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Regulation of the maize *HRGP* gene expression by ethylene and wounding. mRNA accumulation and qualitative expression analysis of the promoter by microprojectile bombardment

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Abstract

The expression of the maize gene coding for a hydroxyproline-rich glycoprotein (HRGP) has been studied by measuring the mRNA accumulation after wounding or ethylene treatment. RNA blot and *in situ* hybridization techniques have been used. The temporal and tissue-specific expression has been observed: the cells related to the vascular system show the more intense *HRGP* mRNA accumulation. Transcriptional constructions of the maize *HRGP* promoter have been tested on different maize tissues by microbombarding. A 582 bp promoter is able to direct the expression of the *gus* gene on calli and young leaves. Constructions having shorter promoter sequences lose this ability. The 582 bp construction retains the general specificity of expression observed for the *HRGP* gene.

Introduction

Little information is available on the regulation of genes coding for cell wall components of monocot species, although several genes encoding cell wall proteins have been cloned in the last two years [3, 8, 14, 19, 25]. In maize, a hydroxyproline-rich glycoprotein (HRGP) rich in threonine has been purified [11] corresponding to a cDNA clone and a gene already identified [25, 26]. The modalities of expression of this maize *HRGP* gene have been characterized both at the mRNA and protein levels [16, 22, 23, 26]. The spatial and temporal pattern of mRNA accumulation can be summarized as follows: (i) accumulation in dividing cells, (ii) transient overexpression in the vascular system and (iii) induction by wounding in young tissues.

The regulatory sequences controlling the expression of the *HRGP* gene in maize are unknown. In dicots, it was shown that a promoter of a tobacco *HRGP* gene was able to induce the expression of a reporter gene during initiation of lateral roots in transformed plants [9]. Similarly, the promoter of a gene encoding a glycine-rich wall protein of French bean was responsible for the

tissue-specific expression of a reporter gene in transgenic tobacco plants [10]: the information necessary for wound-induced expression was contained in a 494 bp promoter. A rape extensin promoter can govern *gus* expression in transgenic rape roots; the expression seems to be localized to the vascular system [24].

The aims of the present study were, first, to define the timing of induction of the maize *HRGP* gene by wounding and to distinguish it from cell division effects. Second, to analyse the effect of ethylene on expression of the maize *HRGP* gene in different parts of the plant. Finally, a primary functional dissection of the promoter was conducted and maize cells were transformed by particle bombardment in order to define regions necessary for the qualitative characteristics of the maize *HRGP* gene expression.

Materials and methods

Plant material

The plant material used for ethylene induction and wounding studies was derived from seeds of *Zea mays* L. cv. W64A pure inbred line. Seeds were germinated in the dark at 25 °C and grown for 7 days in vermiculite. In these conditions the mesocotyl elongates between 5 and 10 cm.

The plant material used for particle bombardment experiments derived from the *Zea mays* cv. LH132 pure inbred line. Seeds were surface-sterilized by a 5 min wash in pure ethanol, then agitated for 20 min in 6% sodium hypochlorite, followed by 5 washes in sterile water. 5 seeds per jar (d = 10 cm, h = 13 cm) were germinated in the dark at 25 °C for two days, then grown in the light for 5 and 13 days in medium containing Murashige and Skoog (MS) salts and vitamins [17], 15 g/l sucrose and 3 g/l Gelrite (Scott Lab.). Calli were induced from immature embryos excised from greenhouse-grown plants according to published techniques [6], but using a medium containing MS salts and vitamins, 20 g/l sucrose, 2 mg/l 3,6-dichloro-methoxybenzoic acid, 100 mg/l casein hydrolysate, 3 g/l proline and 3 g/l Gelrite. The friable callus line used for particle bombardment was isolated from one immature embryo 3 months after culture initiation, and was maintained for 15 months by weekly subcultures in the dark at 25 °C.

