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mRNA accumulation and promoter activity of the gene coding for a hydroxyproline-rich glycoprotein in *Oryza sativa*

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Abstract

The accumulation of the mRNA corresponding to the gene coding for a hydroxyproline-rich glycoprotein has been studies in rice. The patterns of gene expression obtained are similar to those observed in maize in regions rich in dividing cells such as the meristematic zones of roots. However, the gene does not seem to be induced by wounding as it is the case in maize. This effect is correlated with the absence of sequences present in the promoter of the maize gene and that have been described as responsible for ethylene induction on other plant systems. Instead, the promoter has a sequence that corresponds to abscisic acid-responsive elements and, in fact, HRGP mRNA levels can be two-fold increased in rice leaves by ABA. The genes coding for homologous proteins in two cereal species such as maize and rice appear, therefore, to have distinct mechanisms of gene regulation.

Introduction

Hydroxyproline-rich glycoproteins (HRGP) are supposed to be structural components of the plant cell wall. They were encoded in dicotyledonous species by complex families of genes having specific patterns of expression [4]. In cereals, HRGP genes have been identified and characterized in maize [18], sorghum [16] and rice [3]. In the case of maize, a single locus in chromosome 2 contains the HRGP gene and, although related sequences might be expressed in defined organs of the plant [13], the system appears to be more simple in terms of genomic complexity than in dicots. The maize HRGP gene is expressed in organs rich in dividing cells [10], preferentially in provascular cells [18], and it may be induced by wounding of young tissues [10] and by ethylene [19].

A gene homologous to maize HRGP was cloned in rice [3] and sequenced. The protein encoded by this gene has a structure very similar to the maize one, including a signal peptide, a hydrophilic stretch, a highly repetitive fragment rich in threonine and tyrosine and a non-repetitive end. The repetitive sequence has strong similarities with the repetitive domain of the maize HRGP. However, the rice protein has specific distinctive features. For instance, the level of

serine residues is very low (only two serine residues among 369) and it lacks the SPPPP motif, typical of dicot extensins that is present only once in the maize proteins and it is substituted by the sequence GPPPP in rice.

In this report we present the results of the analysis of the accumulation of HRGP mRNA in different parts of the rice plant and in response to wounding as well as the analysis of the corresponding promoter by transfection of tobacco protoplasts and by microbombarding maize leaves. The effects observed appear to be qualitatively different from those reported for the maize promoter and they point to specific ways of controlling the expression of these homologous genes in two cereal species.

Materials and methods

RNA extraction and RNA blot hybridization

Seeds of rice (*Oryza sativa* cv. IR36) were germinated under light at 26 °C in a growth chamber. One-week-old rice plantlets were used to extract root and leaf total RNA as described by Logemann *et al.* [9]. Total RNA was separated in 1.5% agarose-formaldehyde gels [8] and blotted onto Hybond-N membranes (Amersham, UK). A probe containing the coding sequence of rice HRGP gene [3] was labelled with ^{32}P-dATP by random primer labelling to a specific activity of 10^8 cpm/μg. Prior to blotting, the gels were stained with ethidium bromide to ensure that RNA samples contained approximately equal amounts of RNA.

Protoplast preparation and transformation

Mesophyll protoplasts of tobacco leaves were prepared from tobacco (*Nicotiana tabacum* cv. Petit Havanna SR1) as described by Bilang and Schrott [1]. The prepared protoplasts were directly transformed in the presence of polyethylene glycol as described by Negrutiu *et al.* [15] by constructions of the rice HRGP gene promoter, the GUS reporter gene and the NOS terminator taken from pBI101.3 [7] in pUC18. The GUS assay was based on the method of Jefferson [6]. Protein concentration in the extracts was measured according to the Bradford method [2].

Microparticle bombardment transformation

Embryos of maize (*Zea mays* L. cv. W64A) cut from the seeds of plants grown in greenhouse conditions in Barcelona, Spain were germinated onto agar medium to produce ten-day-old plants. The leaves of the plants were bombarded with the same plasmids used in tobacco protoplast transformation by the Biolistic PDS-1000/He Particle Delivery System (BioRad, USA). The histochemical GUS assay of the bombarded tissues was completed as described by Jefferson [6].

