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A gene coding for a basic pathogenesis-related (PR-like) protein from *Zea mays*. Molecular cloning and induction by a fungus (*Fusarium moniliforme*) in germinating maize seeds

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Abstract

Pathogenesis-related proteins (PRs) are plant proteins produced in leaves in response to infection by pathogens including viruses, viroids, fungi and bacteria. Information on the presence and/or expression of PRs in monocotyledonous plants is scarce. Here we report the identification of cDNA and genomic clones coding for a basic form of a protein from germinating maize seeds having a high homology with the group of PR-1 from tobacco.

A cDNA library enriched in aleurone-specific sequences was prepared from maize seeds two days after germination. One clone was found to contain an open reading frame encoding a protein homologous to PR proteins from tomato (p14) and tobacco (PR-1 group). Sequence analysis of the corresponding genomic clone revealed that it was encoded by a single exon. Besides, DNA blot hybridization indicates that this PR-like protein is encoded by a single-copy gene in maize. The accumulation of its mRNA increases after rehydration of desiccated seeds. Furthermore, a relationship was found between its expression and infection by a natural pathogen of maize, the fungus *Fusarium moniliforme*. The possible role of this protein as a response mechanism following fungal infection in cereal seeds is discussed.

Introduction

Pathogenesis-related proteins (PRs) were first described as a group of proteins which are synthesized *de novo* in tobacco plants reacting to infection by viruses, viroids, fungi or bacteria [2, 12, 17, 32, 33]. These proteins share several properties, namely, they are produced in leaves responding to a pathogen and they accumulate in the intercellular fluid. They are selectively extractable at low pH. They are highly resistant to proteolysis, and many of them are also induced by specific chemicals (polyacrilic acid, benzoic acid derivatives) [32]. The most interesting aspect of PRs is their possible protective function in the plant. A relationship between infection and resistance to a subsequent pathogen attack has been proposed [28].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X57095 maize mRNA PRms.

The cloning of cDNAs corresponding to a number of PR proteins has allowed their amino acid sequences to be deduced and homologies between proteins in different plant species to be established [2, 5]. Based on serological relationships and molecular properties, tobacco PRs can now be grouped into at least five groups of closely related proteins. Four of these groups are biochemically well characterized, and each comprises one or more acidic proteins as well as their basic counterparts. The proteins in each group are encoded by multigene families in the tobacco genome. Group 1 contains the PR-1 proteins, whose function is not known. After tobacco mosaic virus (TMV) infection three acidic PR-1 proteins (PR-1a, PR-1b and PR-1c) and one basic protein (G-protein encoded by cluster G cDNA) are found [12, 24]. A basic PR protein from tomato (p14), serologically related to the PR-1 group from tobacco, has been purified and characterized. It appears to accumulate in tomato leaves infected with the viroid of the spindle tuber disease of potato (PSTV) [15]. Groups 2 and 3 contain acidic and basic isoforms of enzymes with β-1,3-glucanase and chitinase activity respectively. Group 4 contains a class of acidic low molecular weight proteins of unknown function. Group 5 contains two acidic proteins, showing extensive sequence similarity to the sweet-tasting protein thaumatin and to a bifunctional inhibitor of α-amylase and protease of insects from maize (seeds) [27], and at least one basic protein which is identical to osmotin.

In spite of the considerable amount of data available on PRs in dicotyledonous plants, a more limited information about PRs in monocotyledonous species is presently available. Proteins serologically related to the PR-1 type proteins were detected in mildew-infected barley and in brome mosaic virus (BMV)-infected maize leaves [35]. The presence of high concentrations of endochitinases in wheat germ and barley flour has been described [14, 18]. In maize, ten PRs, named PRm proteins (m for maize), have been identified in leaves after BMV infection or mercuric chloride treatment and some of them have been purified to homogeneity [22]. According to their serological properties and their biological functions, maize PRs can be divided into at least three families: chitinases, β-glucanases and a family, including PRm2, which shows serological relationships and similarities in its amino acid composition to the PR-1 group of tobacco. However no information is available on the structure and expression of their corresponding genes. We now report the isolation of a cDNA and genomic clones of a gene encoding a basic PR-like protein from maize, which has been named PRms (ms for maize seed). Its mRNA accumulates at high levels in germinating maize seeds upon infection with a natural pathogen of maize, the fungus *Fusarium moniliforme*.