Ethylene treatments

Six separate pools of 15 individual plantlets each, were grown in the conditions described above. Then the flasks were hermetically sealed and were inoculated with a 5% ethylene gaseous stock to a final concentration of 0.1 or 100 ppm. Control flasks were inoculated with air. Ethylene concentration was checked every 2 h by gas chromatography. The samples were collected after 3 h or 9 h of incubation at room temperature and under daylight. Each plantlet was dissected separating the coleoptile, mesocotyl and root tip. One cm long sections of each part were immediately frozen and stored at −80 °C until RNA extraction.

Wounding

Seven-day-old plantlets grown in the dark were wounded in the mesocotyl by a longitudinal incision, 1 cm long, equidistant from the first node and the rest of the seed. After 15 min, 1 h or 2 h the wounded mesocotyl was dissected and immediately frozen in liquid nitrogen. For each time point 15 plantlets were used. The controls consisted of three groups of 15 unwounded plants, dissected in the same zone as the wounded plants.

RNA extraction, separation and blot hybridization

RNA was extracted according to Logemann *et al.* [15], separated in a denaturing agarose gel, blotted and hybridized as described [22]. The probes used were a 512 bp *Sna* BI segment of the 3′ coding region of a genomic clone of the *HRGP* [25], and a 328 bp insert of the histone *H4C14* clone, kindly provided by Dr Claude Gigot, IBMP, Strasbourg [18]. The probes were labelled

by random priming to a specific activity of 10^8 cpm/μg. The comparison between *HRGP* and *H4* mRNA signal was made by hybridizing the same filter with each probe. The autoradiographs were analysed by densitometry, and an arbitrary value of '1' was given to the mesocotyl control at time zero, corresponding to either the *HRGP* or the *H4* probe signals. The signals of the other samples were scaled according to these two reference values. *In situ* hybridization was performed as described [13, 23].

Plasmids

All the plasmid constructions were sequenced before their use in transformation experiments.

p1023GUS chimaeric gene. A 1023 bp *Bam* HI-*Mae* I fragment of the maize *HRGP* gene [20, 25] containing 970 bp upstream of the TATA box and ending 53 bp before the translation initiation codon was ligated into the *Hin*d II site of pUC19. The *Xba* I-*Hin*d III fragment was cloned into the promoterless GUS expression vector pBI101.1 [7]. The *Hin*d III-*Eco* RI fragment containing the whole chimaeric gene was subcloned in *Hin*d III-*Eco* RI sites of pUC18, to give the plasmid p1023GUS (Fig. 4).

p582GUS chimaeric gene. p1023GUS was digested with *Xho* I to eliminate a 441 bp 5′ fragment. The deleted plasmid was religated to give p582GUS (Fig. 4).

p244GUS chimaeric gene. p1023GUS was digested with *Hin*d III and *Xba* I to generate a 1023 bp promoter fragment and a pUC-GUS remainder plasmid. The former was digested with Ava and the 244 bp generated fragment was cloned into the pUC-GUS plasmid, both previously filled by the Klenow enzyme, to give p244GUS (Fig. 4).

p103GUS chimaeric gene. p244GUS plasmid was digested with *Bgl* I, filled by the T4 DNA polymerase and digested with *Bam* HI. A 103 bp fragment was isolated and cloned into the promoterless pUC-GUS expression vector to give p103GUS (Fig. 4).

p35S. The 3 kb *Hin*d III-*Eco* RI fragment of pBI121 [7] containing the CaMV 35S promoter, the GUS gene and the nopaline synthase terminator was cloned into the pUC19.

Particle bombardment device

Just prior to bombardment, small pieces of calli were placed on filter paper (Whatman 4.1), and expanded leaves and unexpanded leaves still in the leaf sheath were dissected and laid on their respective culture media in 6 cm Petri plates.

