Increased Expression of the Calmodulin Gene of the Late Blight Fungus *Phytophthora infestans* During Pathogenesis on Potato

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In order to isolate *in planta*-induced genes encoding putative pathogenicity factors of the late blight fungus *Phytophthora infestans*, a genomic library was differentially screened. For the differential hybridization, labeled first-strand cDNA synthesized on mRNA isolated from *P. infestans*-infected potato leaves and on mRNA isolated from the fungus grown *in vitro* were used as probes. This screening resulted in the isolation of the *P. infestans* calmodulin gene. The gene, designated *calA*, contains an open reading frame of 447 base pairs without introns and is unique in the *P. infestans* genome. The predicted amino acid sequence is 89.9–94.6% identical to calmodulins from higher eukaryotes, whereas the identity to calmodulins of higher fungi is significantly less (60.8–85.1%). Expression studies revealed that the *P. infestans calA* gene is constitutively expressed in *in vitro* grown mycelium. However, during pathogenesis on potato the level of *P. infestans* calmodulin mRNA is increased approximately fivefold.

Additional keywords: calcium-binding protein, differential screening, Oomycetes, pathogenicity, potato late blight.

The fungal plant pathogen *Phytophthora infestans* (Mont.) de Bary (Oomycetes), the causal agent of potato late blight, is one of the most devastating diseases of potato (*Solanum tuberosum* (L.)). Infection of potato leaves and tubers by a virulent strain of this hemibiotrophic fungus initially causes a rapidly spreading lesion followed by necrosis, which results in complete destruction of the infected tissue. The specialization of *P. infestans* on solanaceous plants such as potato and tomato implies a mechanism for recognition that most likely requires the exchange of signals between host and pathogen. On the one hand, infection of the host by *P. infestans* induces specific expression of a particular set of host genes, some of which are thought to be involved in defense (Fritzemeier *et al.* 1987; Taylor *et al.* 1990; Matton and Brisson 1989). On the other hand, growth of the fungus on its host plant can lead to induced expression of genes in the pathogen (Pieterse *et al.* 1992). The products of the *in planta*-induced *P. infestans* genes might be involved in the establishment and maintenance of a compatible interaction between the pathogen and its host plant. Our approach to identify factors that determine the pathogenic abilities of *P. infestans* and factors that are involved in the signaling between host and pathogen is to isolate genes of *P. infestans* that show induced or increased expression during pathogenesis. Identification of the proteins encoded by these genes might elucidate some of the molecular and cellular events involved in plant-pathogen interactions.

To isolate *P. infestans* genes whose expression is specifically induced or increased during colonization of the host plant, we screened a genomic library of *P. infestans* by differential hybridization using labeled cDNA probes synthesized on mRNA isolated from *P. infestans*-infected potato leaves and on mRNA isolated from the fungus grown *in vitro*. This resulted in the isolation of several fungal genes of which the expression is induced during growth of the fungus *in planta*. Characterization of one of the clones led to the identification of an *in planta*-induced gene of *P. infestans* coding for polyubiquitin (Pieterse *et al.* 1991). Here we describe the characterization of another *in planta*-induced gene of *P. infestans* that appeared to encode calmodulin, a calcium-binding protein that has an essential function in various cellular processes.

Calmodulin, which is highly conserved in all eukaryotic organisms, is one of the major intracellular calcium receptors responsible for mediating cellular responses to the calcium fluxes which are generated by a variety of processes. Upon binding of Ca^{2+}, calmodulin undergoes a conformational change, which enables the Ca^{2+}-calmodulin complex to bind to, and in that way to regulate, the activity of a large number of enzymes (Cheung 1980; Roberts *et al.* 1986). It is evident that both the Ca^{2+} and the calmodulin concentration play an essential role in this process. An important class of calmodulin-regulated enzymes are protein kinases whose action is recognized as a primary mechanism for the transduction of extracellular stimuli into intracellular events (Hepler and Wayne 1985; Roberts *et al.* 1986). Thus, through binding of Ca^{2+}, calmodulin can have a regulatory effect on a diverse array of cellular processes.

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In plants, calmodulin and Ca^{2+} are thought to be involved in the regulation of mitosis, polarized cell growth, and cytoplasmic streaming (Marme and Dieter 1983; Hepler and Wayne 1985). In some fungi, e. g. *Metarhizium anisopliae* (St Leger *et al.* 1989), *Ceratocystis ulmi* (Muthukumar and Nickerson 1984), and *Candida albicans* (Paranjape *et al.* 1990), and in the slime molds *Dictyostelium discoideum* (Lydan and O'Day 1988) and *Physarum polycephalum* (Uyeda and Furya 1986), the Ca^{2+}-calmodulin complex has been reported to be involved in growth and differentiation.

