were subcultured to new medium with 60 mg/l hygromycin.

2.6. Challenge of cells with ethylene and elicitors

Maize two DAG shoots and seven DAG seedlings were transferred to 1 l jars and challenged with ethylene (20 p.p.m.) or air 2 h after the bombardment. Ethylene concentration was monitored by gas chromatography. Previously to ethylene treatment, maize transgenic cells were transferred to 125 ml Erlenmeyers containing liquid MSE medium without auxin and hygromycin. After 3 days, aliquots containing 30 mg of cells (FW) were distributed in 13 ml glass tubes tightly closed with a rubber. Ethylene (20 p.p.m.) or air were injected with the aid of a syringe with a needle. Elicitor (15 μg/ml) from *Fusarium moniliforme*, prepared as described [20], or etephon (150 mg/l) were added directly to the culture medium.

3. Results

3.1. Tissue-specific activity of the HRGP promoter

Northern and/or in situ hybridization analyses have previously revealed that the maize *Hrgp* gene is mainly expressed in meristematic and transiently in vascular tissues [2,4]. In addition, the *Hrgp* gene has been shown to be highly expressed, at the protein and/or mRNA level, in plant structures such as the pericarp and the style [9,21]. These analyses have allowed to determine which cell types are responsible for the expression and when the expression takes place. The analysis of transient gene expression in cells transformed by bombardment with microprojectiles may allow to know which maize cell types are expressing the *Hrgp* gene in real time and to study the effects due to transcriptional activation of its promoter. Accordingly, different organs of the maize plant were bombarded with the p719 construct, that contains the promoter fragment of *Hrgp* gene starting at -719 (numbering related to the ATG) in its 5′-end and finishing at +16 within the coding region, fused in frame to the coding region of *uidA* gene in order to drive GUS expression. This promoter sequence was selected because it shows more than 90% identity among homologous *Hrgp* genes from the genus *Zea* [3] and because a transcriptional fusion (ending at position -52 in its 3′-end) of similar length of promoter (the 5′-end at -634) drove, in preliminary experiments, similar levels of GUS activity in maize callus cells as a transcriptional fusion with more than thousand bp of promoter [5]. Nevertheless, this type of constructs showed lower levels of expression compared to translational fusion (p719) used in this work [17]. In Fig. 1 the result of microbombardment of three organs with the p719 construction is presented. These include the immature 14 DAP pericarp and the leaf and the root tip of maize plantlets, in all of which the *Hrgp* mRNA accumulation has been studied. Pericarp and root tip are tissues exhibiting high endogenous *Hrgp* mRNA accumulation [2], while in the elongating and in the most differentiated zones of the primary root the *Hrgp* mRNA levels are gradually decreasing [6]. Correspondingly, after bombardment of the pericarp, GUS expression was found widespread through the surface of the kernel (Fig. 1A) and in different parts of the tip and the elongating zone of the root, with an apparent predominance at the boundary between the cortex and the central pith (Fig. 1C). In the

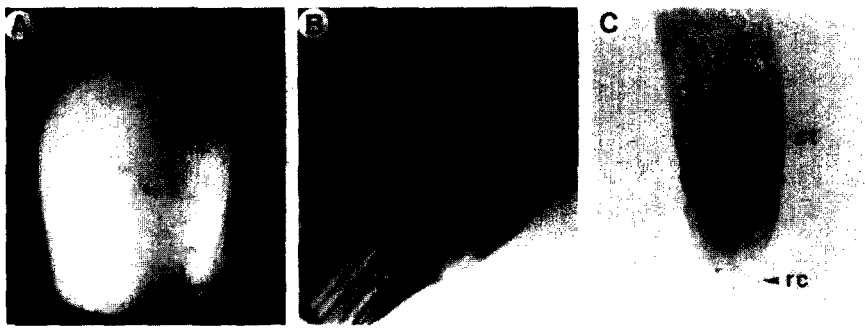

Fig. 1. Expression directed by the *Hrgp* promoter in maize tissues following microprojectile bombardment. Maize tissues were bombarded with 0.66 μg of p719 DNA construct and assayed for GUS expression as described in Section 2. Blue spots correspond to cells where the *Hrgp* promoter is expressed. (A) Maize grain showing blue spots in the pericarp. (B) Leaf showing blue spots in the auricle (au), sheath (sh) and leaf blade (lb) (C) Root tip with blue spots; rc, root cap; ct, cortex.

adult leaf, only a low number of spots was observed, mostly around the veins in good correlation with the low level of *Hrgp* expression observed previously in adult leaves [4,5] and in the auricle (Fig. 1B). This plant structure tolerates high tension to keep the sheath and the stem in almost perpendicular position. It is important to mention that the results here presented are representative examples of systematic investigation about these phenomena. In all cases, at least six independent experiments were always carried out and only reproducible results are here reported.