Materials and methods

Plant material

Maize (*Zea mays* pure inbred line W64A, grown in the greenhouse in Barcelona, Spain) was used as the experimental material. Maize seeds were germinated at 25 °C in the dark for three days followed by a daily cycle of 15 h of illumination for the required time. When required, seeds were surface-sterilized and germinated under sterile conditions. Seeds used for this purpose were rinsed in ethanol for 5 min and in 5% w/v calcium hypochlorite solution for 15 min, washed twice with sterile water and germinated in sterile medium including macro-elements according to Murashige and Skoog [20], micro-elements according to Heller [11], 30 g/l sucrose and 7 g/l agar.

At different stages of germination, seeds were harvested and dissected to obtain the aleurone-enriched fraction as follows. Embryos (including scutellum) were removed from germinating seeds with a scalpel. The surface of the endosperm was carved off with a scalpel and the carvings were immediately frozen in liquid N_2. In all subsequent experiments, aleurone layers do not refer to pure isolated aleurone layers but to aleurone layers with adjacent endosperm tissue.

Maize seeds naturally infected with *F. moniliforme* were harvested from germination trays and

used to obtain conidial suspensions. The conidial suspension was prepared from 4-day-old cultures grown on Bacto-malt agar (Difco). Sterile seeds were germinated for 1 day and then inoculated with the conidial suspension of *F. moniliforme* by adding approximately 50 μl (2000–3000 spores/ml) to each seed. Inoculated seeds (and sterile control seeds) were allowed to continue germination for two more days.

Leaves and roots were obtained from plants grown in a greenhouse for about 6 weeks, and immediately frozen in liquid N_2 after harvesting.

RNA and DNA isolation and construction of libraries

Total RNA was isolated from the aleurone layers obtained from germinating seeds and from adult plant tissues by the procedure described by Mundy *et al.* [19]. Each RNA sample was further purified by sedimentation through a CsCl cushion essentially according to Chirwin *et al.* [4]. Poly(A)$^+$ RNA was selected by oligo (dT) cellulose chromatography. The library was constructed in pUC18 using *Eco* RI linkers and cDNA synthesized according to Gubler and Hoffman [10]. Differential screening was carried out as described [30].

Total DNA was extracted from 6-week-old plant leaves according to Burr *et al.* [3]. Fungal DNA was isolated by the method of Davidson *et al.* [7]. Screening of genomic clones was carried out on a Charon35 library [9].

S1 nuclease mapping and primer extension analysis

The 270 bp *Nae* I-*Eco* RI fragment from the genomic clone λmPR1 was 5′-end-labelled with [γ-^{32}P]ATP and used as probe for S1 nuclease mapping. Hybridizations were carried out for 3 h at 70 °C in 20 μl of 300 mM NaCl, 30 mM trisodium citrate, pH 7.5, 2 mM EDTA containing 10000 cpm of probe and 1 μg each of total RNA isolated from aleurone obtained from 6-day germinated seeds or tRNA. The hybrids were digested with 100 units of S1 nuclease (Boehringer) in 200 μl of S1 buffer (200 mM NaCl, 5 mM $ZnSO_4$, 60 mM sodium acetate, pH 4.5, 100 μg/ml salmon sperm DNA) at 23 °C for 30 min. The products were analysed on a 6% polyacrylamide gel containing 8 M urea.

A 21-mer single-stranded oligonucleotide, complementary to the sequence starting 151 nucleotides downstream from the initiating codon ATG and extending downstream, was synthesized (5′-GCTGCAGCTTCGTGCTCCAGG-3′). The 21-mer was 5′-end-labelled with [γ-^{32}P]ATP and polynucleotide kinase (Boehringer). For primer extension, the radiolabelled 21-mer (50000 cpm) was mixed with 10 μg each of total RNA from aleurone layers from 6-day germinated seeds or adult leaf in 50 mM Tris-HCl, pH 7.5, 60 mM NaCl, heated to 85 °C for 5 min and slowly cooled to 30 °C. The mixture was ethanol precipitated, dissolved in 25 μl of 90 mM Tris-HCl, pH 8.3, 125 mM KCl, 10 mM $MgCl_2$, 1 mM dNTP and then incubated at 42 °C for 60 min in the presence of 100 units of reverse transcriptase (Boehringer).

