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Analysis of regulatory elements of the promoter and the 3' untranslated region of the maize *Hrgp* gene coding for a cell wall protein

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Abstract Hydroxyproline-rich glycoproteins (HRGP) are structural components of the plant cell wall. *Hrgp* genes from maize and related species have a conserved 500 bp sequence in the 5'-flanking region, and all *Hrgp* genes from monocots have an intron located in the 3' untranslated region. To study the role of these conserved regions, several deletions of the *Hrgp* gene were fused to the *β*-glucuronidase (*GUS*) gene and used to transform maize tissues by particle bombardment. The overall pattern of GUS activity directed by sequential deletions of the *Hrgp* promoter was different in embryos and young shoots. In embryos, the activity of the full-length *Hrgp* promoter was in the same range as that of the p35SI promoter construct, based on the strong 35S promoter, whereas in the fast-growing young shoots it was 20 times higher. A putative silencer element specific for young shoots was found in the −1,076/−700 promoter region. Other major *cis* elements for *Hrgp* expression are probably located in the regions spanning −699/−510 and −297/−160. Sequences close to the initial ATG and mRNA leader were also important since deletion of the region −52/+16 caused a 75% reduction in promoter activity. The presence of the *Hrgp* intron in the 3' untranslated region changed the levels of GUS activity directed by the *Hrgp* and the 35S promoters. This pattern of activity was complex, and was dependent on the promoter and cell type analysed.

Keywords Cell wall · Intron · Particle bombardment

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Introduction

Plant cells define their interactions with other cells and with the environment through their wall. Plant cell walls are the result of complex processes that modify their composition and that take place during the development of the plant (Smith 2001). Extracellular and developmental signals, such as pathogen attack and hormones, also trigger changes in the cell wall architecture, influencing the way cells interact with the surrounding environment. The essential components of plant cell walls are complex polymers composed of carbohydrate and protein.

Among the protein components of the plant cell wall, hydroxyproline-rich glycoproteins (HRGP) are structural components with a ubiquitous distribution in plants (Josè and Puigdomènech 1993; Showalter 1993). These proteins may form cross-links with other HRGP, via isodityrosine bridges, increasing the mechanical strength of cell walls (Showalter 1993; Kieliszewski and Lamport 1994). Several reports have shown that expression of HRGP genes in various species is developmentally regulated in a tissue-specific manner (Keller and Lamb 1989; Ye and Varner 1991; Sheng et al. 1993) and also in response to biotic and abiotic stresses (Lawton and Lamb 1987; Showalter et al. 1991; Bradley et al. 1992).

A maize gene coding for a HRGP rich in threonine has been cloned (Stiefel et al. 1988) and its expression is modulated in several ways. In tissues rich in dividing cells, such as coleoptile nodes and root tips, there are high levels of *Hrgp* transcripts (Ludevid et al. 1990; Stiefel et al. 1990; Tagu et al. 1992). A transient accumulation at new vascular sites is observed in embryos and roots (Stiefel et al. 1990; Ruiz-Avila et al. 1991, 1992) and also in response to ethylene and wounding (Tagu et al. 1992).

So far, there have been few studies on the regulatory regions that modulate the expression of genes coding for structural cell wall proteins. Identification of transcriptional regulatory elements within promoter regions from plant cell wall genes may be important in unravelling the network of transcription factors that modulate the changes that take place during the life of the cell. A high level of sequence conservation is observed in the first 500 bp upstream from the coding region of the *Hrgp* genes in maize, teosinte and sorghum (Raz et al. 1992). A previous study of *Hrgp* promoter deletions in maize calli showed that the region between bp −634 and −297 had a profound effect on promoter activity (Tagu et al. 1992). We later found that a 699 bp fragment of the *Hrgp* promoter contained all of the elements essential for controlling the spatial and developmental pattern of *Hrgp* gene expression (Menossi et al. 1997). This construct was able to direct expression in several maize tissues, including the pericarp, endosperm, leaves, roots, silks and immature embryos, according to the pattern of mRNA accumulation (Menossi et al. 1997).

In addition to the 5' upstream region, regulatory elements also occur in introns, where they can modulate gene expression by increasing transcription (Gidekel et al. 1996) and mRNA stability (Mogen et al. 1990; Ohtsubo and Iwabuchi 1994), and which are essential for proper expression in some genes (Callis et al. 1987; Vasil et al. 1989; McElroy et al. 1990; Fu et al. 1995a). The only intron present in the maize *Hrgp* gene is located in the 3' untranslated region—an unusual position (Raz et al. 1992). The *Hrgp* homologues of teosinte, sorghum and rice (Raz et al. 1991, 1992; Caelles et al. 1992) and other genes coding for cell wall proteins in carrot (Chen and Varner 1985) and tomato (Salts et al. 1991) also have an intron located in this region. Surprisingly, the *Hrgp* intron has an overall sequence similarity of 90% when different introns from maize lines and teosinte are compared (Raz et al. 1992). We are unaware of any study that has examined the putative role of these introns located in the 3' region of genes coding for cell wall proteins.