The DNA was precipitated onto tungsten particles with $CaCl_2$ [12] in microcentrifuge tubes. 8 μl of a 5 μg/μl DNA was added to 25 μl of tungsten suspended in 50% glycerol (400 mg/ml). 25 μl of 2.5 M $CaCl_2$ was added, followed by 10 μl of 10 M spermidine. After a brief centrifugation, 55 μl of the supernatant was removed and the rest was mixed and used for 4 bombardments (2 μl per shot).

The particle gun was a modified 22 caliber compressed-air rifle. A steel tube was inserted into the barrel and a stopping plate placed at the end with a plexiglass chamber holding the Petri plate 6 cm from the stopping plate. A 50 mbar vacuum was formed between the teflon macroprojectile and the plexiglass chamber before firing. The macroprojectiles attained a speed of 450 m/s (measured without a stopping plate or vacuum, 1 m from the end of the barrel). Each Petri plate was bombarded once and then incubated in the dark at 25 °C.

GUS enzyme assays

β-glucuronidase assays were conducted after 48 h by transferring the leaves or the calli (on the filter paper) into empty 6 cm Petri plates and adding 4 ml of sterile reaction buffer containing 50 mM NaH_2PO_4, 0.1% Triton X-100, 0.1% X-gluc at pH 8 [7]. Samples were infiltrated under vacuum for 5 min, then incubated for 24 h at 37 °C and 3 days at 25 °C. Samples were then fixed for 1 h with a solution containing 5% formaldehyde, 5% acetic acid, and 45% ethanol, and

rinsed with pure ethanol. Blue cells were counted under a stereomicroscope using 10–15 × magnification.

Results

Induction of HRGP *gene by wounding of mesocotyl*

The pattern of the *HRGP* mRNA accumulation in different parts of the maize plant has previously been studied. mRNAs were present mainly in the coleoptile node, the plumule and the root apex [26]. It has been shown that the gene is induced by stress conditions, like wounding in young leaves and coleoptiles. It was concluded that this cell wall protein is involved in the defence mechanisms of maize [16].

The timing of wound-induced gene expression was studied in mesocotyls from etiolated plantlets since they have a low rate of cell division and a low basal level of *HRGP* mRNA. Mesocotyls of young maize plantlets were wounded by incision and the accumulation of mRNA was measured at different times after wounding (Fig. 1). In the conditions of analysis, *HRGP* mRNA was not detectable in the unwounded mesocotyls but appeared 15 min after wounding, then sharply increased at 1 h and 2 h. This result indicated that the expression of the gene in mesocotyls could be induced by a mechanical stress whereas the basal expression was undetectable. The slight increase in the mRNA accumulation in the control with time might be due to a general activation of metabolism by light, since the plants were grown in the dark until the experiment was performed.

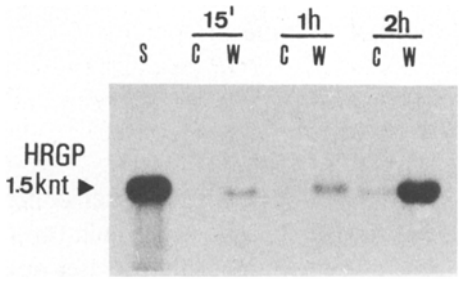

Fig. 1. Accumulation of maize *HRGP* RNA in response to wounding in mesocotyls. Gel blot hybridization of total RNA extracted from mesocotyl at different times after wounding, using a maize *HRGP* probe. S: coleoptile RNA (5 μg), used as a positive control; C: total RNA (10 μg) extracted from unwounded mesocotyls at different times; W: total RNA (10 μg) extracted from wounded mesocotiles at different times (see Materials and methods for details).