Northern and Southern blots

Total RNA (5 μg) was separated by electrophoresis on 1.5% formaldehyde-containing agarose gel and transferred to nylon membranes (Hybond N, Amersham). The *Eco* RI insert from the selected pUC recombinant plasmid (B8A2) was isolated by agarose gel electrophoresis, and used as a hybridization probe.

Total DNA (10 μg) was digested to completion with *Kpn* I, *Sac* I, *Bam* HI, *Eco* RI and *Hind* III, and separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the random primed *Eco* RI insert from the B8A2 cDNA clone. After washing under stringent conditions (0.1 × SSC, 0.1% SDS, at 65 °C) the filter was autoradiographed for three days with an intensifying screen at − 80 °C. Fungal DNA was also digested with *Eco* RI, spotted onto a nylon membrane and probed with the insert DNA from clone B8A2.

DNA sequence analysis

Nucleotide sequence was determined using the dideoxynucleotide chain termination method [24] with [α-^{35}S]dATP. Overlapping clones were sequenced on both strands and restriction sites were confirmed in all cases. Sequence analysis was carried out using Micro-Genie software (Beckman) [26].

Results

Isolation and characterization of the maize seed pathogenesis-related cDNA clone

Differential screening of a pUC library prepared from poly(A)$^+$ RNA extracted from the aleurone layer-enriched material obtained from seeds for two-days germinated (approx. 1500 recombinant clones were screened) resulted in 24 aleurone-specific cDNA clones. These clones showed positive hybridization with the ^{32}P-cDNA probe prepared from the aleurone-enriched fraction obtained from two-day germinated seeds, whereas the hybridization to a labelled cDNA prepared from dry embryos was negative. Initial sequence analysis of one clone of this group (clone B8A2) revealed homology to the pathogenesis-related protein p14 from tomato. This clone was chosen for further study.

DNA sequence analysis indicated that the cDNA insert (537 nucleotides long) contained only one open reading frame encoding a protein homologous to the PRs described in tomato (p14) and tobacco (PR-1 proteins) [5, 16, 24]. Its amino acid sequence begins at residue 2 in the amino acid sequence of the homologous mature proteins (from tomato and tobacco). We have named the protein encoded by this cDNA PRms protein (ms from maize seed). The 5′-end of the mRNA was not present in clone B8A2 and no positive clones longer than this one were found in the cDNA library after further screenings.

The cDNA insert from clone B8A2 has a 3′ untranslated sequence of 105 bp which includes two putative polyadenylation signals, AATAAT (about 20 nucleotide upstream from the polyadenylation site) and AATAAA (located immediately before the poly(A)tail). The existence of multiple putative polyadenylation signals has been observed for other plant genes [8], including PR-1 mRNAs from tobacco [25].

The cDNA insert from clone B8A2 was then used as probe to screen the genomic library. A single positive clone, denoted λmPR1 was isolated from the genomic library. Sequence analysis revealed that the coding sequence was identical to that of the cDNA and that it was encoded by a single exon. A schematic representation of the PRms gene is given in Fig. 1. The nucleotide and deduced amino acid sequences for the PRms gene is shown in Fig. 2.