The silks, the styles of female inflorescence, are the structures of the maize plant where the highest expression of the *Hrgp* gene, analyzed by northern experiments, is attained [8]. However, no data are available so far on the spatial and temporal distribution of *Hrgp* mRNA accumulation in maize styles. In order to gain an insight into those aspects of *Hrgp* expression and to verify whether the DNA sequences present in the p719 promoter are sufficient to direct GUS activity in silks, female inflorescence were bombarded with the p719 construct. The results are shown in Fig. 2. The promoter was found to be particularly active in the emerging silks in the female inflorescence (Fig. 2A). Interestingly, in a single silk the *Hrgp* promoter activity appear to be higher it the hairs of the style, where the reception of pollen takes place in maize [22], than in the body of the silk (Fig. 2B).

The GUS activity directed by the p719 *Hrgp* promoter in maize kernels also had a good correspondence with the mRNA accumulation results observed in the immature kernel [23]. The GUS activity driven by the promoter in the endosperm has been assayed by bombardment into kernels hand-dissected in halves at three stages of development (Fig. 3). In Fig. 3A the result of bombarding an immature kernel of 14 days after pollination (DAP) is shown. At this stage the p719 *Hrgp* promoter is active in the embryo (see

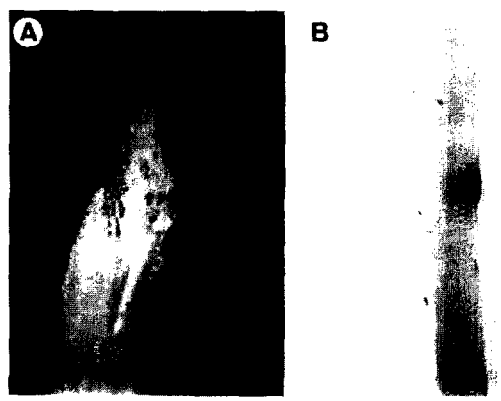

Fig. 2. Expression directed by the *Hrgp* promoter in the female inflorescence of maize. Young maize ears and isolated silks (styles) were bombarded with p719 DNA construct and the GUS activity assayed as described in Section 2. The blue spots correspond to cells where *Hrgp* promoter is active. (A) Young ear showing *Hrgp* promoter activity in the styles of very young pistils. (B) Detail of a silk, collected upon emergency, showing expression in the body style and its hairs.

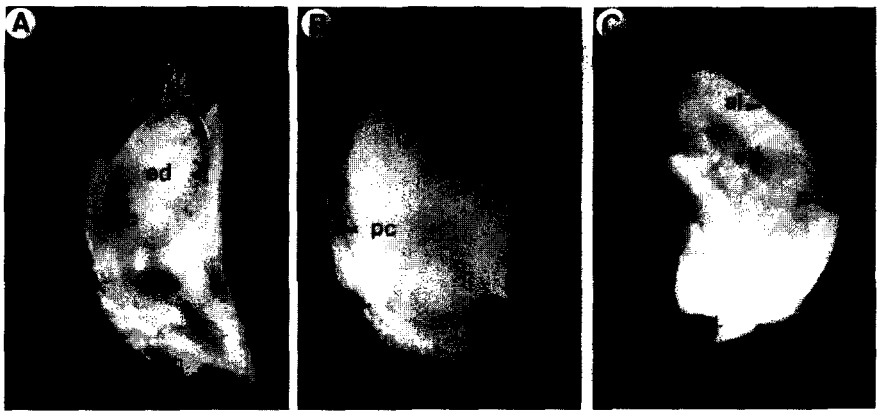

Fig. 3. GUS activity directed by the *Hrgp* promoter in different stages of the development of the maize grain. Maize grains were dissected in halves, bombarded with p719 DNA and developed to the GUS activity by incubation with X-Gluc, as described in the text. Blue spots represent cells where the *Hrgp* promoter is active. (A) 14 days after pollination (DAP) maize grain. *Hrgp* is expressed in the aleurone (al), endosperm (ed) and pericarp (pc). (B) 22 DAP maize grain showing expression in the aleurone and endosperm. (C) 30 DAP maize grain with no *Hrgp* expression.