Ethylene treatments

Ethylene is known to increase under conditions of environmental stress and an increased biosynthesis of this plant hormone was shown to accompany wounding [1, 4]. In the case of fungal infection, ethylene production was correlated with the induction of *HRGP* in melon [21]. In our system, it was of interest to study the effect of ethylene as a possible mediator of the wounding response. Three tissues (namely coleoptiles, roots and mesocotyls) of young maize plantlets were exposed to different concentrations of ethylene as described in Materials and methods, and the mRNA accumulation was measured (Fig. 2). To discriminate between the direct effect of ethylene and its possible activation of cell division, a histone *H4* probe was used. In Fig. 2, the result of the RNA blot is shown using both the maize *HRGP* and histone *H4* probes. For each tissue a quantification of the results for the two probes was done, using as a reference the untreated mesocotyl at time zero. This allowed the quantification of the *HRGP* mRNA accumulated during the ethylene treatment. The highest induction of mRNA accumulation by ethylene was found in the mesocotyl, where the basal expression in an ethylene free atmosphere is very low. A 100 ppm treatment gave a maximum induction. After 9 h of treatment the induction was 25-fold, and a 3 h ethylene treatment produced a 17-fold increase in *HRGP* mRNA. In coleoptiles and roots, a lower induction was detectable (a maximum of 2-fold increase). In these tissues, there is a high basal level of *HRGP* mRNA. Ethylene was able to induce maize *HRGP* gene in mesocotyl where no basal expression is detected suggesting that the observed effect of ethylene could be correlated to the wound response.