Protein and genomic sequences

The translational initiation methionine has been assigned by homology of this region to the corresponding N-terminal regions on the homologous PRs from tomato and tobacco, and it is the first potential methionine codon after the initiation of transcription deduced from S1 mapping and primer extension analysis (see below). Besides, analysis of the sequences surrounding the assigned initiation codon conforms with the consensus sequence for translation start sites in

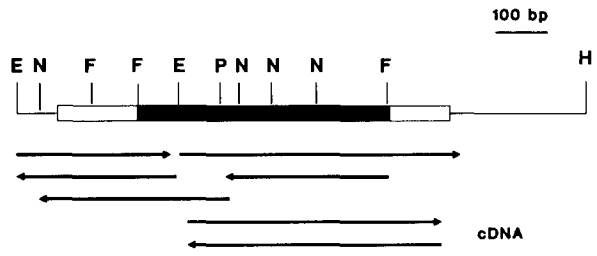

Fig. 1. Restriction map and sequencing strategy for the PRms gene. Enzymes used for mapping were: *Eco* RI (E), *Nae* I (N), *Fok* I (F), *Pst* I (P) and *Hind* III (H). The nucleotide sequence presented in Fig. 2 is represented by a rectangle including the coding sequence (blackened box). The two arrowed lines at the lower level marked as cDNA indicate the nucleotide sequence obtained from the PRms cDNA clone.

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TATTTTTCCAAGCATACCGGAGAAAATTCACATGCTCTC   -35
                                      *
CACTATAAATAGCCCCCACATATTTGCATTATAACATCC     5

ACAAATTAAACTCACGCACACACACAATTAGGTCACATA    44

CACACTTCCCGGCTGCCTCCCTAGGTAGCAGCTTCTACA    83

ATG GAG GCA TCC AAC AAG CTC GCA GTC TTG   113
Met Glu Ala Ser Asn Lys Leu Ala Val Leu    10

CTC CTG TGG CTG GTC ATG GCA GCT GCC ACT   143
Leu Leu Trp Leu Val Met Ala Ala Ala Thr    20

GCC GTG CAC CCT TCC TAC TCT GAG AAT TCG   173
Ala Val His Pro Ser Tyr Ser Glu Asn Ser    30

CCT CAA GAC TAC CTC ACT CCC CAA AAC AGC   203
Pro Gln Asp Tyr Leu Thr Pro Gln Asn Ser    40

GCC CGT GCC GCC GTC GGT GTT GGC CCG GTG   233
Ala Arg Ala Ala Val Gly Val Gly Pro Val    50

ACC TGG AGC ACG AAG CTG CAG CAG TTC GCA   263
Thr Trp Ser Thr Lys Leu Gln Gln Phe Ala    60

GAG AAG TAC GCC GCA CAG AGG GCC GGC GAC   293
Glu Lys Tyr Ala Ala Gln Arg Ala Gly Asp    70

TGC CGT CTC CAG CAC TCG GGC GGG CCC TAC   323
Cys Arg Leu Gln His Ser Gly Gly Pro Tyr    80

GGG GAG AAC ATC TTC TGG GGG TCC GCC GGC   353
Gly Glu Asn Ile Phe Trp Gly Ser Ala Gly    90

TTC GAT TGG AAG GCG GTG GAC GCA GTG CGA   383
Phe Asp Trp Lys Ala Val Asp Ala Val Arg   100

TCG TGG GTA GAC GAG AAA CAG TGG TAC AAC   413
Ser Trp Val Asp Glu Lys Gln Trp Tyr Asn   110

TAC GCC ACC AAC AGC TGC GCC GCC GGC AAG   443
Tyr Ala Thr Asn Ser Cys Ala Ala Gly Lys   120

GTG TGT GGC CAC TAC ACG CAG GTG GTG TGG   473
Val Cys Gly His Tyr Thr Gln Val Val Trp   130

CGC GCC ACT ACA AGC ATC GGC TGC GCG CGC   503
Arg Ala Thr Thr Ser Ile Gly Cys Ala Arg   140

GTC GTG TGC CGC GAC AAC CGT GGC GTC TTT   533
Val Val Cys Arg Asp Asn Arg Gly Val Phe   150

ATC ATC TGC AAC TAC GAG CCC CGC GGC AAC   563
Ile Ile Cys Asn Tyr Glu Pro Arg Gly Asn   160

