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Unusual electrophoretic mobility of maize *Hrgp* mRNA

Nora García-Muniz, José Antonio Martínez-Izquierdo, Pere Puigdomènech*

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

* Author to whom correspondence should be addressed (fax +34 32045904; e-mail pprgmp@cid.csic.es)

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Abstract — Hybridisation bands of mobility lower than expected appear in RNA blot analysis performed using total maize RNA hybridised with an hydroxyproline-rich glycoprotein (HRGP) cDNA probe. This behaviour has previously been observed and some authors have proposed that these bands could be transcripts of genes coding for highly similar genes. However, other data indicate that a single gene coding for HRGP exists in the maize genome. Here, we show that some of the mRNA molecules hybridising with the *Hrgp* probe migrate in agarose gel electrophoresis at a lower velocity than expected. The relative intensity of these low-mobility bands depends on the abundance of the *Hrgp* mRNA molecules loaded in the electrophoresis gels, a result confirmed by using two-dimensional gel electrophoresis. We propose that these hybridisation bands may be the product of different secondary structures or multimers of the 1.5-kb *Hrgp* mRNA probably due to high G+C content and the highly repetitive sequence of these molecules. © Elsevier, Paris

Electrophoretic mobility / hydroxyproline-rich glycoproteins / *Zea mays*

HRGP, hydroxyproline-rich glycoprotein

1. INTRODUCTION

The HRGP (hydroxyproline-rich glycoproteins) are abundant proteins in plant cell walls. The maize HRGP is one of the best known cell wall proteins of monocotyledoneous plants and it has been characterised at protein, cDNA, and genomic levels [18, 22]. Maize HRGP is coded by a single gene as seen by Southern blots [22]. The maize *Hrgp* gene contains a single intron in the 3'-non-translated region [18]. The maize HRGP protein could be divided in three different regions [18, 22]: a signal peptide, a hydrophilic region and a highly repetitive region, which represents most of the protein. The structure of the repetitive region consists of several units of the hexapeptide PPTYTP followed by one to three pentapeptides (SPKPP, ATKPP or TPKPT) partly modified as shown by the analysis of the protein sequence [10, 13]. This protein is rich in tyrosine which may form cross-links between different polypeptides [5].

The maize *Hrgp* mRNA is 1.5 kb long and accumulates in young tissues containing actively dividing cells, such as coleoptile nodes, plumule, root apex or calli [14, 21, 22]. The maize *Hrgp* mRNA is transiently accumulated in provascular cells in germinating embryos, young leaves and roots [19, 22]. Maize *Hrgp* mRNA accumulates at a very low level in elongating and differentiated tissues and is induced in seedlings by wounding and ethylene treatment [14, 23], in response to fungal elicitors and some antioxidant substances [6], and specific stress treatment [12]. The *Hrgp* mRNA is also present in maize pericarps and silks [16]. Comparative studies at protein level showed that the highest HRGP accumulation is located in the protective or supporting organs like maize silks and pericarp [9], a result supported by promoter activity analysis in transiently transformed cells in these tissues [15].

RNA blots performed using RNA extracted from silk tissues detected multiple bands hybridising with the *Hrgp* probe [16]. This fact has been taken as an argument to propose that in maize, HRGP proteins may be encoded by a multigene family, but Southern blot and sequencing data do not agree with this hypothesis. The objective of our work was to elucidate this apparent contradiction. The expression of the *Hrgp* gene in maize inflorescences was studied by RNA blot hybridisation. In addition to a band of the

expected size, we detected bands at higher molecular masses. Further experiments revealed that these slowly-migrating *Hrgp* hybridisation bands are composed of the same 1.5 kb *Hrgp* mRNA molecules and appear as a consequence of inter- or intra-molecular interactions.

