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Induction of mRNA accumulation corresponding to a gene encoding a cell wall hydroxyproline-rich glycoprotein by fungal elicitors

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Abstract

The *Hrgp* (hydroxyproline-rich glycoprotein) gene codes in maize for one of the most abundant proteins of the cell wall. HRGPs may contribute to the structural support of the wall and they have also been involved in plant defense mechanisms. This second aspect has been tested for the *Hrgp* gene in maize where, in contrast with the situation in dicot species, the gene is encoded by a single-copy sequence. *Hrgp* mRNA accumulation is induced in maize suspension-cultured cells by elicitors, isolated either from maize pathogenic or non-pathogenic fungi. The induction of *Hrgp* mRNA accumulation by elicitor extracted from *Fusarium moniliforme* has been studied in detail. The level of induction depends on elicitor concentration and remains high until at least 24 h. Ethylene and protein phosphorylation appear to be involved in the transduction pathway of *Hrgp* gene activation by the *F. moniliforme* elicitor but not by 5 μM methyl jasmonate or 1 mM salycilic acid. Different compounds known to participate in plant stress responses such as ascorbic acid or reduced glutathione have also a positive effect on *Hrgp* mRNA accumulation.

Abbreviations: α-AB, α-aminobutyric acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, amino oxyacetate; BAP, 6-benzylaminopurine; BMS, Black Mexican Sweet; HRGP, hydroxyproline-rich glycoprotein; MeJa, methyl jasmonate; STS, silver thiosulfate; St, staurosporine; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Introduction

The plant cell wall is a dynamic structure, changing with physiological fluctuations caused by environmental stimuli, tissue differentiation and maturation [7, 55, 59]. HRGPs (hydroxyproline-rich glycoproteins) constitute one of the most abundant structural proteins in the plant cell wall [11] and one of the most abundant classes of proteins rich in proline in plants. HRGPs have been described both in mono- and dicotyledonse. In the dicot species HRGPs are often called extensins and they have been studied in more detail than in monocots, albeit the presence of hydroxyproline in cell walls have been detected in different cereal species [33, 63].

Studies in different species have shown that, although extensins are synthesized as soluble precursors, once secreted to the cell wall they become insoluble, presumably due to the formation of covalent cross-linkages [29]. The expression of the extensin genes in dicotyledonous plants is developmentally regulated [31, 69] and mRNA accumulation has been found in phloem and cambium tissues in healthy plants [55]. Expression of extensin is induced in response to mechanical wounding [15, 40, 45, 53, 68], microbial elicitors [15, 54, 68], pathogen attack [3, 15, 37, 54] or ethylene treatment [9, 19, 22, 40].

At least two main functions have been proposed for extensins in plants [66]. First, they may contribute to the mechanical properties of the cell wall by creating a network formed by glycoprotein elements, even though direct functional evidence is absent. Second, they contribute to plant defense, by strengthening the wall upon pathogen attack or mechanical wounding

[55]. It has also been proposed that extensin molecules that have a net positive charge may immobilize pathogens by interacting ionically with the negatively charged surfaces of plant pathogens [34, 39].

Probably the best studied HRGP from monocotyledoneous plants is the one from maize which has been characterized at protein, cDNA and genomic levels [49, 57]. The protein has been shown to be the main protein extracted from maize cell walls using several methods [32, 28] and corresponds to the cDNA and genomic sequences reported [57]. Similar sequences have been reported in rice [6] and sorghum [49]. The maize *Hrgp* mRNA accumulates in young tissues containing actively dividing cells, such as the coleoptile node, plumule, root apex and also in calli [36, 56, 57]. mRNA accumulation is low in adult tissues, especially in elongating and differentiated tissues. The mRNA corresponding to the maize HRGP transiently accumulates in provascular cells present in germinating seedlings, young leaves and roots [51, 57]. The distribution of *Hrgp* promoter activity [42] closely follows the patterns of mRNA accumulation observed.