Promoter construction and sequencing

Deletions of different length of the promoter of the rice HRGP gene were generated by restriction enzyme cutting upstream from the ATG. The constructions kept always the same 3′ site. The promoters with the GUS gene and the NOS terminator were fused into pUC18 vector to obtain transient expression GUS gene in transformed plant tissues. The constructions were checked by restriction mapping and DNA sequencing (ALF, Pharmacia).

Results

RNA blot analysis of mRNA accumulation of HRGP in rice leaves

It has been shown in a number of species that HRGP genes can be either induced by wounding or expressed at defined developmental stages or both. This is the case, for instance, of extensin in bean [4] where a number of specific genes coding for this protein appear to be induced by mechanical stress while other genes are under

developmental control. In the case of cereals, it has been shown in maize that the gene coding for the protein having a large degree of sequence similarity to the rice HRGP here studied can be induced by wounding and ethylene [10, 19]. In order to analyse whether similar effects would be observed in rice, an experiment of wounding was carried out in young rice leaves. Wounding was applied by razor blade incision and the mRNA accumulation was measured by RNA blot (Fig. 1A), but, unlike the expression of this gene in maize leaves [10], the mRNA accumulation hardly increased after 2 h of wounding. It is also possible to observe in the same figure that the steady-state level of HRGP mRNA in young rice roots was much higher than in young leaves and that only one band of 1.5 kb was hybridizing with the HRGP probe in rice leaf RNA (Fig. 1A).

It was of interest to analyze the pattern of expression of the gene in rice and under the effect of specific physiological situations. As it will be later discussed, a short sequence in the 5′ upstream region of the rice HRGP gene has homology to G-boxes responsible for abscisic acid induction [12]. The results are shown in Fig. 1B showing that the expression of HRGP gene in intact young leaves of rice was increased by 100 μM ABA and 200 mM $CaCl_2$. Other factors that may have effects related to stress response such as water deficit, increased NaCl concentration, fungal elicitors or sucrose have no detectable effect on the accumulation of HRGP mRNA (Fig. 2B). Water stress often has the same effect as ABA on the gene expression of some plants. By comparing the effects of water stress and ABA on the expression of HRGP gene, we found that only ABA might increase the accumulation of HRGP mRNA in intact young leaves while water stress did not appear to have any effect (Fig. 2C).