also Fig. 4), pericarp and mainly in the endosperm cells, preferentially in the external layers of the three/four upper region of the 14 DAP endosperm. These regions have the youngest cells, active in cell division, since maize endosperm maturation proceeds from the center to the periphery and from the base to the top [24]. The activity of the promoter is restricted to the outermost layers in endosperm sections of 22 DAP (Fig. 3B), stage where transcriptional activity is practically reduced to the aleurone and sub-aleurone layers of the endosperm. Finally, at later stages (Fig. 3C) transcriptional activity in the endosperm is residual and no GUS activity can be observed using the *Hrgp* construct.

In immature embryos the expression of the maize *Hrgp* gene is subjected to a tissue-specific type of control: the mRNA and protein are abundant in the axis but undetectable in the scutellum [23]. However, when immature 14 DAP embryos were bombarded with the *Hrgp* promoter, the GUS activity was observed not only in the embryo axis but in the scutellum, particularly in the scutellum regions surrounding the axis node (Fig. 4A). A longitudinal section of the same age embryo shows high level of GUS activity in the coleoptile and in regions of the scutellum coincident with the scutellar procambium (Fig. 4B). In fact, when in situ hybridization experiments were performed, allowing a precise spatial detection of expression in immature embryos, a similar pattern was obtained comparing the endogenous mRNA accumulation in scutellar cells [7] and the GUS activity driven by the *Hrgp* promoter in the same cells.

The differential tissue specificity of *Hrgp* expression can be better analyzed in a quantitative way. This can be done dividing the GUS activity driven by the *Hrgp* promoter by the luciferase activity directed by the maize ubiquitin promoter of pAHC18 [12] from the same protein extract obtained from tissues co-transformed with p719 and pAHC18 constructs. In fact, the relative GUS/Luciferase activity driven by the *Hrgp* and 35SI promoters varies significantly from one tissue to another (Table 1). In tissues where the *Hrgp* mRNA level is low, such as leaves and immature embryos, the activity of *Hrgp* promoter is a fraction of the 35SI activity: between 7 and 2 times lower, respectively (Table 1). However, in 2 day old shoots where the mRNA level is the highest compared to the other organs [4,7] the relative activity is also the highest compared to 35SI and 40 to 140 times higher than in embryos or leaves, respectively.

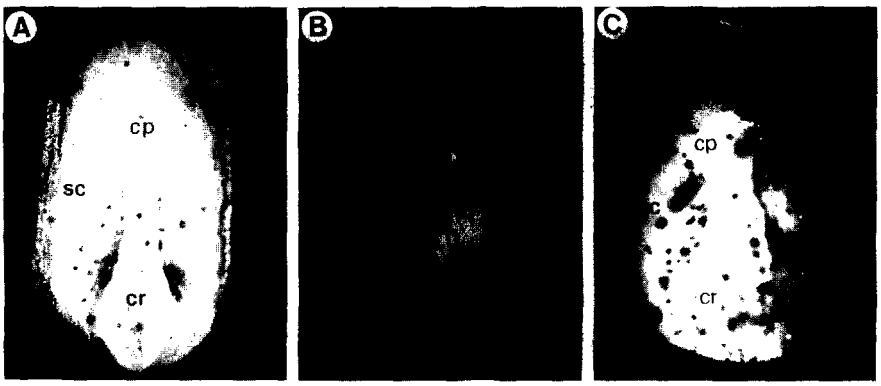

Fig. 4. Maize *Hrgp* promoter expression in immature maize embryos. 14 DAP maize embryos were hand dissected, transferred to MS medium and bombarded with 0.66 μ of p719 or p35SI DNA constructs and assayed for GUS expression as described in Section 2. Blue spots corresponds to cells where the *Hrgp* promoter is active. (A) Top view of a intact 14 DAP maize embryo showing expression of the *Hrgp* promoter in the coleoptile (cp), scutellum (sc) and coleorhiza (cr). (B) 14 DAP maize embryo longitudinally dissected before bombardment showing *Hrgp* promoter expression internally: cp, coleoptile; pl, plumule; sp, scutellar procambium; sc, scutellum; pr, primary root. (C) Embryo, as in A, showing expression of the 35SI promoter in the same tissues as *Hrgp* promoter. Note that tissue relative expression of GUS is different for both promoters, being the ratio of GUS activity in scutellum *versus* embryo axis lower for the *Hrgp* promoter.