In this work, several *Hrgp::β-glucuronidase* (*GUS*) constructs were tested using particle bombardment in order to identify positive and negative regulatory regions involved in controlling gene expression in young shoots and immature embryos. Our analysis of the putative regulatory regions of the *Hrgp* gene showed that the intron in the 3' untranslated region affected gene expression in a tissue- and promoter-dependent manner.

Materials and methods

Plant material

Plants from the maize (*Zea mays* L.) W64A inbred line were grown in a greenhouse and used throughout the gene expression studies employing microprojectile bombardment.

DNA constructs

For convenience, the sequence positions for all the constructs are given considering the A of the *Hrgp* initiation codon as +1. The initial construct was the plasmid pBSFOK144, containing a *Fok*I fragment spanning the region −159/+16 from an AC1503 maize genomic fragment (EMBL accession number AJ131535) cloned in pBluescript KSII. An *Eco*RI/*Bam*HI fragment from pBi101.3 (Jefferson 1987) containing the GUS coding region (*uidA* gene) followed by the nopaline synthase terminator (3'NOS) was cloned in frame after the pBSFOK144 insert to give construct p159.

A *Kpn*I/*Dra*II fragment from p159 (spanning the region −159/−140) was replaced by a *Kpn*I/*Dra*II fragment containing the −699/−140 promoter region from the genomic clone to produce construct p699. A *Bam*HI fragment containing the entire promoter region from p699 was digested with *Alu*I/*Dra*II to yield a fragment containing the −509/−140 promoter region. This region replaced the *Kpn*I/*Dra*II of p159 and produced construct p509. p297 was obtained from partial digestion of p699 (previously linearised with *Eco*RI) with *Hin*dII, and the 2.3 kb fragment containing the −297/+16 promoter fragment plus the *uidA*/3'NOS cassette was cloned in pUC19. A *Sca*I/*Dra*II fragment containing the −1,076/−140 promoter region was obtained from pUC1023 (containing the −1,076/−73 promoter region cloned in pUC18). This fragment replaced the −699/−140 region of p699, producing construct p1076.

To obtain p114, PCR was performed using p297 as the template, with the primers 5'-TGTATATAAGCAGTGGCA-3' (starting at position −114) and 5'-GTCGAGTTTTTTGATTTCACGGG-3' (from the *uidA* gene). The PCR product was cloned in pUC18 and the region −114/+16 was released with *Eco*RI and *Sma*I. This fragment replaced the −297/+16 *Hrgp* promoter fragment from the p297 construct. p104 was obtained in the same way, using the *Hrgp*-specific primer 5'-CAGTGGCAGGGTGAGCG-3' (starting at position −104). To produce p1076/35S, a −1,076 (*Hin*dIII)/−139 (*Dra*II, Klenow) from pUC1023 was cloned upstream of the −72/+6 fragment of the 35S promoter from p-70/35S (kindly provided by Dr. S. Prat), previously cut with *Xba*I, treated with Klenow and digested with *Hin*dIII.

p1076/52 contained the −1,076/−52 promoter region fused to *uidA* and 3'NOS (Tagu et al. 1992). pGN contained only the *uidA* and 3'NOS (kindly provided by Dr. S. Prat). p35S contained the 800 bp 35S promoter region/*uidA*/3'NOS cassette from pBi121.1 (Jefferson 1987) cloned in pUC19. p35SI contained a 450 bp promoter region from the 35S promoter fused to the first intron of the *Adh1-S* gene and the *uidA*/3'NOS region, and was obtained by digesting pCaMVI1NEO (Callis et al. 1987) with *Bam*HI/*Eco*RI and replacing the resulting NPTII/3'NOS fragment with a *Bam*HI/*Eco*RI fragment containing the *uidA*/3'NOS from pBi121 (Jefferson 1987). The constructs used as internal controls were pAH18 (Cornejo et al. 1993), containing the maize ubiquitin promoter and its first intron fused to a luciferase (LUC) coding region, and pC1 and pB-Peru, containing the 35S promoter and the maize *Adh1* first intron fused to the *c1* and *B-Peru* coding regions, respectively (kindly provided by Dr. S. Wessler).