A number of studies have shown that in cereals hydroxyproline levels change upon infection or elicitor treatment [12] but no data are available on the effects of pathogens on specific cell wall proteins in these species, in particular on signalling mechanisms related to these processes. In dicotyledonous species, incorporation of [^{14}C]hydroxyproline in the cell wall in melon seedlings infected with *Colletotrichum lagenarium* reportedly is significantly lowered by the presence of the specific ethylene pathway inhibitors [61]. Although the maize Hrgp gene is induced in seedlings by wounding and ethylene [36, 58], no information has been reported on the induction of the maize *Hrgp* gene in response to biotic or abiotic elicitors or pathogen infection. In the present study the effect of different agents related to pathogen attack on the level of expression of the Hrgp mRNA accumulation was studied. The involvement of different regulatory mechanisms in the defense response is also discussed.

Materials and methods

Plant material

The plant material used in this study derived from seeds of *Zea mays* pure inbred line BMS that were germinated at 25 °C in the dark. Seeds used for this purpose 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.

Suspension cultures

Maize BMS cell suspensions prepared from immature embryos were grown in 250 ml flasks containing 25 ml Murashige and Skoog medium [35] (1 mg/l 2,4-D, 0.02 mg/l BAP) shaken at 120 rpm on a rotary shaker at 27 °C under 900 lux of constant fluorescent lighting. The cultures were maintained with subculturing to fresh medium about every 7 days. Optimal elicitor responsiveness was observed with cells 3–4 days after subculture, a state in which the cells were growing exponentially (ca. 200 000 cells/ml).

Treatment of suspension-culture cells

All treatment on suspension culture cells were done on 25 ml of BMS maize cells growing exponentially. Cells were collected by filtration after 8 h of treatment (except in kinetic studies) in the different conditions described for suspension cultures, and the washed cells were frozen in liquid nitrogen. In studies with inhibitors (amino oxyacetate, AOA; silver thisulfate, STS; staurosporine, St), cells were preincubated for 10 min with inhibitors prior to the addition of elicitor. The fungal elicitor preparation and chemical compounds (except staurosporine) were prepared by diluting 1000× concentrated stocks in water. Staurosporine was diluted in dimethyl sulfoxide. The experiments were performed independently at least three times and the data presented are representative examples of the results obtained.

Elicitor preparation

Conidial suspensions were prepared from *Fusarium moniliforme*, *Tricoderma* spp. and *Penicillium* spp. as previously described [8]. Crude preparations of elicitor from *F. moniliforme* (perfect state, *Gibberella fujikuroi*), *Tricoderma* spp. and *Penicillium* spp. were prepared by standard procedures [2]. The fungus was grown on agar plates (Bacto-malt agar, Difco) at 28 °C until the mycelium had covered the surface of the plate. Fungal mycelium was collected by shaking the plate with 10 ml of sterile water for 2 h at room temperature. The solution containing the whole mycelial suspension was sonicated at 100 W for 15 min in a Labsonic 1510 apparatus (B. Braun, Melsunger A.G.),

then autoclaved at 115 °C (69 kPa) for 40 min and finally concentrated by lyophilization. The elicitor from *Phytophthora megasperma* was obtained as a purified preparation [62] from Dr M.T. Esquerré-Tugayé (Université Paul Sabatier, Toulouse, France). All the elicitors were added to the suspensions from 1000× concentrated aqueous stocks (dry weight of elicitor per ml of water).

RNA isolation and blot analysis

RNA was extracted from suspension-cultured cells using the phenol/SDS extraction and lithium chloride precipitation method [1]. All the RNAs were checked by non-denaturing electrophoresis in 1.5% agarose gels and ethidium bromide staining, and the concentrations adjusted by reading the absorbance at 260 nm. For northern blots, total RNA was separated by electrophoresis on a 1.5% formaldehyde-containing agarose gel and blotted onto a nylon membrane (Hybond N, Amersham) and hybridized to the ^{32}P-labelled by random priming (using a kit from Boehringer) cDNA plasmid probes.

Probes

The Hrgp probe used is a 512 bp *Sna*B1 fragment of the 3′-transcribed and translated region of the genomic clone of maize HRGP [57]. The maize H4 histone probe (provided by Dr Claude Gigot, IBMP, Strasbourg) is a 328 bp insert of H4 C14 clone [47], which covers the whole coding sequence of the protein.