2. RESULTS

2.1. Accumulation of *Hrgp* mRNA in maize reproductive organs

The accumulation of maize *Hrgp* mRNA in reproductive tissues was studied in different parts of the inflorescenses at different stages of development. RNA isolated from maize male and female inflorescences and vegetative tissues was analysed by RNA blot hybridisation (*figure 1*) using a probe corresponding to a *Hrgp* genomic clone [22]. In male inflorescences, we studied immature staminate spikelet (IA) and mature staminate spikelet (MA), before and during anthesis, respectively. In female inflorescences, we studied the whole silk of mature ears (WS), ovary from mature ear (Ov), and three regions of mature silks: basal (LS), middle (MS) and top (US). We consider as mature the ear showing silks longer than 12 cm. We also used total RNA from roots (Ro) and coleoptile (Co) from 7-d-old seedlings as controls. *Hrgp* mRNA accumulates at a high level in silks (lanes LS, MS, US and WS) and less abundantly in ovary (Ov). The accumulation levels were much lower in the vegetative tissues (Co and Ro) and in the male inflorescence (MA). There was no hybridisation signal in immature staminate spikelet RNA, even after long exposures of the autoradiographic films. Transcript accumulation level is higher in basal (LS) and middle (MS) than top (US) silk (*figure 1*). A possible explanation may be that the upper region contained the region undergoing senescence (brown region in the tip of the ear) while the silks located under the leaves enclosing the ear (LS and MS) are green.

In the tissues studied in the previously shown RNA blots, a 1.5-kb band hybridising to the *Hrgp* probe was observed with different levels of intensity but in silks, we also observed some additional hybridisation bands corresponding to higher apparent sizes: 4.4, 3.6, 3.1 and 2.8 kb, respectively. These hybridisation bands could correspond to cross-hybridisation of the *Hrgp* probe with other transcripts, for example other unidentified *Hrgp* genes expressed specifically in silks, although no other *Hrgp* genes has been found in maize when using Southern blot analysis [18, 22]. It was also possible that the *Hrgp* probe cross-hybridises with glycine-rich protein (GRPs) coding transcripts. GRPs mRNAs are rich in glycine codons (GGC), which are complementary to the proline codon (CCG) of which the *Hrgp* is specially rich.

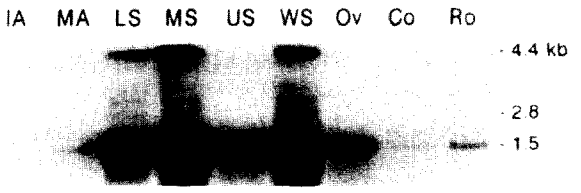

Figure 1. *Hrgp* mRNA accumulation in maize inflorescence tissues. Northern blot analysis of 10 µg total RNA extracted from different maize tissues and hybridised with a *Hrgp* gene probe. (IA), Immature staminate spikelet; (MA), mature staminate spikelet; (LS), basal part of mature silk; (MS), middle part of mature silk; (US), upper part of mature silk; (WS), whole silk; (Ov), mature ovary; (Co), coleoptile of 7-d-old seedlings; (Ro), roots of 7-d-old seedlings.

2.2. Analysis of the mobility of the *Hrgp* mRNA band

In order to identify the nature of the low-mobility RNA electrophoretic bands, we performed RNA blot assays using three different cDNA probes corresponding to the coding, 3'- and 5'-non-translated regions (*figure 2*). The same bands, including those running with a lower mobility, were highlighted using any of the three probes (*figure 3*). Because the 3'-non-translated regions are normally gene specific, this result may indicate that the hybridisation bands do not correspond to transcripts of different *Hrgp* genes or to *Grp* genes.

The low-mobility hybridisation bands only appear in tissues in which the *Hrgp* mRNA is very abundant. So, we decided to study whether there was a correlation between the abundance of the *Hrgp* mRNA and the intensity of the low-mobility hybridisation bands. We performed RNA blots loading 15, 12.5, 10, 7.5 or 5 µg mature silk total RNA per lane in a 1.5 % agarose 2.2 M formaldehyde gel (*figure 3*). The RNA blot was hybridised with the *Hrgp* probe and, as a control, with a probe corresponding to the histone H4 gene [17]. Our results showed that, when the quantity of RNA loaded decreases, unexpectedly the intensity of the 1.5-kb hybridisation band increases. At the same time, the intensity of the low-mobility hybridisation bands decreases. This result suggested that the low-mobility bands were composed of the same molecular species than the 1.5-kb ones but the existence of interactions