The *Hrgp* 3' untranslated region from nucleotide +973 (relative to ATG; containing the last 12 bp of the coding region and the stop codon) to the second A in the putative polyadenylation site, was obtained by PCR from the MC56 construct containing a cDNA clone (Stiefel et al. 1988) using the primers 5'-CACCTTACTACTAGAAACCGATGCC-3' and 5'-CGAATTCTCCTTGCATTTTTTCCACATTTATT-3'. The amplified insert was cloned in the *Sma*I site of pUC18, to produce the pTc construct. pTg was obtained as described for pTc, using genomic DNA as the template. The *Sac*I/*Eco*RI fragment containing the NOS terminator from the constructs p699, p35S and p35SI was replaced with a *Sac*I/*Eco*RI fragment from pTc to give constructs containing the *Hrgp* terminator without the intron. Another set of constructs containing the *Hrgp* terminator with the intron was obtained in the same way using a *Sac*I/*Eco*RI fragment from pTg.

Particle bombardment and quantification of promoter activity

Six young maize seedlings at 2 days after germination (DAG) were placed in the centre of a 5.5 cm diameter Petri dish containing 0.6% agar in water. Immature embryos from surface sterilised 14 DAP (days after pollination) ears were hand-dissected and transferred to

Petri dishes (nine embryos per dish) containing Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose and 2.4 g/l Gelrite (pH 5.8). Black Mexican Sweet (BMS; Chourey and Zurawski 1981) maize suspension cells were collected 3–4 days after subculturing in MSE medium (MS medium containing 1 mg/l 2,4-dichlorophenoxyacetic acid, 0.02 mg/l 6-benzylaminopurine, 3% sucrose, pH 5.8). The cells were filtered through a 500 μm mesh, and 0.5 ml packed cell volume was placed on a 5.5 cm disc of filter paper. The cells were kept in the dark for 4–5 days before bombardment. The number of replicates in the experiments varied from 6 to 14, as indicated in the figure legends.

Particle preparation, DNA coating and bombardment were carried out as described elsewhere (Menossi et al. 2000). Histochemical detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) as substrate. Fluorometric detection of GUS activity was done as described by Jefferson (1987), using 25% (v/v) methanol in the reaction buffer to decrease endogenous GUS activity (Kosugi et al. 1990). To quantify LUC activity the Luciferase Assay System (Promega, Madison, Wis.) was used. Tissues were extracted with the reporter lysis buffer supplied and LUC activity was quantified in a luminometer by mixing 20 μl protein extract with 100 μl luciferase assay reagent. The GUS/LUC ratio of each sample was the quotient between GUS activity (fluorescence units/min) and the integral of the mV produced in 10 s in the luciferase assay.

Results

Analysis of 5' deletions of the *Hrgp* promoter

Although no significant sequence similarity was found between the promoter of the *Hrgp* gene and other gene promoters, two potential transcription factor binding sites were found using Matinspector software (Quandt et al. 1995). One putative binding site was in the regions −540/−527 and −508/−493, which contained two sequences (in opposite orientation) that differed by only one base from the octamer motif CGCGGATC found in plant histone promoters that show high activity in tissues rich in dividing cells (Chaubet et al. 1986; Meshi et al. 1998). The other binding site, located in the region −462/−468 (minus strand), also differed by only one base from the AGCCGCC box found in genes that are up-regulated by ethylene (Ohme-Takagi and Shinshi 1990). A nuclease hypersensitive site is located in the region −205/−173 (Vallés et al. 1991). To investigate sequences involved in expression of the *Hrgp* promoter, a series of 5' deletions was fused in frame to the *uidA* gene. The 5' flanking region of the *Hrgp* promoter with the end-points of the deletions and the relevant sequences are shown in Fig. 1.

Using particle bombardment, the constructs represented schematically in Fig. 2A were transferred to immature embryos and young shoots to assess the relevance of distinct promoter regions. A gradual decrease in GUS activity was detected in immature embryos upon progressive deletion of the *Hrgp* promoter (Fig. 2B). The p1076 construct showed 50% of the activity of the p35SI construct based on the strong 35S promoter. The highest changes in GUS activity occurred between constructs p699 and p509 and between constructs p297 and p159, indicating the presence of positive regulatory elements in these regions. The relevance of the sequence between