ATT GCC GGG ATG AAG CCC TAC TGA TATATTG   594
Ile Ala Gly Met Lys Pro Tyr End

TGCCTGCGAACGATGGCAACTGATATTATATCTAGACAC   633

GGGCTTGTTGCAGCGCATGACTTTCCACGTGATATATGA   672
                    *
ATAATATTTTATAAATAAATCAAAGAGGTTTGTTATTTA   711
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Fig. 2. Nucleotide sequence of the PRms gene. Coding region is shown with the translated amino acid sequence. In the promoter region, the putative TATA-box is underlined, and the start site of the mRNA is indicated by an asterisk (nucleotide number 1). In the 3′-untranslated region the putative polyadenylation signals (AATAAT and AATAAA) are underlined, and the site of polyadenylation of the cDNA clone is shown by an asterisk.

plants [16]. The deduced protein contains 167 amino acids with an N-terminal sequence of 27 amino acids which is in agreement with the characteristics of a signal sequence [34]: expected signal peptide size (20–30 amino acids long), hydrophobic central core and charged residues in the N-terminal region. Besides, the pattern of amino acids near the putative signal sequence cleavage site fullfil the rules for the prediction of signal sequence cleavage sites [35].

A comparison of the amino acid sequences of PRms protein with the amino acid sequences of PR-1 proteins (PR-1a, PR-1b and G-protein) from tobacco and PR protein p14 from tomato was performed (Fig. 3). The homology of PRms protein with the G-protein from tobacco (G-protein amino acid sequence deduced from the cluster G cDNA clones) was found to be 63%. Homology with PR-1a and PR-1b proteins from tobacco was 55%, and with protein p14 from tomato 53%. The excess of basic residues over acidic residues in the mature protein (11% Arg + Lys, 6% Asp + Glu) in the PRms protein closely resembles the composition of the G-protein from tobacco (10% Arg + Lys, 5% Asp + Glu) and protein p14 from tomato (10% Arg + Lys, 6% Asp + Glu). On the other hand, PR-1a and PR-1b from tobacco contain an excess of acid residues over basic residues (6% Arg + Lys, 12% Asp + Glu; 6% Arg + Lys, 10% Asp + Glu, respectively).

The coding sequence is 63.5% G + C while the 3′ and 5′ untranslated regions have lower G + C content, viz. 35% and 48% respectively. In general, the codon usage pattern in this gene shows an overall preference for G + C content in the codon position III; this is commonly found in plant genes, particularly in monocots [21].

S1 nuclease protection and primer extension analysis were performed to determine the site of transcription initiation (Fig. 4). S1 nuclease mapping analysis was carried out with the ^{32}P-labelled 270 bp *Nae* I-*Eco* RI fragment of λmPR1 as the probe. Fig. 4a shows that a major protected band was detected in the gel (some minor bands also appear). Judging from the intensities of the protected bands, we concluded that a major transcription start site was 82 nucleotide upstream from the assigned initiation codon ATG. To confirm this result, primer extension analyses was carried out (Fig. 4b). Primer extension using the

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                          20                  40                  60
PR-ms       ENSPQDYLTPQNSARAAVGVGPVTWSTKLQQFAEKYAAQRAGDCRLQHSGGPYGENIFWG
Cluster G   Q.......N.H.A..RQ.....M..DNR.AA..QN..N.......M..........LAA-
PR-1a       Q..Q....DAH.T...D...E.L..DDQVAAY.QN..S.L.A..N.V..H.Q....LAE.
PR-1b       Q..Q....DAH.T...D...E.L..DNGVAAY.QN.VS.L.A..N.V..H.Q....LAQ.
p14         -.......AVH.D...Q.....MS.DAN.ASR.QN..NS.....N.I...A--...LAK.