3.2. Effect of ethylene on HRGP promoter activity

Hrgp gene expression can be induced by mechanical wounding [5,6] and by placing maize plantlets in an ethylene atmosphere [5]. The presence of the sequence GGGAAGCCTCC in the *Hrgp* promoter, which is very similar to the motif GAGAAGCCGCC found in the ethylene-induced chitinase gene from bean [25], prompted us to investigate, in maize transformed cells by particle bombardment, whether the promoter constructions containing such a sequence are responsive to ethylene or ethylene-producing compounds. In Table 2, the GUS activity of the *Hrgp* promoter is shown, compared to the activity driven by the cauliflower mosaic virus (CaMV) 35S promoter in mesocotiles or in two DAG shoots, in air or in an ethylene atmosphere. Surprisingly, the activity driven by the 35S promoter was always lower in the presence of ethylene than without. This negative effect of ethylene on the 35S activity was considerably reduced after only 4 h of ethylene treatment. A similar effect is observed for the *Hrgp* promoter: A slight reduction of activity in 24 h ethylene treated two DAG shoots versus 66% increment in shoots treated for only 4 h. Nevertheless, the highest increment of the activity directed by the *Hrgp* promoter upon ethylene treatment was taking place on mesocotiles (Table 2) in accordance with what has been observed for the endogenous gene [5].

Table 1
Expression of the p719 *Hrgp* gene promoter in different organs relative to p35SI promoter

Tissue	Relative expression[a]	Promoter compared
Leaves	0.15	35SI
14 DAP embryos	0.5	35SI
Two DAG seedlings	20	35SI

[a] Values represent the quotient of GUS activity driven by p719 *Hrgp* promoter and luciferase activity directed by pUBI promoter, relative to the quotient of GUS activity driven by p35SI promoter and luciferase activity directed by pUBI promoter in independent experiments.

The effect of ethylene on *Hrgp* promoter activity was also analyzed in stably transformed BMS maize cells. BMS cells were co-bombarded with the DNA construct having the *Hrgp* gene promoter (or the 35SI promoter, as a control) and a plasmid containing the gene coding for hygromycin resistance. Cells were allowed to prolif-

Table 2
Activity of the p719 *Hrgp* gene promoter in different organs in the presence or absence of ethylene

Organ	Construction	GUS activity[a] (pmoles/min per mg protein)	
		Air	Ethylene
Mesocotiles	p719	4.92 ± 1.86	10.22 ± 3.97
	p35SI	9.57 ± 2.23	7.46 ± 4.11
2 DAG shoots (24h)	p719	593.1 ± 212.1	461.0 ± 226.0
	p35SI	14.22 ± 2.48	5.72 ± 2.39
2 DAG shoots (4 h)	p719	496.3 ± 184.8	826.3 ± 366.5
	p35SI	48.89 ± 35.74	46.02 ± 26.84

[a] The results are the average of eight different experiments.

erate and to form calli in the presence of hygromycin. This protocol of transformation can give rise to chimeric calli. Nevertheless, the proportion of true transformed cells was increased by prolonging the selective regime. The different transformed calli were checked for the presence of *Hrgp* (or 35SI) promoter stably integrated into the maize genome (results not shown). Different compounds which caused increased ethylene production such as fungal elicitors [26], ethephon [27] or gaseous ethylene itself produce different effects on activities driven by *Hrgp* and 35SI promoters (Figs. 5 and 6). After treatment of transgenic cells of 35SI promoter construct with those compounds, an overall reduction of GUS activity driven by the 35SI promoter, compared to the non-treated cells is observed (Figs. 5 and 6). The opposite effect can be seen when transgenic cells of *Hrgp* promoter construct were exposed to fungal elicitors, ethephon or ethylene atmosphere: a general increase of GUS activity driven by the *Hrgp* promoter, compared to the non-treated cells was observed. Interestingly, the activity was higher at 6 h than at 24 h of ethephon treatment (Fig. 6). These results are compatible with a negative effect of ethylene on the overall transcription rate after long exposures to ethylene, reflected by the reduction of 35SI promoter activity, that could be due to a certain level of cell death in the tissues [28].