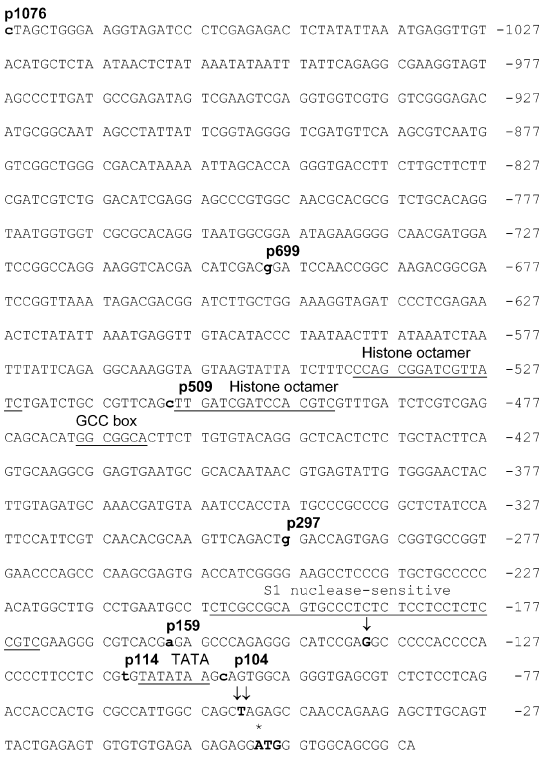

Fig. 1 Nucleotide sequence of the 5'-flanking region of the maize *Hrgp* gene. Nucleotides are numbered relative to the ATG of the translation start site (*asterisk*). Putative regulatory sites are underlined. The 5' end of the seven translational fusion deletions are indicated in bold lower case letters. The 3' ends of the two transcriptional fusions, p1076/35S and p1076/52, are indicated with single and double *arrows*, respectively

−159 and −115 bp was inferred by the 39% decrease in GUS activity when p159 and p114 were compared. Further deletion of the 5' flanking sequence −114/−105 (TG*TATATAA*G) containing the putative TATA box (italicised) reduced GUS expression to the background level seen in the promoter-less pGN.

To evaluate whether the activity driven by the *Hrgp* promoter regions was modulated differently in other tissues, the same constructs were assayed in young shoots (Fig. 2C). The pattern of GUS activity directed by the *Hrgp* deletions in coleoptiles was different from that in immature embryos. Progressive 5' deletions in the promoter did not cause a gradual decrease in GUS activity: p699 was 60% more active than p1076. This result suggested the presence of a negative regulatory element. As in immature embryos, the regions −699/−510 and −297/−160 also had an important role in promoter expression in shoots since their deletion caused reductions of 62% and 78%, respectively. Deletion of region −159/−115 caused a 36% decrease in GUS activity, similar to that in 14 DAP embryos. Only a background level of expression was observed upon deletion of the region −114/−105, as also observed in 14 DAP embryos. In the fast-growing maize cells from coleoptiles, p1076 was far more active than the p35SI construction, in contrast to

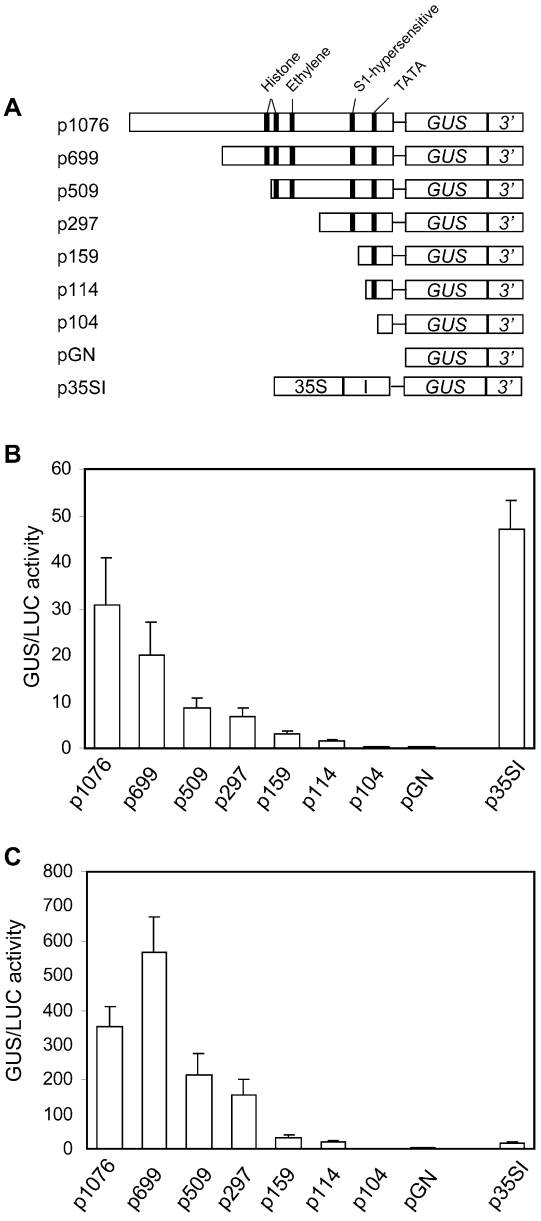

Fig. 2A–C *Hrgp* promoter analysis in maize embryos and seedlings. **A** The promoter deletions used in the transient expression assays are shown schematically. The *black boxes* indicate the different regulatory elements in the *Hrgp* promoter. *GUS* and *3'* indicate the *β*-glucuronidase coding region and the nopaline synthase (NOS) terminator, respectively. Constructs were transferred by particle bombardment to **B** maize embryos 14 DAP (days after pollination) (*n*=6), and **C** seedlings 2 DAG (days after germination) (*n*=14). In both cases, a luciferase (LUC) internal control (pUBILUC) was included to normalise the efficiency of transfection. GUS/LUC activities were measured 24 h after particle bombardment. Columns represent means ±SD

expression of these two constructs in immature embryos (Fig. 2A). The level of GUS activity directed by construct p114, which contained only 2 bp before the TATA box, was similar to that observed with the p35SI promoter in coleoptile cells.