                          80                  100                 120
PR-ms       SAGFDWKAVDAVRSWVDEKQWYNYATNSCAAGKVCGHYTQVVWRATTSIGCARVVCRDNR
Cluster G   -.YPQPH.AG..KM......F...NS.T....N...........NSVRL.....R.N-.G
PR-1a       .D..MTA.K-..EM......Y.DHDS.T..Q.Q...........NSVRV.....Q.N-.G
PR-1b       .D..MTA.K-..EM......Y.DHDS.T..Q.Q...........NSVRV.....K.N-.G
p14         GGD.TGR.--..QL..S.RPS......Q.VG..K.R.......-.LGG--.R..--.N-.G

                          140                 160
PR-ms       GVFIICNYEPRGNIAGMKPY
Cluster G   WY..T...D.P..WR.QRTVILKSNIPLIPSWNFQLMSSNNGLRDQIMNKSFVMC
PR-1a       .YVVS...D.P..YR.ES..
PR-1b       .YVVS...D.P..VI.QS..
p14         WW..S...D.V..WI.GR..
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Fig. 3. Comparison of the amino acid sequences of the PRms protein with the amino acid sequence of the G-protein (deduced from the nucleotide sequences of the cluster G cDNA clones), PR-1a and PR-1b from tobacco and p14 from tomato. Residues are numbered beginning with the first amino acid of the mature proteins. Amino acids which are different from the PRms protein are shown; identical amino acids are indicated by dots (•); gaps are indicated by dashes (–).

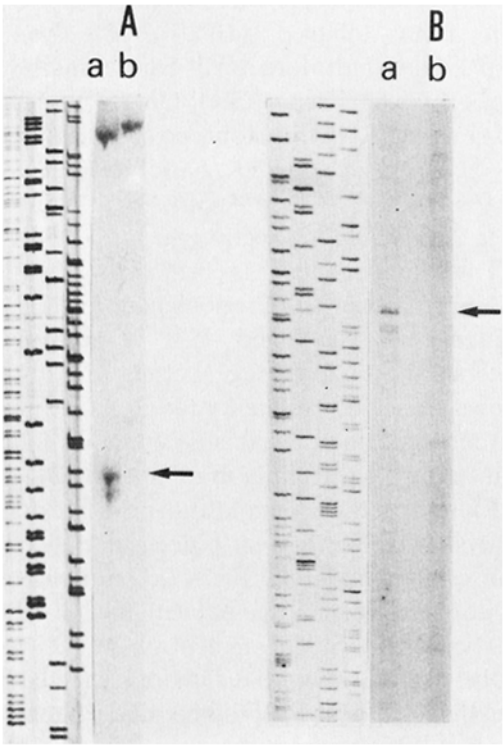

Fig. 4. Determination of the transcriptional initiation site. A. S1 nuclease mapping of mRNAs prepared from aleurone obtained from 6-day germinated seeds (lane a) and tRNA (lane b) as a control assay. S1 nuclease mapping was performed as described in Materials and methods using the 270 bp *Nae* I-*Eco* RI fragment from the genomic clone λmPR1 as a probe. Bands on the top correspond to the full-length probe. B. Primer extension analysis with total RNA isolated from aleurone layers obtained from 6-day germinated seeds (lane a) and adult leaf (lane b). Samples were analysed on a 6% polyacrylamide/8 M urea gel that also included sequencing reaction products.

oligonucleotide 5′-GCTGCAGCTTCGTGCT-CCAGG-3′, yields a fragment extending 255 bp in the 5′ direction to a point 82 bp upstream from the initiation codon ATG.

Analysis of the 5′-flanking region of the PRms gene indicates the presence of a putative TATA-box (*TATAAATA*) at 32 nucleotides upstream of the transcription start point. In contrast to what has been found for the PR-1 a gene from tobacco [6], the PRms gene does neither contain repeats (18 and 36 bp) nor any sequence resembling the heat shock consensus sequence upstream from the putative TATA-box. Furthermore, the 5′-flanking region of the PRms gene does not have recognizable CAAT-box.

Organization of the PRms protein gene

Total DNA isolated from maize leaves was digested with *Kpn* I, *Sac* I, *Bam* HI, *Eco* RI and

Hind III. Probing the genomic blot with the *Eco* RI cDNA insert from clone B8A2 revealed a single band for all digestions (Fig. 5a). The bands observed in the Southern blot coincide with those of the genomic clone, in the cases where both are available. In addition, the cDNA insert was also used as probe for hybridization to DNA purified from conidia of *Fusarium moniliforme* (Fig. 5b). The results obtained indicate that the cDNA insert hybridizes specifically to the maize DNA demonstrating that this clone derives from a mRNA species of host origin. The genomic blot results suggest that, at least at the level of stringency used for the analysis, the maize genome contains a single PRms gene. The existence of a single PRms gene in the maize genome contrasts with the complexity found for the PR-1 genes in the tobacco genome. Indeed, in addition to eight genes encoding acidic PR-1 proteins, the Samsun NN genome contains also approximately eight genes encoding the basic PR-1 protein (G protein) [2, 6].