4. Discussion

The pattern of expression of maize *Hrgp* gene is an interesting system to be explored in comparison to the patterns of expression of the corresponding genes in dicotyledonous plants. While in dicotyledonous species hydroxyproline-rich glycoproteins, called extensins, are encoded by complex gene families [1,29], in maize a single gene appears

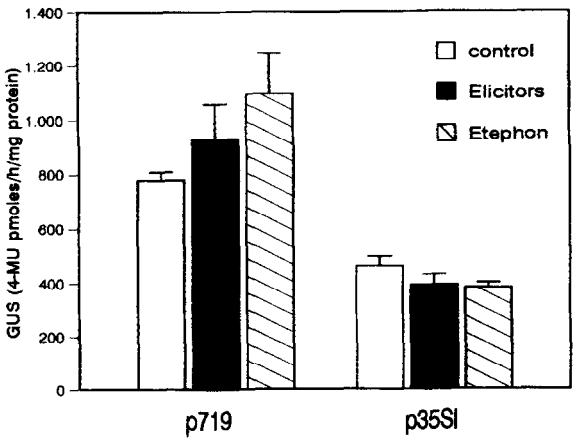

Fig. 5. Modulation of *Hrgp* expression in response to elicitors and etephon. Transgenic maize cells transformed with the construct p719 were incubated in MS medium with *Fusarium moniliforme* elicitors (15 μg/ml) and ethephon (150 mg/l). Control cells were incubated with MS medium only. Cells transformed with the p35S construct were used as non-inducible control. 24 h after challenge with the three treatments, cells were collected and assayed for GUS activity as described in Section 2. Bars correspond to the S.E.M. ($n = 8$, two independent experiments).

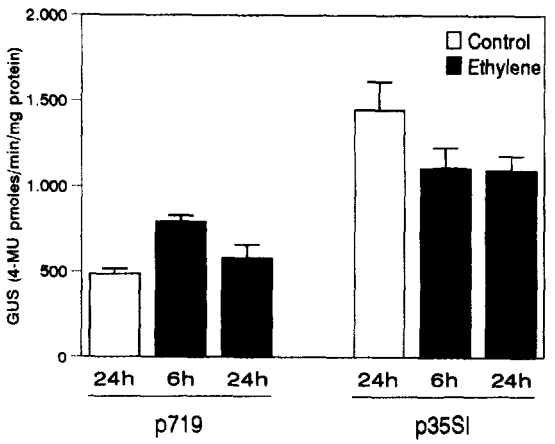

Fig. 6. Modulation of *Hrgp* expression in response to gaseous ethylene. Transgenic maize cells transformed with the construct p719 were incubated in MS media containing ethylene as described in Section 2. Cells transformed with the p35S construct were used as non-inducible control. Cells aliquots were collected after 6 and 24 h, as indicated under the bars, and assayed for the GUS activity. Bars correspond to the S.E.M. ($n = 8$, two independent experiments).

to encode HRGP [2,3]. Correspondingly, while in the dicotyledoneae analyzed so far, specific genes appear to respond to either developmental or stress signals [1] in maize the same gene is playing several roles. In fact, maize *Hrgp* gene expression is not detected in the scutellum of immature embryos but it is high in meristems, coleoptile and protecting or supporting organs like silks [2,4,7–9]. The same gene is also induced by mechanical wounding, ethylene treatment [5] and by fungal elicitors. Several attempts, in this laboratory, to study the expression of the *Hrgp* promoter in transgenic tobacco plants either with transcriptional or translational fusion constructs failed to produce any activity driven by the promoter. One possible explanation is that tobacco *Hrgp* genes present different expression patterns than maize *Hrgp* gene being, therefore, under distinct control mechanisms. It was concluded that the activity of the *Hrgp* promoter should be studied in maize or related species rather than in heterologous systems. In fact, preliminary results [5] indicated that particle bombardment could be used with success in the analysis of *Hrgp* promoter activity in maize.