Tissue prints

Tissue prints of cross-sections of etiolated maize seedlings were made using the procedure of Cassab and Varner [10]. Two replicates of three cross-sections of mesocotyl and coleoptile were printed. The freshly cut sections (1 mm thick) were dabbed on paper tissues to remove excess cellular debris and with pressure were pressed onto the nitrocellulose membrane (Schleicher & Schuell) for 15 to 20 s. Membranes were stored at room temperature in the dark, between blotting paper. The same membrane was stained with India ink [27] for total protein determination. The HRGPs were detected using antiserum raised against the purified protein [36] and goat anti-rabbit immunoglobulin G (IgG)-peroxidase conjugates using the ECL (enhanced chemiluminescence) assay [5].

Results

Effect of fungal elicitors on the level of Hrgp transcripts in suspension-cultured maize cells

Hrgp mRNA levels were investigated in response to different fungal elicitors in maize suspension-cultured cells. Crude preparations of fungal mycelia have been used as elicitors to induce plant response to pathogens as it has been used in a number of previous studies [17]. Also, we used cell suspension cultures in order to avoid erratic results due to differences in the uptake of inducing or blocking agents, and to have a response from the maize cells that could be as homogeneous as possible. Suspension-cultured cells were incubated with 20 and 30 μg/ml of elicitor preparations extracted from *Fusarium moniliforme*, *Tricoderma* spp., *Penicillium* spp. and *Phytophthora megasperma*. The first three fungi have been isolated from mycoflora present in maize seeds and *F. moniliforme* has been reported as a maize pathogen [30]. *P. megasperma glycinea* is a soybean pathogen and *P. megasperma* elicitors have been shown to stimulate ethylene production in a number of species [22, 62]. The RNA isolated after 8 h of treatment was analyzed by RNA blot hybridization with a probe corresponding to a genomic clone of maize the *Hrgp* gene. A clear increase in *Hrgp* mRNA level was detected in the cells treated with all four elicitors (Figure 1D). The increment in mRNA accumulation depends on the source of the elicitor and its concentration. The highest increment was obtained with 20 μg/ml of *F. moniliforme* elicitor. For this reason this elicitor was chosen for further investigations.

Suspension-cultured cells were incubated with 0, 2, 4, 10, 20 and 30 μg/ml of *F. moniliforme* elicitor. As can be seen in Figure 1C, an increasing concentration of *F. moniliforme* elicitor produced increasing accumulation of *Hrgp* mRNA up to 20 μg/ml of elicitor, where the highest level of *Hrgp* mRNA was found. At 30 μg/ml the mRNA level was lower. *Hrgp* transcript levels were measured between 6 and 24 h of induction with 20 μg/ml of *F. moniliforme* elicitor (Figure 1A, B) being *Hrgp* mRNA detectable already 3 h after elicitor treatment.

It has previously been shown that ethylene treatment induces an increase in the *Hrgp* mRNA level in maize seedlings [58]. It was of interest to study whether the effect observed by fungal elicitors could be related to ethylene in maize suspension-cultured cells. Maize cells were treated with different sub-