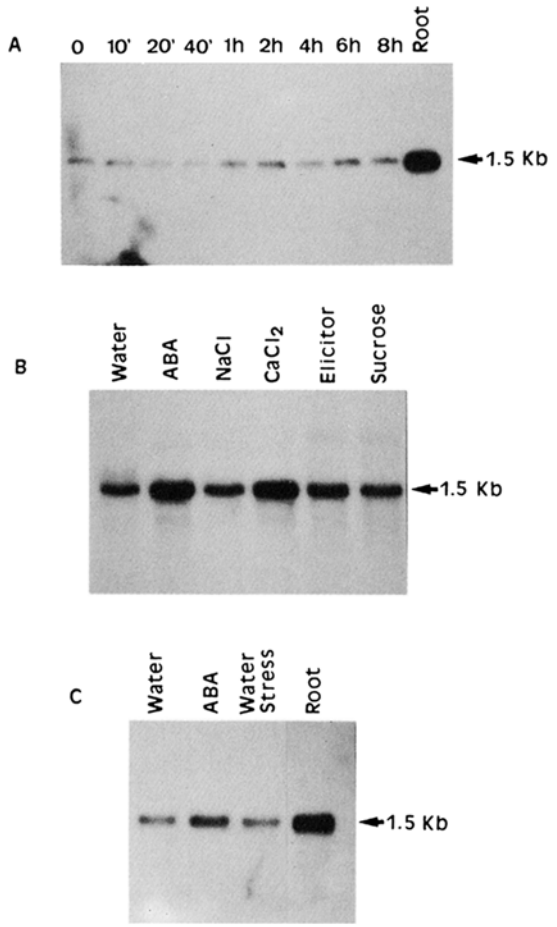

Fig. 1. Effect of inducible factors on the accumulation of hydroxyproline-rich glycoprotein mRNA in rice young leaves. A. Total RNA (10 μg) from one-week-old rice leaves wounded by cutting from 10 min to 8 h and normal leaves and roots were loaded on each lane. B. Total RNA (20 μg) from intact leaves, which were treated by water, 100 μM of ABA, 200 mM of NaCl, 200 mM of $CaCl_2$, 100 μg/ml of elicitor and 400 mM of sucrose, were loaded on each lane. C. Total RNAs (20 μg) from ABA- and water-stress-treated leaves and from normal leaves and roots were loaded on each lane. Water: incubated in water for 24 h; ABA: 100 μM incubated for 24 h; water stress: incubated without water at 25 °C for 24 h. A 430 bp coding sequence of hydroxyproline-rich glycoprotein gene was used as a probe.

The accumulation of HRGP mRNA in different parts of intact young rice roots

The expression of the HRGP gene in maize is developmentally regulated and in this species it shows an accumulation of its mRNA related to the presence of dividing cells [10]. We divided whole young roots into three parts. The first part, which is from root tip up to 1 cm, includes meristematic and elongating tissues, the second part from 1 cm to 2 cm is elongated tissue and the third part, from 2 cm to 3 cm, is a region where

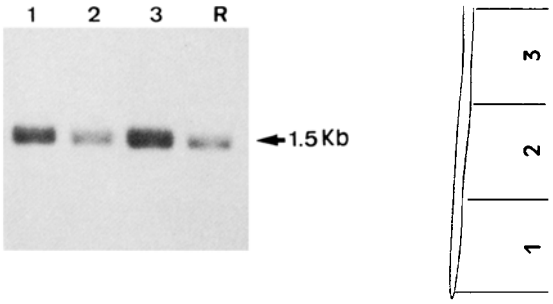

Fig. 2. Expression of the hydroxyproline-rich glycoprotein gene in the different parts of young root. Total RNAs (10 μg) isolated from different parts of young root were loaded on each lane. 1, from the tip of root to 1 cm; 2, from 1 cm to 2 cm; 3, from 2 cm to 3 cm; R, total root RNA. 430 bp coding sequence of rice hydroxyproline-rich glycoprotein gene was used as a probe.

many lateral roots may be observed and therefore it may also contain potential meristematic tissues. Previous results indicated that a high level of accumulation of HRGP mRNA was related in root tip with meristematic tissue [10]. In this study, a 430 bp coding sequence of rice HRGP was used to hybridize total RNAs from the three parts. The result of RNA blot analysis showed that the parts (first and third) which contain meristematic tissues have higher accumulation of HRGP mRNA (Fig. 2) than the second one where tissues are mainly in an elongating state. To determine whether the steady-state level of HRGP mRNA may be correlated with meristematic tissue, a probe corresponding to the maize histone H3 gene as meristematic marker was used. The result showed that the third part which may produce lateral roots had the higher accumulation of histone H3 mRNA (result not shown) in accordance with what is observed with HRGP.

The promoter structure of rice HRGP gene and transient activity by protoplast transfection and microprojectile bombardment

The fragment of the promoter of the rice HRGP gene used in this study is a 947 bp DNA segment derived from the genomic clone of the gene [3]. It contains a TATA box motif and one G-box motif. A second region of the sequence having some similarity to a TATA box can be found at position −560. The G-box motif is a highly conserved DNA sequence that has been identified in the 5′-upstream region of plant genes exhibiting regulation by a variety of environmental signals and physiological situations [12]. Deletion of G-box sequences from two light-regulated plant promoters dramatically reduced their expression levels [11, 20]. Deletion of a 62 bp region containing a G-box motif from the 5′-upstream region of the wheat Em gene decreased the ABA responsiveness of this gene in rice protoplasts [11]. Several reports showed that the G-box sequence motif (CCACGTGG) functions as an ABA-responsive element to modulate reporter gene induction in transient expression system [11, 20].