Indeed, the *Hrgp* promoter sequence of the p719 construct seems to contain the elements that are essential to the observed pattern of expression of the endogenous gene [2,4–7]. On the one hand, p719 has been shown to contain the region of the promoter directing the highest GUS expression in two DAG maize coleoptiles cells [17], tissue with high *Hrgp* mRNA accumulation [4]. On the other hand, all the tissue-specific features of the expression of the gene already known are conserved. This is the case for young leaves [2,4], pericarp [8] and partly roots, in the sense that mRNA accumulation is found mainly in the root tip [4]. Interestingly, new patterns of *Hrgp* expression, previously unknown, are revealed with our experimental system, transient expression in cells transformed by bombarding with microprojectiles. In the case of the root, activity is observed in cortical cells, that would require reinforcement of their cell walls as a consequence of being put under mechanical stress by adjacent tissues, as has been reported for the *Nicotiana plumbaginifolia* extensin [30]. Similar reinforcement of cell walls is probably required in the cells of silk hairs, where high *Hrgp* promoter activity is shown (Fig. 2) and where the pollination takes place in maize [22]. *Hrgp* promoter activity is regulated developmentally, as expected, in the endosperm, being their highest activity simultaneous with active cell division at the early-mid stages of development (Fig. 3).

The only apparent contradiction between tissue-specific *Hrgp* promoter activity obtained by particle bombardment and previous published results is in the embryo. In this organ, when studied by RNA blot experiments [23] the gene seems to be repressed in the scutellum although division activity is present in this immature organ. Indeed, our microbombardment experiments show that both the axis and the scutellum actively express the *Hrgp* construct. In the scutellum, GUS activity driven by *Hrgp* promoter is specially high in the scutellar node (Fig. 4A), this region seems to be active in cell division, as has been shown by the high accumulation of histone mRNA [7], but no endogenous *Hrgp* mRNA have been detected. Other scutellar places with high *Hrgp* activity seen by bombardment correspond to the sculellar procambium. The seeming contradiction between the low levels of *Hrgp* expression in the scutellum,

assessed by Northern analysis, and the expression observed in certain scutellar cells transformed by bombardment could be reconciled if we took in account that there are few scutellar procambium cells compared to the total number of scutellar cells, and that the mRNA corresponding to endogenous *Hrgp* would be too much diluted in the pool of mRNA to be observed by Northern experiments. An alternative explanation to the apparent discrepancy between the mRNA accumulation results and the microbombarding data may be interpreted taking into account that after 2 h of placing immature embryos in in vitro culture the scutellar cells start *Hrgp* expression as a consequence from switching from an immature embryo programme to a callogenesis programme (José-Estanyol, M. personal communication). Consequently when the embryos are bombarded, scutellar cells have already become competent for *Hrgp* expression.

The *Hrgp* promoter of the p719 construct tested, as the endogenous *Hrgp*, is also responsive to ethylene, or ethylene-producing compounds as etephon or fungal elicitors. Therefore, a *cis*-acting element (or elements) involved in ethylene responsiveness must be present downstream nucleotide 719. A putative ethylene responsive element is the motif GGGAAGCCTCC present in the p719 promoter, which is having one imperfect (TCC) and one perfect GCC box, shown to be responsive to ethylene in tobacco [31]. To demonstrate the importance of this motif with respect to ethylene response either mutation of the GCC box or constructs without the motif should be tested in the future.