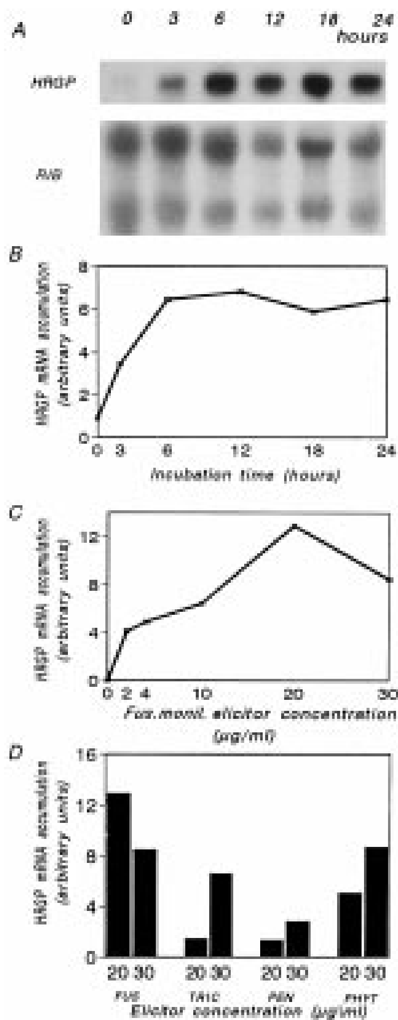

Figure 1. *Hrgp* mRNA accumulation in response to fungal elicitors. A. Time course accumulation of *Hrgp* transcript. RNA blot analysis of RNAs obtained from BMS maize cells incubated with 20 μg/ml of *F. moniliforme* elicitor during 0, 3, 6, 12, 18 and 24 h. Each lane was loaded with 10 μg of total RNA. The blot was hybridized with the *Hrgp* (HRGP) probe and a maize ribosomal (RIB) probe [38]. B. Graphical representation of the time course accumulation of *Hrgp* transcript. C. Graphical representation of *Hrgp* mRNA accumulation in response to different concentrations of *F. moniliforme* elicitor. BMS maize cells were incubed for 8 h with 0, 2, 4, 10, 20 and 30 μg/ml of *F. moniliforme* elicitor. Values are plotted taking the value obtained by densitometric scanning of cells incubated in the absence of *F. moniliforme* elicitor as a reference. D. Graphical representation of *Hrgp* mRNA accumulation in response to fungal elicitors. BMS maize cells were incubated for 8 h with 20 or 30 μg/ml of elicitors: *F. moniliforme*, *Tricoderma* spp., *Penicillium* spp. and *Phytophthora megasperma*. As a control, untreated BMS cells with elicitor were used. Values are plotted taking the value obtained by densitometric scanning of cells incubated in the absence of any elicitor as a reference. In the graphical representation of *Hrgp* mRNA accumulation (B, C and D), the *Hrgp* mRNA bands observed in the different treatments in RNA blot autoradiograms were quantitized by densitometric scanning and the *Hrgp* mRNA level normalized to ribosomal rRNA level in each sample. The untreated control is serving as the reference and has been given a value of 1 in B and D and a value of 0.

Figure 2. Role of ethylene and protein phosphorylation in the elicitor transduction pathway activating *Hrgp* expression. Northern blot analysis of 10 μg of total RNA extracted from BMS maize cells were incubated for 8 h with 20 μg/ml of *F. moniliforme* elicitor (El) as positive control, amino oxyacetate 0.1 mM (AOA), silver thioshulfate 50 μM (STS), 50 μM STS plus 20 μg/ml of *F. moniliforme* elicitor (STS + El), 1-aminocyclopropane-1-carboxylic acid 50 mM (ACC), 0.1 mM AOA plus 20 μg/ml of *F. moniliforme* elicitor (AOA + El), α-aminobutyric acid 5 mM (α-AB), staurosporine 0.4 μg/ml (St), staurosporine 0.4 μg/ml and 20 μg/ml of *F. moniliforme* elicitor (St+El). A control of cells in the absence of any treatment is included. The blot was hybridized with probes corresponding to *Hrgp* (HRGP) and histone (H4).

stances having an effect in the synthesis or the action of ethylene: aminooxyacetic acid (AOA), an inhibitor of ethylene biosynthesis; silver thiosulfate (STS), an inhibitor of the ethylene action; 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor in the synthesis of ethylene; and α-aminobutyric acid (α-AB), an inducer of ethylene production. Suspension-cultured cells were incubated with 0.1 mM AOA, 50 μM STS, 50 mM ACC or 5 mM α-AB. AOA and STS treatments were also performed in the presence of 20 μg/ml of *F. moniliforme* elicitor. RNA isolated after 8 h of treatment was analyzed by northern blot hybridization using the same *Hrgp* probe as above. As seen in Figure 2, AOA and STS treatments inhibit the accumulation of the *Hrgp* mRNA in elicited cells. On the other hand, treatments with ACC and α-AB increased *Hrgp* mRNA levels in unelicited cells. The RNA blot was also hybridized with a probe corresponding to histone H4 gene [47] as a measure of abundance of dividing cells. The H4 mRNA accumulation observed (Figure 2) does not correlate with *Hrgp* mRNA accumulation.