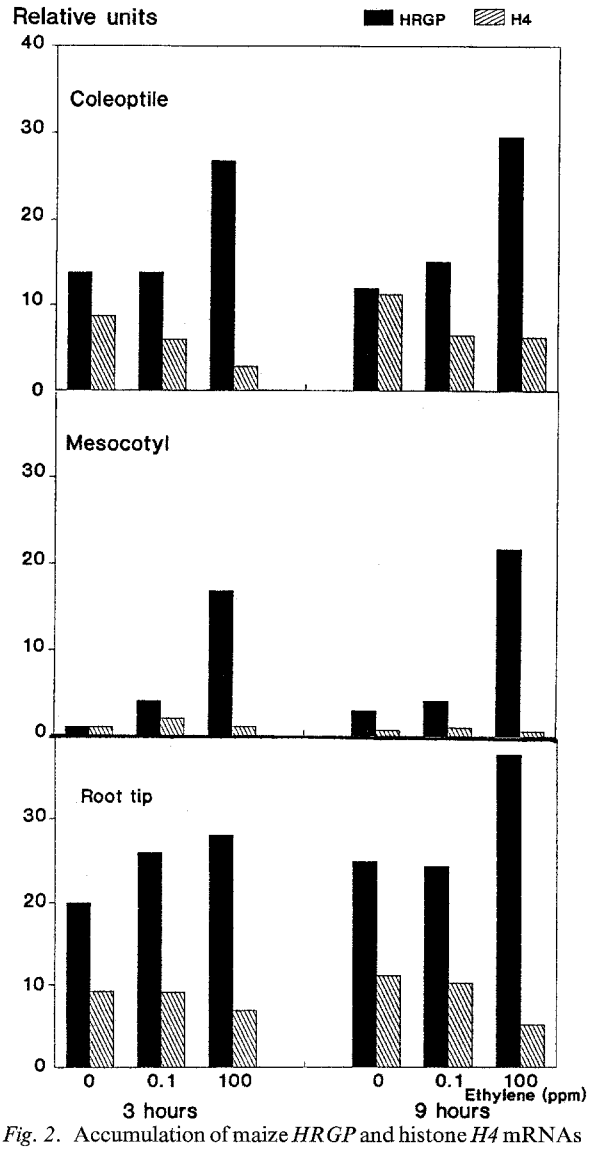

Fig. 2. Accumulation of maize *HRGP* and histone *H4* mRNAs in response to ethylene. The autoradiographs of a gel blot hybridized consecutively with *HRGP* and *H4* probes were analysed by densitometry: the values were scaled by assigning an arbitrary unit of 'one' to the densitometry units obtained for the *HRGP* and *H4* hybridizations in untreated mesocotyls at time zero. The plants were grown in the dark for 7 days in 6 separate pools of 15 individuals each. The recipients were injected with 5% ethylene gas so that two different atmospheres were created (0.1 mg/l and 100 mg/l of ethylene). The control batches were injected with an equivalent volume of air. One series of 0, 01 and 100 ppm of ethylene was collected after 3 h of treatment, and the other after 9 h. Each plantlet was manually dissected into sections representative of the coleoptile (1 cm section equidistant to the node and to the tip), mesocotyl (1 cm section equidistant to the node and to the seed), and root tip (1 cm section).

In situ *hybridization*

The *HRGP* mRNA shows a very defined pattern of accumulation when it is observed by *in situ* hybridization. In normal developing tissues, it shows a strong hybridization in provascular cells both in immature embryos and in germinating plantlets [23, 25]. It was of interest to know whether the induction of the *HRGP* mRNA upon ethylene treatment could be localized to specific cell types. To this end, *in situ* hybridization experiments were carried out in maize coleoptiles. The result showing a general overview and the region around the main vascular bundles in the presence and in the absence of ethylene is described in Fig. 3. The cells responsible for the mRNA induction were localized around the main xylematic vessels in the mesocotyl.

Functional analysis of the 5′ upstream DNA sequences

It was previously observed that the mRNA accumulation of the maize *HRGP* in dividing or wounded tissues and in vascular differentiation was related to a nuclease-sensitive structure in the chromatin of the 5′ region of the corresponding gene [27]. These findings allowed us to perform a functional analysis of the upstream DNA sequences of the maize *HRGP* gene. The 2.5 kb region immediately upstream of the TATA box was sequenced and the comparison of the 5′ regions of 4 different *HRGP* genes from a teosinte, sorghum and two varieties of maize revealed the presence of two zones [20]. The proximal region of ca. 500 bp (including the TATA box) was highly homologous among the four compared sequences, but the distal region showed less than 40% similarity. These findings were taken into account for the chimaeric gene constructions.

Four promoter fragments with 3′ end-points located at −53 bp from the first codon were ligated to the GUS-coding region. The polyadenylation signals were provided by a nopaline synthase 3′ end fragment. The resultant recombinant plasmids, p1023GUS, p582GUS, p244GUS and