The deleted promoter fragments of rice HRGP gene were fused with the GUS gene and NOS terminator and they were analysed by transient expression in tobacco protoplasts and in maize leaves by microprojectile bombardment experiments. The −580 promoter contains the TATA box but no G-box. A repeated AGCCAACAT sequence motif is found two times between −330 and −580. The −78 promoter fragment does not contain any sequence where a function could be attached by similarity to a reported sequence.

Deleted promoters fused with the GUS gene and NOS-ter were integrated in the pBI101.3 plasmid in order to get the appropriate open reading frame (Fig. 3). The chimeric fragments were recombined into pUC18 plasmids to be used in the experiments of tobacco protoplasts and young maize leaves transformation. When tobacco protoplasts were transformed by PEG treatment in presence of the plasmid DNA, an overall steady decrease of GUS activity was found correlated with reduction of promoter length. It also reproducibly appeared that the −580 promoter has a dramatically lower activity than −330 promoter construction. Deletion of the promoter to the −78 produced a complete reduction of all the activity (Fig. 4). The longest promoter used in this study had a 16% of activity relative to 35S promoter (result not shown).

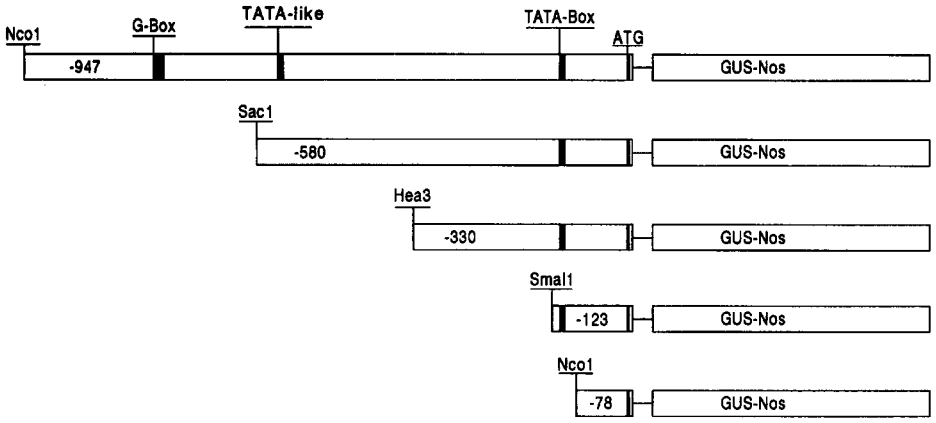

Fig. 3. Schematic drawing of the sequence and deleted promoter constructions of rice hydroxyproline-rich glycoprotein gene promoter.

These chimeric constructions were also used to transform maize leaves by particle bombardment. To measure the transient activity of the promoter, blue spots on the bombarded leaves were counted. In these experiments, the −947 promoter deletion showed higher activity than the 35S promoter. A very low activity of the −580 promoter was observed in maize leaves as it was the case in the protoplast transfection experiments. The −123 bp fragment is long enough to direct a reduced level of expression of the GUS gene in the transient expression system of maize leaves (Table 1). The same constructions used for the transient expression experiments shown here were also transferred to tobacco plants via *Agrobacterium tumefaciens* Ti plasmids. Although transgenic plants were obtained as checked by kanamycin resistance and Southern blot analysis, no GUS activity could be detected by histochemical methods and only a low GUS activity could be observed in protein extracts from the transgenic

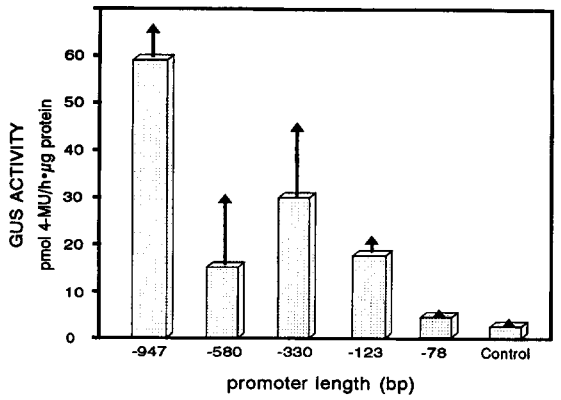

Fig. 4. Transient expression of GUS gene directed by the promoter of rice hydroxyproline-rich glycoprotein gene in tobacco leaf protoplasts. 5×10^5 protoplasts prepared from tobacco leaves were transformed with 20 µg of plasmid DNAs as described in Materials and methods. Four repeat samples for each plasmid were done.