In this study we describe the isolation and characterization of the *P. infestans* calmodulin gene (*calA*) and show that the expression level of this gene increases severalfold during pathogenesis. The significance of the *in planta*-induced expression of the *P. infestans calA* gene will be discussed.

RESULTS AND DISCUSSION

An *in planta*-induced gene of *P. infestans* codes for calmodulin.

In order to achieve a 99% probability of having any DNA sequence represented, 80,000 recombinant bacteriophages of the *P. infestans* genomic library were differentially screened. This resulted in the selection of 20 recombinant phage clones containing putative *in planta*-induced genes of the fungus. The number of positive clones does not provide information on the number of *in planta*-induced *P. infestans* genes. One recombinant phage can easily contain a constitutively expressed gene in the proximity of a differentially regulated gene; the latter will not be detectable in the differential screening procedure. In a dot blot experiment, DNA of 10 clones hybridized relatively strongly to the cDNA probe derived from mRNA isolated from infected leaves, whereas hybridization to the cDNA probe synthesized on mRNA isolated from *in vitro* grown mycelium gave relatively weak signals. When the differentially hybridizing DNA fragments from these clones were used as probes on northern blots, it appeared that eight of these 10 clones contain a gene of which the expression is significantly increased during growth of the fungus *in planta* (data not shown). There was no cross-hybridization between these eight clones, indicating that they contain different genes.

In one of the eight positive clones, DHC-C, the coding region of the gene responsible for the differential hybridization was mapped on a single 5.0-kb *Sst*I fragment, which was subsequently subcloned into pTZ19U. The other *Sst*I fragments from DHC-C did not hybridize to the cDNA probes. In Figure 1, the restriction map of the resulting plasmid, designated pPiCM-S, is depicted. Additional differential hybridization experiments indicated that the gene is located on two linked *Pst*I fragments of 1.2 and 1.5 kb, respectively. Dideoxy sequencing of various overlapping subclones within the two *Pst*I fragments resulted in the nucleotide sequence shown in Figure 2. In the sequenced region, a single open reading frame (ORF) of 447 nucleotides coding for a protein of 149 amino acids was found. Comparison of the ORF with the nucleotide sequence data library revealed that the gene codes for a protein that is highly similar to calmodulin, a calcium-binding protein present presumably in all eukaryotes. The deduced amino acid sequence of the gene contains four EF-hand calcium-binding domains (indicated by ★★★ in Fig. 5 below) which is typical for calmodulins. From these data it was concluded that the *in planta*-induced gene present in the recombinant bacteriophage DHC-C encodes calmodulin. Therefore the gene was designated *calA*.

Analysis of the *calA* gene locus.

On a Southern blot containing genomic *P. infestans* DNA digested with various restriction enzymes, a probe derived from the coding region of the *calA* gene (a 342-bp *Pvu*II/*Hin*dIII fragment from pPiCM-S, see Fig. 1) hybridized to single *Bam*HI and *Sst*I fragments of 6.0 and 5.0 kb, respectively (Fig. 3). Sequencing data revealed that the coding region of the *calA* gene contains an *Eco*RI and a *Pst*I restriction site, and as a result, the *calA* probe hybridizes to two *Eco*RI (2.6 and 17 kb) and two *Pst*I fragments (1.2 and 1.5 kb). The length of the hybridizing *Sst*I fragment is in agreement with the length of the 5.0-kb *Sst*I fragment found in the recombinant bacteriophage DHC-C. The same holds for the two *Pst*I fragments and the 2.6-kb *Eco*RI fragment that are present in pPiCM-S (Fig. 1). The sizes of the other hybridizing fragments could not be verified as they overlap the 5.0-kb *Sst*I fragment on either one side (17-kb *Eco*RI fragment) or both sides (6.0-kb *Bam*HI fragment). From these data it can be concluded that the genome of *P. infestans* contains a single copy of the calmodulin gene. The occurrence of one calmodulin gene per haploid genome is common in most organisms, but in a few cases calmodulin gene families have also been reported (Chung and Swindle 1990; Chien and Dawid 1984; Fischer *et al.* 1988). Each member of these calmodulin gene families may be regulated differently (Fischer *et al.* 1988) but within one organism all calmodulin genes encode identical calmodulin proteins.