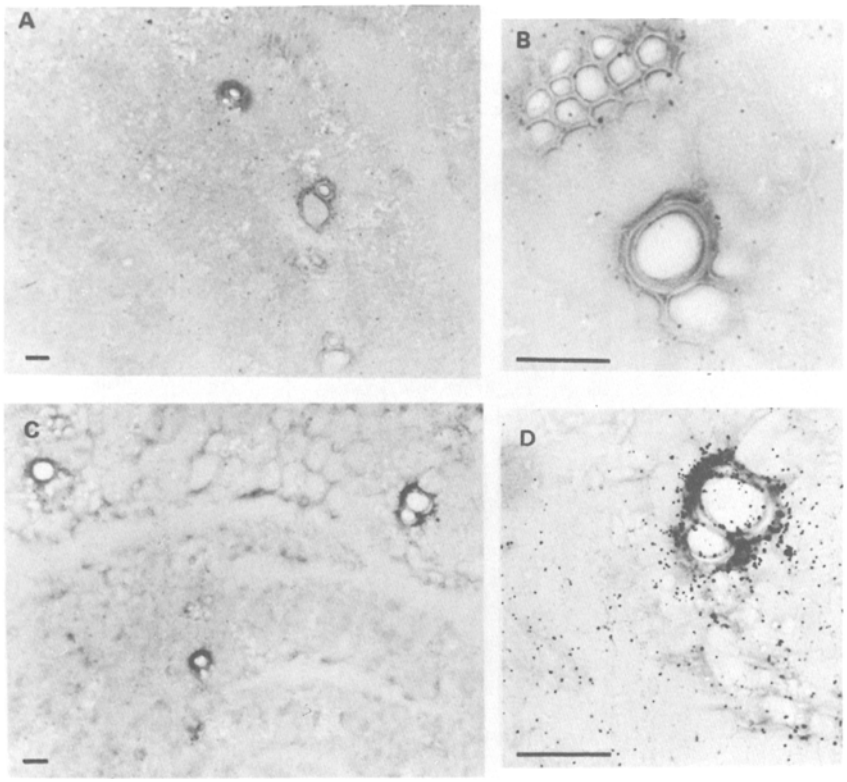

Fig. 3. In situ hybridization of sections of maize mesocotyls. Effect of ethylene. Sections of maize mesocotyl from plants prepared as indicated in Fig. 2 in the presence (C and D) or in the absence (A and B) of ethylene. A general view of the section (A and C) and a detail of the vascular tissues (B and D) are shown.

p103GUS, were introduced into maize calli by particle bombardment. The transient expression of the *gus* gene was analysed by a histochemical assay which allows the determination of the number of the transiently transformed cells (seen as a blue staining as described in Materials and methods). We assumed that the number of blue cells was correlated to the level of expression of the chimaeric gene and the results are summarized in Fig. 4. Two patterns of expression were observed. The first group (the first three lanes in the figure) including p1023GUS and p582GUS, gave a comparable high number of transformed cells. The 441 bp region deleted from the 1023 bp promoter did not quantitatively change the response. In contrast, the second group (p244GUS and p103GUS, lanes C and D) gave significantly lower levels of GUS expression in the maize calli transformed by the DNA particle bombardment. This strongly suggested that the 388 bp region absent from p244GUS contained an important element involved in the expression of the *HRGP* gene. From a quantitative point of view, the minimum functional promoter is restricted to a sequence included in p582GUS.

To analyse the qualitative characteristics of the expression of the maize *HRGP* promoter, p1023GUS was introduced into different types of tissues, namely calli, expanded and unexpanded leaves from 7-day-old plants. In order to perform comparisons between different tissues, the level of GUS activity given by p1023GUS was expressed relative to the 35S promoter (it is considered that the 35S promoter is expressed at similar levels in the different tissues) (Fig. 5). The relative expression of p1023GUS was highest in calli followed by unexpanded leaves, with no significant difference between them, and lowest sig-

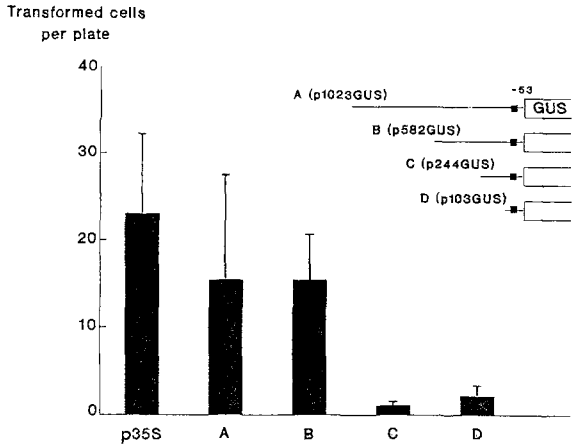

Fig. 4. Transient expression of the GUS gene in maize calli transformed by particle bombardment. Transcriptional *gus* fusions with a series of four maize *HRGP* gene promoter fragments (A to D) have been introduced into maize callus cells by particle bombardment. A *gus* fusion under the transcriptional control of the promoter 35S (p35S) was used as a reference. 48 h after transformation, the GUS activity was assayed as described in methods. Eight separate plates were bombarded with each construct in two independent experiments. The highest and the lowest values were not considered, and the mean of the number of transformed cells is shown on the Y axis. Horizontal bars represent the standard deviation. C and D are significantly different from p35S, A and B at $p < 0.005$. The black box on the construction drawings represents the putative TATA box of the *HRGP* gene.