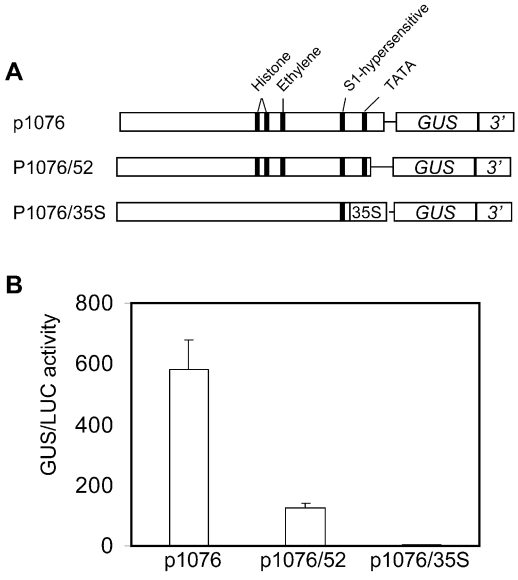

Fig. 3A, B Influence of deletions in the 3' end of the *Hrgp* promoter in maize shoots. **A** Schematic representation of the three constructs. The three relevant boxes are represented by *black boxes* inside the promoter region. *35S* −72/+6 35S deletion. **B** Shoots were bombarded with the constructs shown in **A**, together with a LUC internal control (pUBILUC) used to normalise the efficiency of transfection. GUS/LUC activities were measured 24 h after particle bombardment. Columns represent means ±SD (*n*=6)

To further evaluate the relevance of sequences most proximal to the TATA box, the activity of the translational construct p1076 was compared to that of constructs p1076/52 and p1076/35S (Fig. 3). Deletion of region −52/+16 in construct p1076/52 caused a 75% reduction in promoter activity. The substitution of the sequence −139/+16 by the truncated −72/+6 35S promoter in the p1076/35S construct abolished the promoter activity since GUS activity decreased to background levels.

Influence of the *Hrgp* intron on gene expression

The presence of an intron in the untranslated 3' region of several cell wall genes (Chen and Varner 1985; Raz et al. 1991, 1992; Salts et al. 1991; Caelles et al. 1992), prompted us to study the effects of the *Hrgp* intron on gene expression. For this, the 3' untranslated region spanning the nucleotides from +973 (relative to ATG, and containing the last 12 bp from the coding region as well as the stop codon) to the second A in the putative polyadenylation site was PCR-amplified from both cDNA and genomic DNA. The sequences obtained had the same nucleotide composition, but that amplified from genomic DNA had a 167 bp sequence insert representing the *Hrgp* intron (Fig. 4). There was strong sequence identity between the 5' and 3' splice sites of the *Hrgp* intron (AG/GTACGT and GACAG/GT, respectively) and the consensus maize sequences AG/GTAAGT and TGCAG/GT (Luehrsen and Walbot 1994). The A+T content was

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Genomic  ccaccttactacTAGAAACCGATGCCTACCATACCACACTGCTGTCAGTC
cDNA     ccaccttactacTAGAAACCGATGCCTACCATACCACACTGCTGTCAGTC
                                                        ↓
         TCTGGAGCATTTCATACCACACTGCTGTCAGTCTCTGGAGCATTTAGGTA
         TCTGGAGCATTTCATACCACACTGCTGTCAGTCTCTGGAGCATTTAG---

         CGTACTAATACTACGTACAACGGTACAAGAATGGAGCATGCAATATGCAT
         ----------------------------------------------------

         GCACACTACATACATTTAGTATGCTTGTGTCAAATGTATCGTCAGTATCA
         ----------------------------------------------------

         TACTGATCTCCTGGCATAGTCTGGCACTAACCATAGGCTCTCCTTTTCTT
         ----------------------------------------------------
                        ↓
         TTGTGTTGGGACAGGTGGTCTCGATCGATGGAAGAATTGTGTCCTAGCCA
         ----------GTGGTCTCGATCGATGGAAGAATTGTGTCCTAGCCA