In the maize plant, the relative increase in *Hrgp* mRNA level varies from one tissue to another after ethylene treatment. In tissues with high basal expression level of the *Hrgp* gene, such as shoots (coleoptiles) or root tips, the relative increase is not high. Higher levels of induction are observed after ethylene treatment when the basal expression level of *Hrgp* gene is low, as in the mesocotyl [5]. A similar effect has been also observed both in stably or transiently transformed maize cells by bombardment. Nevertheless, the level of induction achieved by the p719 promoter, either in stably or transiently transformed maize cells, upon ethylene treatment was in most of the cases lower than for the endogenous gene. For example, in mesocotyl bombarded with p719 the level of induction of promoter activity produced after 24 h of ethylene treatment was twice, and even a slight reduction of activity was observed in shoots, whereas a level of induction of five or two times, respectively, was observed for the endogenous gene after 9 h of treatment with ethylene [5]. The GUS activity driven by the CaMV 35S promoter shed light on understanding these differences. In fact, the GUS activity driven by the CaMV 35S promoter suffered a gradual reduction with time after ethylene treatment. Because, the 35S promoter, as a classical constitutive promoter is not regulated by ethylene, the well documented senescent effect of ethylene [28] may account for the lower activity observed by reducing the number of cells contributing to the total promoter activity. If this would be the case, a higher increment of *Hrgp* promoter activity should be expected with shorter ethylene treatment. In fact, this is the case for two DAG bombarded shoots treated for 4 h (Table 2) or stably transformed BMS maize cells treated with ethylene for 6 h. Therefore, it is concluded that p719 *Hrgp* promoter is responsive to ethylene either in stably or transient transformed maize cells by particle bombardment.

In conclusion the p719 promoter of the maize *Hrgp* gene, used in the experiments of transient expression, appears to contain the essential elements needed to define the spatial and developmental pattern of expression of the gene. In addition, the experimental model used: Transient expression in transformed cells transformed by particle bombardment, has revealed new, previously unknown, cell types related to the reinforcement of the cell wall. These data offer new insights into the understanding *Hrgp* gene expression control and the role of HRGP in the maintenance of the cell wall.

Acknowledgements

The authors are grateful of the financial support of Dirección General de Política Científica (grant PB93-0043) and Plan Nacional de Inves-

tigación Científica y Técnica (grant BIO 94-0734). The work has been carried out within the framework of Centre de Referència de Biotecnologia de la Generalitat de Catalunya. M.M. is a fellow of CNPq-Brazil.

References

[1] M. Josè, P. Puigdomènech, Structure and expression of genes coding for structural proteins of the cell wall, New Phytol. 125 (1993) 259–282.

[2] V. Stiefel, L. Ruíz-Avila, R. Raz, M.P. Vallés, J. Gómez, M. Pagès, J.A. Martínez-Izquierdo, M.D. Ludevid, J.A. Langdale, T. Nelson, P. Puigdomènech, Expression of maize cell wall hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation, Plant Cell 2 (1990) 785–793.

[3] R. Raz, M. José, A. Moya, J.A. Martínez-Izquierdo, P. Puigdomènech, Different mechanisms generating sequence variability are revealed in distinct regions of the hydroxyproline-rich glycoprotein gene from maize and related species, Mol. Gen. Genet. 233 (1992) 252–259.

[4] V. Stiefel, L. Pérez-Grau, F. Albericio, E. Giralt, L. Ruiz-Avila, M.D. Ludevid, P. Puigdomenech, Molecular cloning of cDNA encoding a putative cell wall protein form *Zea mays* and immunological identification of related polypeptides, Plant Mol. Biol. 11 (1988) 483–493.

[5] D. Tagu, N. Walker, L. Ruiz-Avila, S. Burgess, J.A. Martínez-Izquierdo, J.J. Leguay, P. Netter, P. Puigdomènech, Regulation of the maize *Hrgp* gene expression by ethylene and wounding. mRNA accumulation and qualitative analysis by microprojectile bombardment, Plant Mol. Biol. 20 (1991) 529–538.

[6] M.D. Ludevid, L. Ruíz-Avila, M.P. Vallés, V. Stiefel, M. Torrent, J.M. Torné, P. Puigdomènech, Expression of a cell wall protein genes in dividing and wounded tissues of *Zea mays*, Planta 180 (1990) 524–529.

[7] L. Ruiz-Avila, S. Burgess, V. Stiefel, M.D. Ludevid, P. Puigdomènech, Accumulation of a hydroxyproline-rich glycoprotein gene mRNA is an early event in plant cell wall differentiation, Proc. Natl. Acad. Sci. USA 89 (1992) 2414–2418.

[8] J.M. Murphy, E.E. Hood, Molecular basis for extensin size heterogeneity in two maize varieties, Plant Mol. Biol. 21 (1993) 885–893.

[9] K.R. Hood, R.A. Baasiri, S.E. Fritz, E.E. Hood, Biochemical and tissue print analysis of hydroxyproline-rich glycoproteins in cell walls of sporophytic maize tissues, Plant Physiol. 96 (1991) 1214–1219.