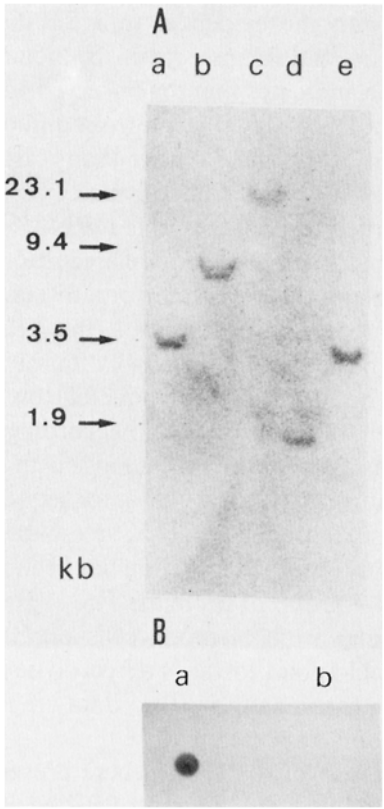

Fig. 5. A. Genomic Southern blot analysis of the PRms gene. Maize genomic DNA (10 μg) was digested with *Kpn* I (lane a), *Sac* I (lane b), *Bam* HI (lane c), *Eco* RI (lane d) and *Hind* III (lane e). Digested DNA was subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with the ^{32}P-labelled *Eco* RI insert from the cDNA clone. B. *Eco* RI-digested DNA from maize (a) and the fungus *Fusarium moniliforme* (b) (1 μg each) were spotted into a nylon membrane and probed with the insert of the cDNA clone.

Fungal infection induces the expression of PRms protein gene during maize seed germination

RNA blot analysis of total RNA samples isolated from aleurone layers obtained from unsterilized germinating maize seeds, at different stages of germination (day 1 to day 6) indicates that the B8A2 cDNA insert hybridizes specifically to an mRNA of approx. 700 bp (Fig. 6a). No signal was observable on northern blots of RNA extracted from embryos isolated from dry seeds, confirming the results of the initial screening. From Fig. 6a it appears that PRms mRNA is barely detectable at day 1 of germination while its level of expression increases drastically from day 1 to day 2 of germination, and progressively increases until the latest stage of germination here analysed (day 6). Furthermore, no expression or

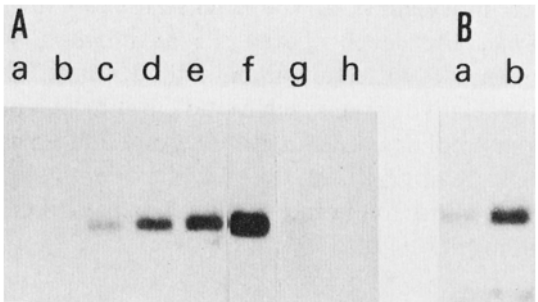

Fig. 6. RNA blot analysis of the maize seed pathogenesis-related protein (PRms). A. Total RNA isolated from dry seed embryos (lane a), from aleurone layers obtained from 1 (lane b), 2 (lane c), 3 (lane d), 4 (lane e) and 6 days (lane f) after maize seed germination, or from root (lane g) and leaf (lane h) from adult (6-week old) plant. B. Total RNA isolated from 3-day germinated seeds without (sterile seeds) (lane a) or with inoculation with the conidial suspension of *F. moniliforme* (lane b).

a very low level of expression was found when analysing RNA samples of adult (6-week-old) plant leaves or roots. Equally, there is no expression in radicle or coleoptile from 6-day germinated seeds (results not shown).