         GCCGGCAAAGGTGACCTGCTGATGATGATGATGAGAGGCGAGTCCTACGC
         GCCGGCAAAGGTGACCTGCTGATGATGATGATGAGAGGCGAGTCCTACGC

         CCTAGTCCTACTACTACCCTCTTTGTGTGCTGCCATCCATCCGTCCCCGC
         CCTAGTCCTACTACTACCCTCTTTGTGTGCTGCCATCCATCCGTCCCCGC

         TAGACGATCGAGGAGAGAATAACGCAGAGCTCTGTGCTCCCGGCCTCTGT
         TAGACGATCGAGGAGAGAATAACGCAGAGCTCTGTGCTCCCGGCCTCTGT

         CTTCTGCCGTCCCGGGCCTTTAATTTATAGTCTCTACTGTGTGTTCGTCC
         CTTCTGCCGTCCCGGGCCTTTAATTTATAGTCTCTACTGTGTGTTCGTCC

         CATGTGTTTAGCAGCAGCAGCAGGTGTGTTGTGCGGGTATGTAATGGTAT
         CATGTGTTTAGCAGCAGCAGCAGGTGTGTTGTGCGGGTATGTAATGGTAT

         TGCAACTATATTGGGTGTAAAACCATAATAAATGTGGAAAAAATGCAAGG
         TGCAACTATATTGGGTGTAAAACCATAATAAATGTGGAAAAAATGCAAGG

Genomic  AGAA  (603 bp)
cDNA     AGAA  (436 bp)
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Fig. 4 Sequence alignment of the 3' untranslated region from genomic and cDNA clones of the *Hrgp* gene. *Lower case letters* Last 12 bp of the coding region, *bold letters* stop codon and polyadenylation signal, *underlined sequences* PCR primers, *arrows* intron processing sites. The length of each sequence is indicated

58%, in agreement with the overall A+T content of 59% in monocotyledonous plant introns (Simpson and Filipowicz 1996).

Two sets of constructs were obtained by replacing the NOS terminator of constructs p699, p35S and p35SI with the *Hrgp* genomic terminator (containing the intron) and the *Hrgp* cDNA terminator (without the intron) (Fig. 5). These constructs were transferred to maize cells by microprojectile bombardment and the GUS activity was measured.

In fast-growing coleoptiles, construct p699 containing the genomic *Hrgp* terminator showed a higher level of GUS activity than the construct containing the *Hrgp* cDNA terminator (Fig. 5). This positive effect was not restricted to the *Hrgp* promoter, since constructs based in the p35S and p35SI regulatory regions showed the same pattern. Nevertheless, the increase in gene expression due to the *Hrgp* intron in p699-based constructs was higher than in the other two sets of constructs. The presence of the Adh1-S intron in the 5' untranslated region of p35SI-based constructs did not alter the positive effect of the *Hrgp* intron.

Expression analysis in maize BMS suspension cells showed that the overall pattern of the *Hrgp* intron effects was quite different. In BMS cells, the presence of the intron in p699-based constructs was deleterious since the intron-containing construct had a lower level of GUS activity when compared to the intron-less *Hrgp* terminator (Fig. 6). In p35SI-based constructs, the level of expression was similar in constructs with or without the intron.

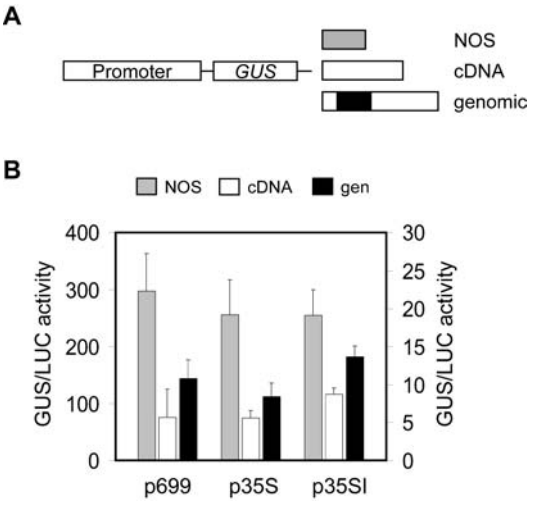

Fig. 5A, B Effects of the *Hrgp* intron on gene expression in 2 DAG maize shoots. **A** Schematic representation of the constructs. **B** Two 2 DAG maize shoots were bombarded with the constructs depicted in **A** and the GUS/LUC activities were determined essentially as described in Fig. 2. *Gray*, *white* and *black bars* represent the mean values (±SD) from constructs containing the 3'NOS terminator and the *Hrgp* terminator from cDNA and from genomic DNA, respectively (n=12). The x-axis on the left applies only to p699-based constructs