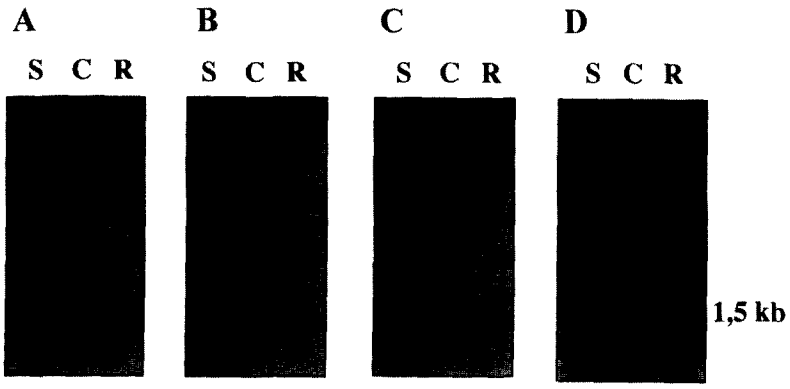

Figure 2. *Hrgp* mRNA accumulation in maize tissues using three different cDNA probes. RNA blot analysis of 10 µg total RNA extracted from S, mature silk; C, coleoptyle of 7-d-old seedling; R, roots of 7-d-old seedlings was carried out. The same total RNAs were loaded in three different blots and hybridised independently with three probes from different domains of the *Hrgp* gene: **A**) 5'-domain (positions -699/+16); **B**) coding region (positions +530/+983); and **C**) 3'-non-translated domain (positions +1065/+1382). The autoradiograms were exposed for 12 h. **D** is the same blot as in **A** but exposed during 24 h.

between *Hrgp* mRNA molecules retarded their migration in the electrophoresis gels.

2.3. Two-dimensional gel electrophoresis of *Hrgp* mRNA

In order to verify the previous hypothesis, a two-dimensional electrophoresis of total RNA extracted from silks was performed. RNA electrophoresis was performed in a 0.8 % agarose/2.2 M formaldehyde gel, the same denaturing conditions used in previous assays (*figures 1, 3*). After running the first dimension, the gel was soaked with shaking in a 1× MOPS buffer/2.2 M formaldehyde bath during 1 h at 65 °C. The heat treament can remove, at least in part, possible secondary structures or interactions with other molecules. After heat treatment, we ran again the gel rotating it by 90 degrees (second dimension). The RNA was blotted onto a nylon membrane and hybridised with the *Hrgp* probe (*figure 4*). The same pattern of *Hrgp* transcripts as in previous experiments was found in the diagonal line. However, molecules having the 1.5-kb mobility were found in the second dimension of the diagonal and were produced by the low-mobility species (black arrow). This result indicates that after heat treatment at least some of the mRNA molecules corresponding to the low-mobility bands of the first dimension changed their electrophoretic mobilities to that of the 1.5-kb *Hrgp* mRNA.

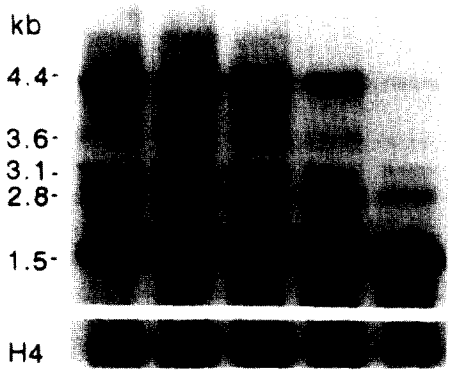

Figure 3. Effect of the amount of total RNA loaded in northern blot analysis on the electrophoretic mobility of *Hrgp* mRNA. RNA blot analysis loading of 15, 12.5, 10, 7.5 and 5 µg (from left to right) total RNA extracted from mature silks. The blot was hybridised with *Hrgp* genomic probe. Hybridisation with a probe corresponding to the histone H4 gene (H4) [17] was used as a control.

3. DISCUSSION

The maize *Hrgp* mRNA is highly accumulated in silks, this is shown here by RNA blot. It had previously been observed for the same gene at the protein level [8]. The high accumulation of HRGP protein could be explained by the fact that this protein act as a physical support for long silks. It has been proposed that HRGP proteins may also function by creating a physical barrier against pathogens [11]. Silks can be easily used by pathogens as a way of entry and many defence-related genes have been shown to be constitutively expressed in these tissues [4].