We also tested if the protein kinase inhibitor staurosporine (St) interacts with the elicitor signal transduction pathway. Suspension-cultured cells were incubated in the presence of 0.4 μg/ml of St, 20 μg/ml of *F. moniliforme* elicitor or both. As can be seen in Figure 2, staurosporine blocks the accumulation of *Hrgp* mRNA in response to the elicitor but does not affect the accumulation of histone H4 mRNA.

Figure 3. *Hrgp* mRNA accumulation in maize cells treated by compounds related to defense response. RNA blot analysis of 10 μg of total RNA extracted from BMS maize cells were incubated for 8 h with: methyl jasmonate 5 μM (MeJ), 20 μ,g/ml of *F. moniliforme* elicitor (El) as positive control, suspension-cultured maize cells maintained in control medium, reduced glutathione 0.1 mM (GSH), ascorbic acid 1 mM (AA), H_2O_2 1 mM (H_2O_2), salicylic acid 1 mM (SA), acetyl salicylic acid 1 mM (AcS). The RNA blot was hybridized with probes corresponding to *Hrgp* (HRGP) and histone (H4).

Figure 4. Effect of different compounds on the induction of *Hrgp* gene expression by ascorbic acid. RNA blot analysis of 10 μg of total RNA extracted from BMS maize cells were incubated for 8 h (except lanes 6 and 7) with: ascorbic acid 1 mM (AA), 0.1 mM AOA plus ascorbic acid 1 mM (AA+AOA), 50 μM STS plus ascorbic acid 1 mM (AA+STS), untreated cells as a control, 20 μg/ml of *F. moniliforme* elicitor at 3 h of treatment (3hEl), ascorbic acid 1 mM at 3 h of treatment (3hAA), 20 μg/ml of *F. moniliforme* elicitor (El). The RNA blot was hybridized with the *Hrgp* probe (HRGP).

Effect of different compounds on the level of Hrgp *transcript*

We examined the effect on *Hrgp* mRNA accumulation of different compounds that have been previously suggested to participate in plant defense transduction signal pathways. Maize suspension-cultured cells were incubated in the presence of 5 μM methyl-jasmonate (MeJa), 0.1 mM reduced glutathione (GSH), 1 mM ascorbic acid or 1 mM hydrogen peroxide. As can be seen in Figure 3, ascorbic acid and reduced glutathione induced the accumulation of *Hrgp* mRNA, whereas salicylic acid, acetylsalicylic acid, MeJa and hydrogen peroxide had no detectable effect. The increase in the *Hrgp* mRNA level induced by ascorbic acid and reduced glutathione was several-fold higher than the increase produced by 20 μg/ml of *F. moniliforme* elicitor. Again, no correlation was observed between the accumulation of the *Hrgp* mRNA and histone H4 mRNA. We also tested if ethylene is involved in the induction of Hrgp mRNA accumulation in response to ascorbic acid. Suspension-cultured cells were incubated in the presence of 1 mM ascorbic acid and in combination with 0.1 mM AOA or 50 μM STS. The inhibitors of ethylene biosynthesis (STS) and action (AOA) have no effect in the *Hrgp* mRNA accumulation induced by ascorbic acid (Figure 4).

Finally, we compared the kinetics of accumulation of the *Hrgp* mRNA in response to ascorbic acid and fungal elicitor. Suspension-cultured cells were incubated in the presence of 1 mM ascorbic acid or 20 μg/ml of *F. moniliforme* elicitor. RNAs were isolated after 3 and 8 h of ascorbic acid treatment and subjected to northern blot hybridization analysis. As can be seen in Figure 4, the *Hrgp* mRNA accumulation in response to ascorbic acid was detectable after 3 h of treatment but showed lower levels of accumulation than at 8 h treatment or 3 h of treatment with elicitor. Confirming the results described in Figure 1B, the level of induction in response to *F. moniliforme* elicitor after 3 h of treatment was slightly lower than the level attained after 8 h.