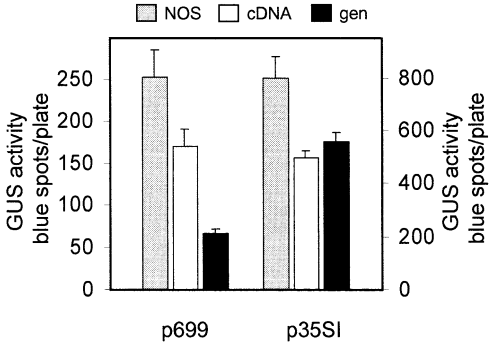

Fig. 6 Effects of the *Hrgp* intron on gene expression in Black Mexican Sweet (BMS) maize cells. Samples were bombarded with p699- and p35SI-based constructs and the blue spots in each Petri dish were quantified 24 h later using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) as substrate (n=6). Columns represent the mean ±SD. Left and right x-axes apply to p699- and p35SI-based constructs, respectively

Discussion

Expression of the maize *Hrgp* is under developmental, organ-specific and environmental regulation. This gene is expressed mainly in tissues rich in dividing cells and can be induced by wounding and exposure to ethylene (Stiefel et al. 1988; Ludevid et al. 1990; Tagu et al. 1992; Menossi et al. 1997).

The search for regulatory elements that modulate the expression of genes encoding cell wall proteins may shed light on the complexity of the changes in cell wall composition. Findings on the regulatory regions may also

help to identify the protein factors that trigger *HRGP* expression. Because several HRGP proteins seem to be involved in defense reactions, these factors would be interesting targets for biotechnological purposes. In a previous study on the promoter regions involved in this complex regulation, Vallés et al. (1991) found nuclease-hypersensitive sites located in the first 300 bp. Particle bombardment of maize calli using four promoter deletions transcriptionally fused to the *GUS* gene indicated that the region located in the first 600 bp was the most relevant to expression of this promoter (Tagu et al. 1992).

In the present work, we performed a more detailed deletion analysis of the *Hrgp* 5' flanking region and also evaluated the effect of the intron located in the 3' flanking region. A gradual decrease in promoter activity with increasing deletion of upstream promoter regions was observed in immature embryos, similar to that observed by Xu et al. (1995) in the promoter of the rice gene encoding a glycine-rich cell wall protein. The overall pattern of GUS activity driven by the various constructs was different in young embryos and shoots, indicating the presence of tissue-type or developmental regulatory elements. Our results indicate that the region −1,076/−700 contains elements that modulate *Hrgp* expression in coleoptiles in a manner contrary to that seen in embryos. Whereas the GUS activity in 14 DAP embryos decreased after deletion of this region, an increase was observed in shoots, suggesting the presence of a negative regulatory region (NRR) modulated by the tissue type. Elliott and Shirsat (1998) also observed an NRR in the *extA* promoter of an extensin gene from *Brassica napus*. In this case, the effects of the NRR on *extA* promoter activity were also observed only in certain tissues.

A strong decrease in promoter activity was observed in both embryos and shoots following deletion of the region spanning nucleotides −699/−510, which eliminated one of the two octamer motifs. We suggest that both motifs are needed to sustain high levels of *Hrgp* expression. The small decrease observed when the deletion proceeded from p509 to p297, eliminating the other histone octamer, supports this view. In addition, the region between p509 and p297 contains the GCC box, suggesting that ethylene may not be a key factor under these assay conditions.

Deletion of the −297/−160 region markedly decreased GUS expression in embryos and shoots and highlighted the role of the S1 nuclease hypersensitive site (Vallés et al. 1991) in *Hrgp* expression. Likewise, in 14 DAP and young shoots, GUS activity fell to background levels when the sequence TGTATATAAG was eliminated, supporting the inference that the sequence TATATAA is the TATA box. Indeed, this sequence, together with the four upstream bp (TCCG), had only four mismatches with the plant consensus TATA box, TCACTATATATAG (Joshi 1987).

Besides the putative TATA box, the *Hrgp* promoter region closest to the coding region certainly has other elements relevant to *Hrgp* expression since promoter activity was four times lower in the absence of the −52/+16 region. In support of this point, when the −139/+16 region was replaced by a truncated version of the 35S promoter (p1076/35S construct), only background GUS activity was detected, whereas the p114 deletion restored GUS activity to levels similar to those of the p35SI construct in shoots. Thus, the proximal region of the promoter plays a major role in *Hrgp* expression since it contains sequences able to direct expression in embryos and shoots. These sequences were also necessary for the activation directed by sequences located upstream from this region. Salvà and Jamet (2001) also reported that the promoter-proximal region has a key role in the regulation of the *Ext 1.4* promoter of an extensin gene from tobacco.

The activity of the p1076 construct in young shoots was 20 times greater than that of p35SI, in sharp contrast to that observed in immature embryos, in which p1076 showed only half of the activity of p35SI. Since our previous work showed a strict correlation between the messenger RNA levels and cell division, with high levels of *Hrgp* and histone transcripts in young shoots (Ludevid et al. 1990; Tagu et al. 1992), the strong *Hrgp* activity in young shoots probably reflected the high growth rate observed in this tissue, which almost doubled the fresh weight every 2 days (data not shown; see Kiesselbach 1980).