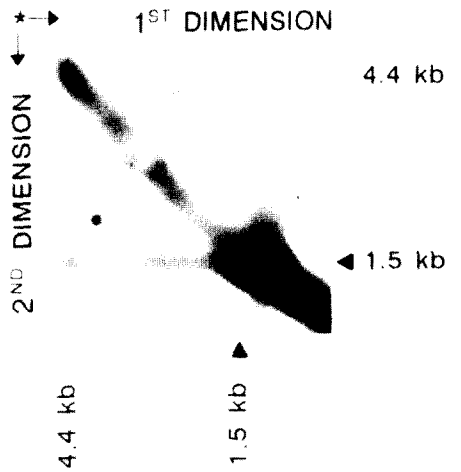

Figure 4. Two-dimensional agarose gel electrophoresis of total RNA extracted from silks. RNA blot analysis loading 50 μg total RNA extracted from mature silk and hybridised with a *Hrgp* probe. Electrophoresis in the first dimension was performed in a 0.8 % agarose/2.2 M formaldehyde in 1× MOPS buffer. After first dimension, the gel was soaked in 1× MOPS, 2.2 M formaldehyde bath during 1 h at 65 °C with shaking. For the second dimension, the gel was rotated 90° and run in 1× MOPS buffer.

In addition to the band corresponding to the expected *Hrgp* mRNA size, several others have been observed when RNA blot analysis was performed using total RNA extracted from maize silks and hybridised with a *Hrgp* probe. Similar results had already been reported in other maize lines [16], but also in *Brassica napus* [20]. These authors proposed that different transcripts could be encoded by other *Hrgp* or highly similar genes in the two species.

In the results presented here, it has been shown that, at least for the case of maize, there is no need to invoke the existence of more than one *Hrgp* gene to explain the presence of additional bands in RNA blots. Several indications support this hypothesis. On the one hand, all the efforts to detect additional genes by Southern analysis or library screenings were unsuccessful [22]. Moreover, the same hybridisation bands appear using probes corresponding to the 5'- or 3'-non-coding regions of the gene, while these non-coding regions usually are gene specific in most multigene families.

If the lower mobility bands do not correspond to additional genes, their electrophoretic retardation could be caused by ultrastructural effects due to secondary or quaternary structures formed by the RNA. The maize *Hrgp* mRNA is very rich in G and C nucleotides, especially in the region coding for the repetitive domain [18, 21, 22]. This fact could produce strong intra-molecular interactions. The analysis of the theoretical folding capacity of the *Hrgp* mRNA shows that it has a strong capacity to generate secondary structures. The structure presented in *figure 5* is the most stable structure for this sequence and has a Gibbs free energy of $-180.4\ \text{kcal}\cdot\text{mol}^{-1}$. Several other possible structures with similar free energies can also be generated. These single-stranded molecules with strong secondary structure are usually expected to have increased mobility, but some examples in which their mobility was decreased have been found in some RNA molecules transcribed in vitro from various cloned yeast mitochondrial DNA fragment with palindromic regions [2]. It is also possible that two or more mRNA molecules hybridise generating complex multimolecular structures. *Hrgp* mRNA could also hybridise with other transcripts, such as *Grp* mRNAs. Each of these possibilities, or a combination of them, could reduce the mobility of the mRNA, generating, in RNA blots, bands of an apparent higher molecular mass. More than one such interaction is possible, and if this is the case, more than one additional band could be expected with different intensities as it has been observed (*figures 1, 2*).

Different data support the hypothesis of molecular interactions as the origin of the additional bands. We have shown that, at least part of the low-mobility RNA molecules shift their mobility to the expected one after heat treatment, which can eliminate in part the intra- or intermolecular interactions. The fact that the relative abundance of the low-mobility bands compared with the intensity of the 1.5-kb band depends on the quantity of RNA loaded supports the hypothesis of intermolecular interactions as this is a concentration-dependent phenomenon.

In conclusion, we have shown that the detection of additional bands in RNA blot hybridisations with a different mobility than expected in the case of *Hrgp* mRNA does not always mean the existence of additional genes, as it has been sometimes suggested. An open question is whether these secondary structures are only artefacts of the RNA blot technique, or whether they exist and play a role in vivo. For example, it has been shown that tightly folded G+C rich DNA sequences display distinct replication patterns during cell cycle [7], it is not known whether such a mechanism exists in plant genes.