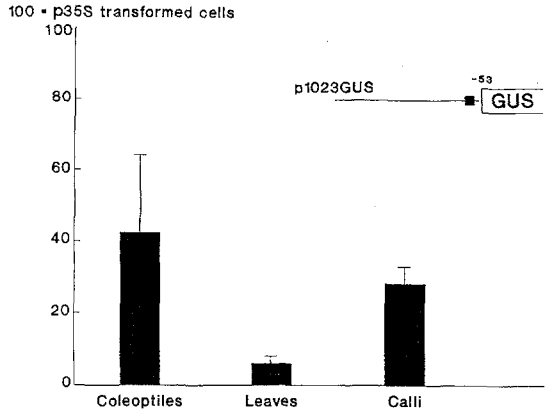

Fig. 5. Transient expression of the *gus* gene in different maize tissues transformed by particle bombardment. The p1023GUS plasmid was used to transform maize coleoptiles, leaves and calli. Plasmid p35S was used as a reference. Eight separate plates were bombarded with each construct, and the GUS activity was assayed 48 h after transformation. For each tissue, the mean of the number of cells transformed with p35S was given the value '100', and the values obtained for the transformation with p1023GUS were scaled accordingly. Horizontal bars represent the standard error of the means. The values obtained for unexpanded leaves and calli were not significantly different, and the value obtained for leaves was significantly different from the other two at $p < 0.05$. The black box on the p1023GUS drawing represents the putative TATA box of the *HRGP* gene.

nificantly different level of expression in expanded leaves. These data indicate that the *HRGP* promoter has a tissue specific expression in dividing cells (calli and unexpanded leaves), but not in the older leaves, where the divisional activity is highly reduced.

The analysis of the GUS expression driven by the 582 bp promoter (Fig. 6) showed that in 7-day-old leaves and calli, the cells containing the intense blue deposits were randomly distributed. However, in expanded leaves from 14-day-old plants, very few blue cells were observed, and all of them were localized in the vascular system, giving rise to a light blue colour diffusing in the vessels. With the 35S promoter blue cells were randomly distributed in leaves from 14-day-old plants (data not shown).

Discussion

The maize *HRGP* gene has previously been shown to have a well-defined pattern of expression. Its mRNA is accumulated in tissues rich in dividing cells, can be induced by wounding and accumulates preferentially in provascular cells [25]. It also has a tissue specificity in defined parts of the immature embryo [22, 23]. One of the interesting features of this gene in cereals is that this family of HRGPs are coded by a single gene in both maize [25], sorghum [19] and rice [3].

In the present paper the induction of the maize *HRGP* gene in relation to the defence responses associated to ethylene in maize has been addressed by analysing the mRNA accumulation using RNA blot and *in situ* hybridization techniques. Functional analysis of the promoter was carried out by transient expression using microprojectile transformation of maize cells.

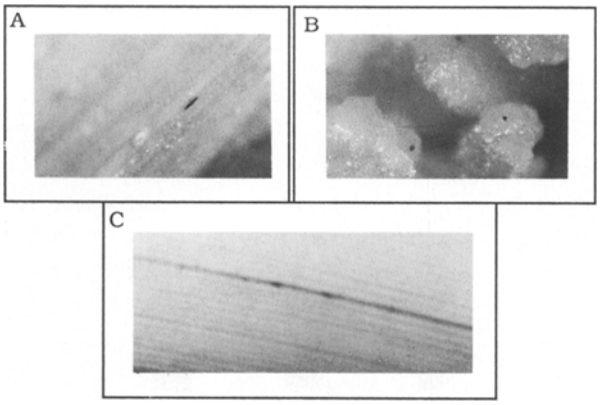

Fig. 6. Transient expression of the *gus* gene in different maize tissues transformed by particle bombardment. The p582GUS plasmid was used to transform expanded leaves from 7-day-old plants (A), calli (B) and expanded leaves from 14-day-old plants (C). Magnification: 225 ×.