Several authors have pointed out the difficulty of finding conserved regions between promoter sequences of plant cell wall genes. Our results on the *Hrgp* promoter highlighted several regions containing positive and negative regulatory elements. The finding that these elements are regulated in different ways depending on the cell type reinforces the view that a complex network is used to modulate the changes in plant cell walls. Moreover, this mapping of relevant regions in the *Hrgp* promoter will help future studies such as gel shifts, to find the protein factors that are interacting with these regulatory regions.

Our results show that the intron located in the 3' flanking region of the maize gene is involved in regulation of *Hrgp* gene expression. Eukaryote genomes usually have a large amount of non-coding sequences. Most of these sequences seem to be functionless and to find experimental evidence of their functionality is a great task (Duret and Bucher 1997). Tagle et al. (1988) proposed that phylogenetic comparisons could identify regulatory elements in homologous genes, because their sequence would be conserved. The striking conservation among the introns of maize, rice and teosinte indicates that they could be important for gene expression (Raz et al. 1992). Introns can increase gene expression by facilitating RNA transport to the cytoplasm, by protecting nascent RNA from attack by nucleases via a spliceosome-mediated mechanism, by facilitating the addition of the poly-A tail during intron processing and even increasing transcription (Callis et al. 1987; Buchman and Berg 1988; Huang and Gorman 1990; Niwa et al. 1990; Deyholos and Sieburth 2000). Most plant introns that do not interrupt the coding region are located in the 5' untranslated region. However, several plant genes coding for plant cell wall proteins have an intron located in the 3' untranslated region, including the *Hrgp* homologues from teosinte,

sorghum, rice and another maize line (Raz et al. 1991, 1992; Caelles et al. 1992), and from carrot (Chen and Varner 1985), tomato (Salts et al. 1991) and possibly tobacco (Keller and Lamb 1989).

A positive effect of the *Hrgp* intron was clearly observed in young shoots since the p699-based construct containing the intron was twice as active as the construct without the intron. Although the overall positive effect of the *Hrgp* intron on the activity of the promoters tested was lower than that observed for other introns, such as those for the *ADH1-S* and *Act1* genes (Callis et al. 1987; McElroy et al. 1991), a positive effect over a similar range was observed for the intron from the *st-es1* potato gene (Leon et al. 1991). To our knowledge, the present report is the first to describe the effect of an intron located in the 3' untranslated region of a plant cell wall gene.

The use of constructs containing different promoters led to the identification of a promoter-dependent effect of the *Hrgp* intron on gene expression. In young shoots, the intron increased GUS activity driven either by the *Hrgp* promoter or the 35S promoter. However, in the construct with the *Hrgp* promoter, the intron doubled the GUS activity, while in the 35S-based constructs the increase was 40–60%. Similarly, the positive effect of intron 1 of the maize *Adh1-S* gene was higher in the *Adh1-S* promoter than in the 35S promoter (Callis et al. 1987). The opposite effect of the intron in the p35SI-based and p699-based constructs observed in BMS cells suggested another level of control, i.e. depending on tissue type. A similar effect was also observed with intron 1 in the *Adh1-S* gene, which had a lower effect on gene expression in BMS cells when compared to endosperm and aleurone protoplasts (Gallie and Young 1994). Fu et al. (1995a, 1995b), working with the *Sus3* and *Sus4* sucrose synthase genes also found that plant introns could both up- or down-regulate gene expression in a tissue-specific manner.

The p699 construct, which lacks the *Hrgp* intron, controls an activity pattern in maize tissues that is closely correlated with the accumulation pattern of the endogenous mRNA (Menossi et al. 1997). This fact indicates that the intron is not absolutely necessary for gene activity in most tissues. However, this intron does modulate gene expression in some tissues, as observed in shoots and BMS cells.

The mechanism by which introns enhance gene expression in maize seems to be correlated to the splicing of the transcript rather than to the presence of transcriptional enhancers (Sinibaldi and Mettler 1992). However, taking into account the differences found between the *Hrgp* and the 35S promoter in BMS cells, the possibility of other effects on gene expression cannot be ruled out. In fact, some introns may contain transcriptional enhancers, as observed in the *AGAMOUS* second intron from *Arabidopsis* (Deyholos and Sieburth 2000). Further studies with the conserved introns in the 3' untranslated region of *Hrgp* genes will certainly result in interesting findings on the regulatory mechanisms that take place in plant cell wall genes.

In summary, we have identified quantitative elements in the *Hrgp* promoter and have shown a strict dependence of the most upstream elements affecting gene activation on sequences located in the neighbourhood of the TATA box. We have also found that the intron located in the 3' flanking region modulates gene expression and that this modulation is affected by the promoter used and the type of tissue involved, reinforcing the view of non-coding genome regions as a source of regulatory elements.

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