[10] J. Callis, M. Fromm, V. Walbot, Introns increase gene expression in cultured maize cells, Genes Dev. 1 (1987) 1183–1200.

[11] R.A. Jefferson, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol. Biol. Rep. 5 (1987) 387–405.

[12] A.H. Christensen, P.H. Quail, Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotydonous plants, Transgenic Res. 5 (1996) 213–218.

[13] T.M. Klein, M. Fromm, A. Wissinger, D. Tomes, S. Schaaf, M. Sletten, J.C. Sanford, Transfer of foreign genes into intact maize cells with high-velocity microprojectiles, Proc. Natl. Acad. Sci. USA 85 (1988) 4305–4309.

[14] T.M. Klein, L.T. Gradziel, M.E. Fromm, J.C. Sanford, Factors influencing gene delivery into Zea mays cells by high-velocity microprojectiles, Bio Technology 6 (1988) 559–563.

[15] W.J. Gordom-Kamm, T.M. Spencer, M.L. Mangano, T.R. Adams, R.J. Daines, W.G. Start, J.V. O'Brien, S.A. Chambers, W.R. Adams, N.G. Willetts, T.B. Rice, C.J. Mackey, R.W. Krueger, A.P. Kausch, P.G. Lemaux, Transformation of maize cells and regeneration of fertile transgenic plants, Plant Cell 2 (1990) 603–618.

[16] S. Kosugi, Y. Ohashi, K. Nakajima, Y. Arai, An improved assay for β-glucuronidase in transformed cells: Methanol almost completely supresses a putative endogenous β-glucuronidase activity, Plant Sci. 70 (1990) 133–140.

[17] M. Menossi, Estudio de la expresión del gen *hrgp* de una proteina de la pared celular del maiz en células transformadas mediante bombardeo con microproyectiles, PhD Thesis, University of Barcelona, Barcelona, Spain, 1995.

[18] R. Bilang, S. Iida, A. Peterhans, I. Potrykus, J. Paskowski, The 3'-terminal region of the hygromycin-B resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*, Gene 100 (1991) 247–250.

[19] D.A. Walters, C.S. Vetsch, D.E. Potts, R.C. Lundquist, Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants, Plant Mol. Biol. 18 (1992) 189–200.

[20] D. Raventós, A.B. Jensen, M-B. Rask, J.M. Casacuberta, J. Mundy and B. San Segundo, A 20 bp *cis*-acting element is both necessary and sufficient to mediate elicitor response of a maize *PRms* gene, Plant J. 7 (1995) 147–155.

[21] E.E. Hood, K.R. Hood, S.E. Fritz, Hydroxyproline-rich glycoproteins in cell walls of pericarp from maize, Plant Sci. 79 (1991) 13–22.

[22] T.A. Kiesselbach, The Structure and Reproduction of Corn, University of Nebraska Press, USA, 1990.

[23] L. Ruíz-Avila, M.D. Ludevid, P. Puigdomènech, Differential expression of a hydroxyproline-rich cell-wall protein in embryonic tissues of *Zea mays* L, Planta 184 (1991) 130–136.

[24] R.V. Kowles, R.L. Phillips, Endosperm development in maize, Inter. Rev. Cytol. 112 (1988) 97–136.

[25] K.E. Broglie, P. Biddle, R. Cressman, R. Broglie, Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco, Plant Cell 1 (1989) 599–607.

[26] Th. Boller, Chemoperception of microbila signals in plant cells, Annu. Rev. Plant Physiol. Plant Mol. Biol. 46 (1995) 189–214.

[27] K.E. Lawton, S.L. Potter, S. Uknes, J. Ryals, Acquired resistance signal transduction in *Arabidopsis* is ethylene independent, Plant Cell 6 (1994) 581–588.

[28] S. Picton, S. Barton, M. Bouzayen, A. Hamilton, D. Grierson, Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene, Plant J. 3 (1993) 469–481.

[29] A.M. Showalter, Structure and function of plant cell wall proteins, Plant Cell 5 (1993) 9–23.

[30] C. Tiré, R. De Rycke, M. De Loose, D. Inzé, M. Van Montagu, G. Engler, Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*, Planta 195 (1994) 175–181.

[31] M. Ohme-Takagi, H. Shinshi, Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element, Plant Cell 7 (1995) 173–182.