In many plants, the induction of a wound response has been related to the production of ethylene [4, 5]. To test this phenomenon in maize, the induction of the *HRGP* gene in different organs has been examined in plants grown with atmospheres containing a defined amount of ethylene. The results indicate that ethylene is able to induce the expression of the *HRGP* gene in coleoptiles with a number of interesting features. First, the accumulation of *HRGP* mRNA is not correlated with the accumulation of histone *H4* mRNA and therefore it is possible to assume that the response is independent of cell division. The fact that wounding produces a fast reaction (detectable at 15 min) with a maximum of expression between 1 and 2 h also argues in that direction. Second, an increase in the mRNA level occurs in the different organs examined but in different proportion. Tissues such as mesocotyl, where the basal level of expression is lower, show the largest relative increase. This is also an indication of processes controlled by the ethylene action, independently from those controlling the *HRGP* gene expression during normal plant growth.

The cells competent for the *HRGP* mRNA accumulation upon ethylene action are located around the vascular system, the same cells which accumulate *HRGP* mRNA in coleoptiles during normal plant growth [26]. This result could also be interpreted as the consequence of a better accessibility to ethylene through xylem. However, the results of microbombardment discussed later argue in favour of these cells being those harbouring the factors necessary for the induction of the *HRGP* gene expression.

To better understand the regulation of the expression of the maize *HRGP* gene, primary functional studies of the promoter were carried out. To define the regions of the promoter that could play a role in the control of the gene expression, a series of 5′ deletions of the promoter were produced. The deletions were designed taking into account the results obtained by comparing the 5′ flanking sequences of the *HRGP* genes from different varieties of maize, teosinte and sorghum. This comparison allowed the definition of a zone of ca. 500 bp where significant homology could be found among these genes, whereas large genomic reorganizations and no homology were observed upstream from that region [20]. The constructions were used in transient expression experiments using microbombardment. It is a useful procedure to introduce and express genes in species such as maize where other approaches for transformation are difficult or impossible. For our purpose, it is an appropriate system to a tissue specific analysis of a promoter that probably carries sequences able to direct the induction by wounding, although it is difficult to separate the wounding effect produced by the microparticles from other effects, such as cell division.

The results of microbombarding experiments show that the most distal region of the promoter is unnecessary at least for the quantitative expression. The remaining 582 bp carrying a sequence almost identical to the ethylene regulating region of the bean chitinase gene [2] appear to be sufficient to induce the transcription of the *gus* reporter gene. It has been shown that this 5′-flanking region contains the main hypersensitive sites for nucleases in the chromatin [27]. When the construction having only 244 bp was tested, the expression of the reporter gene was severely reduced, indicating that at least essential quantitative *cis*-elements were lost.

The expression driven by the 582 bp fragment

was analysed in order to investigate which one of the features of the *HRGP* expression patterns observed by RNA hybridization methods were still conferred by the chimaeric constructions. The results indicate that some of the qualitative aspects of the *HRGP* expression may be reproduced in the transient expression experiments. For instance, a higher expression is observed in youngest leaf tissues. In particular in 14-day-old leaves the expression appears to be limited to cells located around the vascular system. This result agrees with the observation of the accumulation of *HRGP* mRNA in these cells as seen by *in situ* hybridization. Therefore, it can be concluded that the quantitative elements and at least some of the qualitative elements directing the transcription of the *HRGP* gene are conserved in the 582 bp flanking region, in agreement with the definition of the *HRGP* promoter obtained by sequence comparisons [20]. If microbombarding allows a qualitative functional analysis of the *HRGP* promoter into maize cells, further experiments have to be carried out in order to precisely analyse the promoter regions responsible for the quantitative expression.

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