Accumulation of HRGP protein in etiolated maize seedlings

The effect of fungal elicitor was also studied on HRGP accumulation at the protein level. For this purpose young seedlings were selected. The HRGP protein level was analyzed in sections of seedlings by tissue printing and immunodetection of the protein. Tissue printing is a simple technique [10] that has been used to localize HRGP protein distribution in pea [9, 10, 11], in maize [28] or mRNA *Hrgp* localization [69] in soybean. Maize seeds were grown in the dark with the presence or absence of different concentrations of *F. moniliforme* elicitor (0, 30, 60 and 120 μg/ml) in the growing medium. After 7 days, elicitor-treated seedlings presented important morphological alterations with mesocotyls thicker and shorter than in untreated controls (data not shown). Because the highest morphological alteration was obtained with 120 μg/ml of *F. moniliforme* elicitor, this concentration was chosen for the analysis of HRGP accumulation by tissue printing. In the Figure 5 the result of 120 μg/ml *F. moniliforme* elicitor-treated and a control of untreated plantlets is shown. Similar results using western blotting have shown that no unspecific epitopes that could be detected by preimmune sera appear in maize germinated seedlings in the presence of *F. moniliforme* elicitors [44]. For the tissue printing experiment, seedlings were collected and cross-sections of the mesocotyl and coleoptile were printed on the

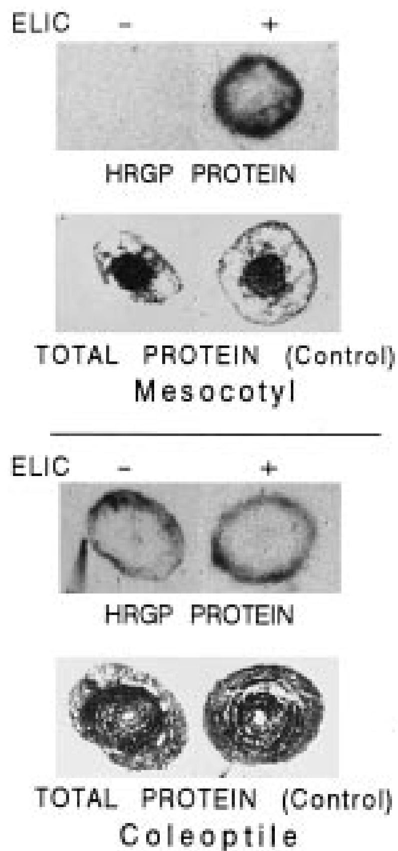

Figure 5. Tissue-print detection of maize HRGP protein in cross-sections of mesocotyl (A) and coleoptile (B) in 7-day old etiolated maize BMS seedlings growth without (left lane, −) and with 120 μg/ml (right lane, +) *F. moniliforme* elicitor. Immunodetection of maize HRGP with a polyclonal antibody against maize HRGP protein [36] (marked as HRGP PROTEIN). India Ink stain of total protein (marked as TOTAL PROTEIN).

same membrane. Tissue prints were stained with India ink for total protein staining and immunoassayed with an antibody against maize HRGP protein [36]. As shown in Figure 5, treatment with elicitor dramatically increases the level of HRGP in mesocotyls. Also, no increase was detected in coleoptile. The cells that accumulate more HRGP in response to elicitor in mesocotyl are the epidermal and perhaps external cortical cells.

Discussion

It has previously been shown that the expression of the *Hrgp* gene in maize is regulated during plant development, is located in tissues containing high rates of cell division activity, especially around provascular cells, and is also induced by ethylene and wounding [36, 58]. In contrast with what is found in dicotyledonous plants, HRGPs in cereals in most of the cases are encoded by low-copy or single-copy genes that must be able to respond to both developmental and environmental factors. No information is available on the effect of pathogen attack on the expression of these genes in cereals. To address this question we have examined the effect of different compounds related to the response of plants to fungal attack on the expression of the maize *Hrgp* gene. In this study crude preparations of fungal mycelia have been used as elicitors to induce plant response to pathogens as it has been used in a number of previous studies [17]. The effect has been analyzed in cell suspension cultures in an attempt to have a response as homogeneous as possible from maize cells.