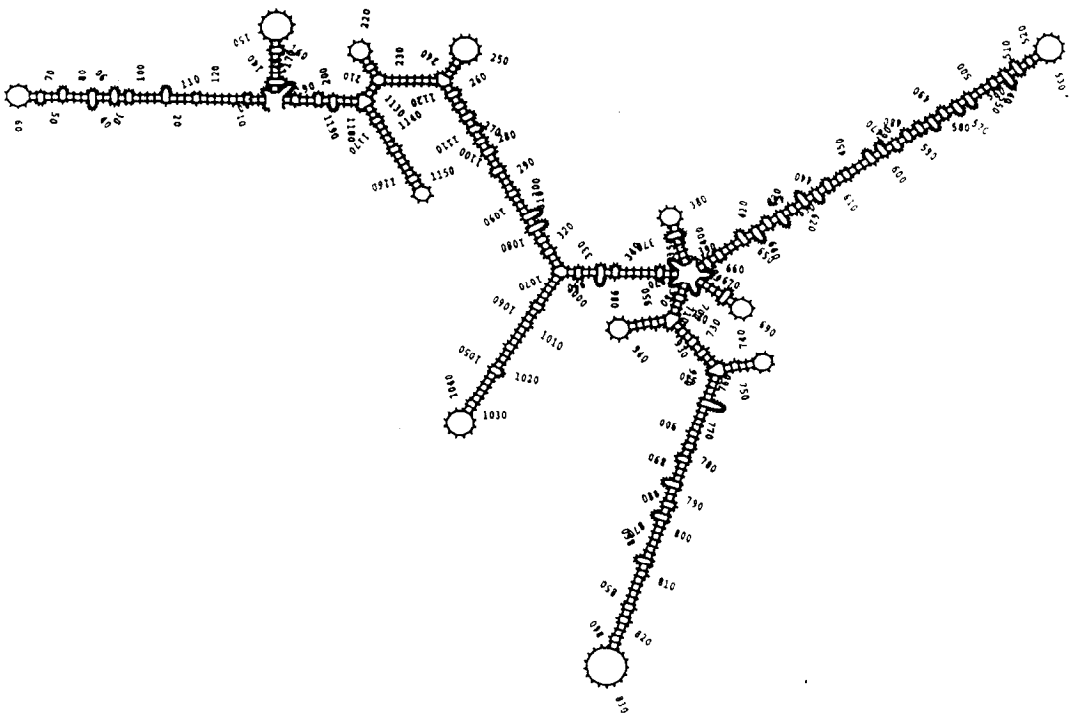

Figure 5. The hypothetical most stable secondary structure of *Hrgp* mRNA. The secondary structure was generated by the FOLD program of the GCG package [3].

4. METHODS

4.1. Plant material

The plant material used in this study derived from seeds of *Zea mays* pure inbred line W64A that were grown in greenhouse conditions. Seeds were rinsed in ethanol for 5 min in 5 % (w/v) calcium hypochlorite solution for 15 min, washed twice with sterile water and germinated in damp vermiculite. Seven-d-old seedlings were obtained from seeds germinated in a growth chamber at 25 °C in the dark.

4.2. RNA isolation

RNA was extracted using the phenol/SDS extraction and lithium chloride precipitation method [1]. All RNA samples were checked by non-denaturing electrophoresis in 1.5 % agarose gels and ethidium bromide staining. The concentrations were determined by reading the absorbance at 260 nm. Non-denaturing gels were prepared in TAE buffer (40 mM Tris-HOAc, pH 8.0; 2 mM EDTA).

4.3. RNA blots

RNA samples were denatured by heating at 65 °C for 5 min in the presence of formaldehyde 2.2 M and formamide, and rapid cooling on ice. RNA samples were then separated by electrophoresis on a 1.5 % agarose/2.2 M formaldehyde gel in the presence of 1× MOPS buffer, blotted onto nylon membranes (Hybond N, Amersham) and hybridised to a [^{32}P]-labelled by random priming (using a kit from Boehringer) plasmid probe.

4.4. Probes

The *Hrgp* gene probe used is a 512-bp *Sna*B1 fragment of the 3'-transcribed and translated region of the genomic clone of maize HRGP [22]. The maize H4 histone probe (provided by Dr Claude Gigot, IBMP, Strasbourg) is a 328-bp insert of H4 C14 clone [17], which covers the whole coding sequence of the protein.

4.5. Computer analysis

Theoretical secondary structures were generated by the FOLD program of the GCG package [3] corresponding to the transcribed strand.

Acknowledgments

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