This northern blot analysis indicates that the PRms gene is expressed at high levels in germinating seeds and not in any other tissue tested. Due to the difficulty in isolating pure aleurone layers from maize seeds it is not possible to ascertain that PRms displays an aleurone-specific expression. However, during germination, endosperm tissues are essentially devoid of transcription activity [1]. Therefore aleurone cells are the probable source for the mRNA detected.

The effect of fungal infection on the expression of the PRms gene was tested by measuring the levels of PRms mRNA both in sterilized germinating seeds (control seeds) and in sterilized germinating seeds that had been inoculated with *Fusarium moniliforme*. Expression of the PRms mRNA on total RNA samples isolated from their aleurone layers was analysed (Fig. 6b). Sterilized seeds show a lower level of expression of PRms gene although its expression is not completely suppressed. This is probably due to the fact that the method of surface sterilization is not completely effective when host invasion has already occurred and the pathogen has penetrated into the seed tissues. However, when sterilized seeds were inoculated with the fungus *Fusarium moniliforme*, the level of the PRms mRNA was increased. No accumulation of the PRms mRNA in vegetative tissues such as radicle and coleoptile from seedlings or leaf and root from adult plants could be attributed to the lack of susceptibility of these tissues to the colonization by this particular pathogen.

Discussion

Pathogenesis-related proteins are specific proteins produced in plants in response to infection by pathogens (viruses, viroids, fungi or bacteria) and by different chemical agents. PRs were first described in leaves from tobacco plants reacting to infection. Since then, serologically related proteins have been found to accumulate in leaves of infected plants from several species and different families: Solanaceae, Amaranthaceae, Chenopodiaceae and Gramineae [36]. However, no sequence or genomic information was available for monocot species.

In contrast to the various already characterized PR proteins whose expression is induced in leaves, very little information is available on seeds producing and/or accumulating PR proteins. In this paper, we describe cDNA and genomic clones from maize representing a mRNA whose expression is induced by fungal infection in germinating seeds. Analysis of expression of its mRNA, together with the evidence from Southern blot analysis that the PRms protein is encoded by a single gene, indicates that the observed variations in PRms mRNA signal result from differences of expression of this gene, rather than arising from a complex of several related genes.

The protein encoded by this mRNA is related to the known pathogenesis-related PR-1 group from tobacco and p14 from tomato leaves. The PR-like protein here described (the PRms protein) is a basic protein. The isolation of PR-proteins has been traditionally directed towards the purification of acidic proteins: accordingly, these proteins have been mainly analysed in alkaline non-denaturing gels and, as a consequence, the basic forms of the PRs may have escaped detection in many cases. No function has been attached to the PR-1 group of proteins. The expression of the PRms gene in aleurone cells during germination would point towards a hydrolytic function for these proteins although no data are available on this point at present.

The interest of the protein here presented lies not only in the expression of a PR-like protein in seeds but also in the relationship we have found between its expression and infection of maize seeds by a fungus (*Fusarium moniliforme*). *F. moniliforme* is a common pathogen of maize throughout the world that causes stalk and ear roots [13]. The fungus reduces seedling stands in crops through seed decay, damping-off and seedling blight [23]. The increased expression of

this gene after fungal infection indicates that its expression could be part of a defence mechanism against pathogens during seed germination as an adaptative response against potential aggressions coming from the environment. It is possible that in the case of graminaceous plants, the protection of the seed is particularly important for the survival of the species.

The mechanism of induction of the accumulation of this mRNA remains unknown. The presence of high concentrations of endochitinases in wheat germ and barley flour [14, 18], and the increase in endochitinase mRNA levels in the aleurone layers during the incubation of barley seeds [31], together with the expression of the PRms protein here presented, may represent the existence of a coordinate expression of a combination of specific genes for maximum protection against fungi in cereal seeds. A more thorough study on the distribution of this protein and its mRNA in different parts of the cereal plant (in particular, in different seed tissues) and investigations on the regulation of the PRms gene expression would contribute to a better understanding of the possible role of this protein against fungi in cereal seeds. Now, the availability of the genomic clone will allow us to elucidate, by plant transformation procedures, the regulatory sequences involved in its induction by fungi.

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