Application of elicitors from different fungi to maize suspension-cultured cells increases *Hrgp* mRNA accumulation. The mRNA accumulation was observed using elicitor preparations from either pathogenic or non-pathogenic maize fungi. The level of *Hrgp* gene induction depends on the preparation tested and the concentration used (Figure 1). These results are in agreement with the fact that chemical composition of the fungal elicitors is complex and that the components able to activate the elicitor inducible genes are diluted in the preparations [2, 17, 62]. Moreover, different components of the same elicitor could induce different signal transduction pathways [4, 18]. The fact that the *Hrgp* mRNA level declined using more than 20 μg/ml of *F. moniliforme* elicitor suggests the existence of suppressors of *Hrgp* activation in the elicitor mixture as it has been reported in bean cells in response to pectic fragments of high molecular weight [4].

The role of ethylene in the elicitor signal transduction pathway of *Hrgp* mRNA accumulation was examined, because one of the earliest events in plant-pathogen interaction is a rapid increase of ethylene biosynthesis and many plant defense genes are regulated by ethylene [19]. For example, ethylene induces the expression of cell wall extensin genes in pea [9], melon [21] and carrot [19]. This is also the case in maize [58] for the *Hrgp* gene. In agreement with the activation of *Hrgp* gene by ethylene, the application of ACC to suspension-cultured cells induces *Hrgp* mRNA accumulation. This effect is probably secondary to the increase of ethylene production taking into account that ACC is the immediate precursor in the synthesis of ethylene (Figure 2) and increases eth-

ylene production in maize suspension-cultured cells [41]. In this sense, the application of α-aminobutyric acid α-AB), an inducer of the production of ethylene, also increased *Hrgp* mRNA accumulation. On the other hand, the fact that both the application of ethylene biosynthesis inhibitor (AOA) and ethylene action (STS) block the effect of the elicitor on *Hrgp* gene activation suggests that ethylene plays a role in the regulation of *Hrgp* gene expression in the elicitor transduction pathway. One of the elicitors here used, the *Phytophthora megasperma* elicitor, is able to induce ethylene biosynthesis in melon, tobacco and soybean [22]. It has been shown here that it is able to induce the expression of the maize *Hrgp* gene (Figure 1D) suggesting again that ethylene is involved in the signal transduction pathway of elicitor action on *Hrgp* gene. In the case of pathogenesis-related proteins the action of ethylene as a signalling component in the response to pathogens has been proposed [13, 35, 50, 51]. On the other hand, our results indicate that the protein kinase inhibitor staurosporine prevents *Hrgp* mRNA accumulation in the presence of elicitor. This result suggests that protein phosphorylation is involved in the elicitor transduction pathway. It has previously been shown that the pattern of protein phosphorylation is altered in plant cells responding to elicitors and protein kinase inhibitors prevent certain elicitor responses such as the induction of phenylalanine-lyase activity and ethylene production in tomato cells [23, 26]. Furthermore, staurosporine prevents elicitor-induced changes in protein phosphorylation as well as ethylene biosynthesis [23] and blocked the elicitor signal transduction pathway that results in an increase of a PR-like gene expression in maize aleurone cells [48].