Table 1. Transient expression of GUS reporter gene directed by the promoter of rice hydroxyproline-rich glycoprotein gene in bombarded young maize leaves. The leaves from 10-day-old maize plants were bombarded three times with total 25 µg of plasmid DNA. The data presented are the average of two independent experiments consisting in three shoots each for every construction. – C, negative control, was done by rice HRGP gene promoter with a wrong reading frame in the GUS gene.

Promoter fused with GUS gene	GUS gene activity (number of blue spot on the leaves)
CaMV 35S	44
– 947	89
– 580	0
– 330	59
– 123	11
– 78	0
Control	0

tobacco roots (result not shown). The low activity observed made it impossible to use these plants for promoter analysis.

Discussion

The HRGP gene from rice had been previously identified by screening a genomic library with a maize HRGP gene probe [3]. The homology of the sequence both at the nucleotide and the amino acid residue level may allow to suppose a similar function of the two proteins encoded by this gene in two cereal species such as maize and rice. When the mRNA accumulation is measured in defined organs of the plant or in different physiological situations, the RNA blot appears to detect only one RNA species in rice as it was also the case in maize. Some of the general features of mRNA accumulation in rice appear to follow what was observed in maize. The root is the part having the highest level of HRGP mRNA in the somatic organs of rice analyzed. In different parts of the root, the HRGP mRNA accumulation follows the accumulation of histone mRNA as it was also the case in maize indicating that the protein is needed in early steps of cell wall formation.

In contrast with what happens in developing organs, the induction of HRGP mRNA that was observed in maize upon wounding does not seem to occur in rice. The results of these experiments reproducibly show no response of the HRGP gene in young rice tissues upon mechanical stress. In similar conditions, a clear response was observed in maize [10]. This observation may be correlated with the differences observed in the promoter sequences. While in maize a box that has been proposed to be responsible for ethylene induction is present, no such box exist in rice and, instead, a G-box with the consensus of the abscisic acid-responsive element is present. In accordance to this observation, an increase in HRGP mRNA level is produced upon application of ABA to rice leaves. No induction of the HRGP gene could be observed by water stress and only by $CaCl_2$ treatment.

No GUS activity was observed in transgenic tobacco plants when analysed by histochemical methods. The lack of promoter activity of monocot promoters in dicot plants is not surprising considering the large evolutionary distance existing between rice and tobacco. It is also possible that this result might reflect the differences in HRGP expression between monocots and dicots. While in dicot species multiple extensin genes seem to be expressed specifically in distinct physiological situations, in cereals the different kinds of HRGP expression appear to be carried out by a simple gene family, possibly even by a single gene.

The analysis of the promoter indicates a complex structure of the sequences regulating rice HRGP gene expression that may include distinct boxes responsible for induction or repression of the gene. The promoter of the rice HRGP gene can be analysed by transient expression in tobacco protoplasts and in maize leaves by microprojectile bombardment. A decrease in the promoter activity is observed upon successive deletions and it is specially important in the −580 deletion. This may reflect the presence of successive positive and negative enhancing elements in the region.

The rice HRGP gene codes for a protein having strong homology with the maize HRGP gene. The gene appears to be expressed in similar organs as the maize gene, however it does not seem to respond to wounding in the same way as it occurs to the maize gene. The differences in the promoter sequence are correspondingly high. It appears that these two genes coding for homologous proteins have very distinct types of regulation. This effect may correspond to different strategies for defense response in these two cereals.

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