Several other compounds are able to induce the expression of different plant defense genes and it was of interest to test the effect of some of them on the accumulation of *Hrgp* mRNA. Salicylic acid produces enhanced resistance to pathogens and accumulation of some PR proteins [25, 64, 65]. Methyl jasmonate is involved in the wounding response [14, 16]. However, neither 1 mM salicylic acid nor 5 μM methyl jasmonate produced an increased in maize *Hrgp* mRNA accumulation. This lack of response is in agreement with the hypothesis that several different molecular mechanisms underlay the defense and stress responses. It has been reported that extensin transcript levels were slightly enhanced after wounding and salicylic acid treatment in *Nicotiana plumbaginifolia* leaves [60]. On the other hand, in our experiments both ascorbic acid and reduced glutathione enhanced *Hrgp* mRNA accumulation. The presence of these antioxidant substances have been reported to induce plant defense genes [20, 72] in other plants. For example, glutathione supplied to bean suspension-cultured cells increased HRGP and phytoalexin biosynthetic enzymes (PAL and CHS) transcript levels [67]. The accumulation of *Hrgp* mRNA by the antioxidant substances is not correlated with the accumulation of histone H4 mRNA and therefore this response is independent of cell division, as has also been observed for the elicitor response of *Hrgp* gene. In contrast to the elicitor-mediated response, ethylene does not seem to play a role in the induction of *Hrgp* gene by ascorbic acid, because AOA and STS did not block *Hrgp* accumulation in response to ascorbic acid. Furthermore, the accumulation of *Hrgp* mRNA produced by fungal elicitor is a response faster than the one produced by ascorbic acid. Both results taken together suggest the existence of at least two different signal pathways in the induction of maize *Hrgp* gene, the elicitor ethylene-mediated pathway and the etylene-independent ascorbic acid pathway not dependent on ethylene. The induction of the same gene by different pathways would allow the activation of specific groups of defense genes in response to changing biological conditions. In dicot species where several extensin genes are found, they are regulated differentially. For instance, carrot extensin genes are regulated differentially by wounding and ethylene in carrot [19], and bean extensin genes by fungal elicitor, wounding or fungal infection [15]. In cereals with a reduced number or even single genes coding for the protein homologous to extensin, the same gene appears to be under the control of the conditions where its expression is necessary.

It has been proposed [24] that mRNA accumulation of ascorbate-free radical reductase is increased dramatically in response to wounding. Wounding also induces ascorbate-dependent prolyl hydroxylation, a reaction required for the accumulation of HRGP in the cell wall. In addition, ascorbate-free radical reductase may contribute to maintain high levels of ascorbic acid giving protection against wound-induced free radical-mediated damage. These data suggest the possibility that ascorbic acid-induced accumulation of *Hrgp* mRNA may be related with the wounding response of the *Hrgp* gene. However, with the present data it is not possible to exclude that ascorbic acid and glutathione induction may reflect an internal role of these substances in the response to pathogens here analyzed.

Maize HRGP accumulation in response to elicitors has been also studied in maize plants. In this case the accumulation of HRGP protein in response to elicitor was investigated in cross-sections of etiolated maize seedlings by the tissue printing immunoblot technique [10]. It has been shown that tissue printing is a quick and easy method for assessing developmental regulation of *Hrgp* gene by mRNA and/or HRGP detection in different plant tissues from a number of plant species [69, 70]. The results obtained by tissue printing immunoblot technique in etiolated maize seedlings show that elicitor-induced HRGP protein accumulation also occurs in tissues of intact plants (Figure 5). The application of elicitor to germinating seedlings produced morphological changes similar to those produced by ethylene [71], in agreement with the hypothesis that elicitor response is ethylene-mediated. HRGP accumulation produced by elicitor is regulated in a tissue-specific manner. The highest increases in HRGP accumulation by presence of elicitor (+) were observed in mesocotyls, the increase of HRGP content inversely correlates with the basal level of HRGP in control seedlings (−) (Figure 5), therefore the lower the HRGP content in untreated seedling (−) the higher increment of HRGP in treated seedling (+). In mesocotyl the basal level of protein is very low and it showed the highest relative increase. The accumulation of maize HRGP protein by elicitor seems to be higher in the epidermis and, possibly, external cortical cells of mesocotyls. This result is in contrast to the mRNA accumulation around the vascular system observed by *in situ* hybridization in ethylene-treated seedlings [58] but in agreement with the results observed in pea epicotyls [9]. The difference may be explained either by the difference in treatment (growing the seedlings in the presence of elicitors instead of application of ethylene athmosphere for a limited time) or by the detection system (protein instead of mRNA). The inducibility by the elicitor and the tissue specificity suggest that HRGP may be involved in plant defense by forming a physical barrier against invading pathogens.

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