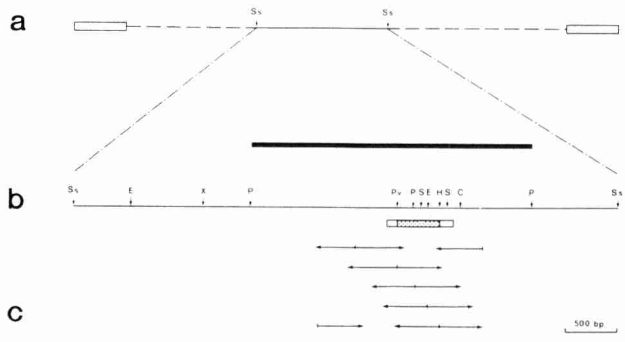

Fig. 1. Restriction map and sequencing strategy of the *Phytophthora infestans calA* gene. **A,** Partial restriction map of the genomic λ clone DHC-C. **B,** Restriction map of the 5.0-kb *Sst*I insert of pPiCM-S. C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, P = *Pst*I, Pv = *Pvu*II, S = *Sal*I, Ss = *Sst*I, and X = *Xba*I. *Bam*HI does not cut in the 5-kb insert. The closed bar indicates the approximate location of the coding region of the *calA* gene as determined by differential hybridization experiments. The open bar indicates the coding region of the *calA* gene as determined by dideoxy sequencing. The hatched area in this bar represents the 342-bp *Pvu*II/*Hin*dIII fragment which was used as probe for northern and Southern blot hybridizations. **C,** Sequencing strategy for *calA*. Arrows indicate the direction and the extent of sequencing.

Further characterization of the *calA* gene.

The nucleotide sequence of the *calA* gene as shown in Figure 2 includes 720 bp upstream of the translation initiation site and 188 bp downstream of the stop codon. The coding region of the *calA* gene is not interrupted by introns. The putative transcription initiation site of the *calA* gene was determined by primer extension using an oligonucleotide complementary to the *calA* sequence at position 9–25 relative to the ATG start codon. The oligonucleotide was annealed to and extended on poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium. A single primer extension product of 71 nucleotides was found, indicating that the *calA* transcription initiation site is located at the T at position −46 relative to the ATG start codon (Fig. 4). The oligonucleotide used in this primer extension experiment is 82% homologous to the corresponding region in potato calmodulin mRNA (Jena *et al.* 1989). To check whether the oligonucleotide hybridized to potato calmodulin mRNA, the primer extension experiment was also performed on poly(A)$^+$ RNA isolated from the potato-*P. infestans* interaction (potato cv. Ajax, 3 days postinoculation). Again, only one primer extension product was detected that is identical in length to the one produced on poly(A)$^+$ RNA isolated from *in vitro* grown mycelium (Fig. 4). Apparently the same *calA* transcription initiation site at position −46 is functional during growth *in vitro* and *in planta*. Potato calmodulin mRNA has a leader sequence of at least 81 nucleotides (Jena *et al.* 1989). Thus the expression level of the potato calmodulin gene is either too low to be detected or the oligonucleotide does not hybridize to potato calmodulin mRNA under the hybridization conditions used.

The transcription initiation site of the *calA* gene is preceded by the sequence TCAT. Interestingly, four other *P. infestans* genes, i.e., the polyubiquitin gene *ubi3R* (Pieterse *et al.* 1991), the actin gene *actA* (Unkles *et al.*

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       -710                  -690                  -670                  -650                  -631
TCGGCAAAAATCAACCAAAGCAGAGTCCCTAATTTTCCCCCCAAAAATGATAAAATAGCTGTTTTACAAACATCAGGACCATTAATACAT
       -620                  -600                  -580                  -560                  -541
GTATTAATAACAGATGCTGTAAACAGCTCTGCACCGACCGCCTCAAATCCCCGAGCAGGAAATAGGCCCTAAGGTTTGAAAAATTAAAAA
       -530                  -510                  -490                  -470                  -451
TATACTAAAAATATCTATTGTCGTTGTGGCAACTAAATGCTGGCGCCATCAAATATTAACACGCTAAGAAGCTACCACATCGAGGTGACT
       -440                  -420                  -400                  -380                  -361
CTCTCGTCGCTCAAGCCTTTCATGCATTATATCTGACTGGAATGTGATCTTTATGAAAGGTCAATTGCATGTAGCAATCAGTTTTTTTTC
       -350                  -330                  -310                  -290                  -271
TGCGATCTTCGCTGTGTCGTTGACTGTTCCGTTCTGTCCACAATCCATCGTTTTGTCAAGCCGTGTGCGCCACACCAATGTGCAAAACCA
       -260                  -240                  -220                  -200                  -181
AAAATATTTGTACGCACCACATCATGGTCGTACATGATATGGTCGTTCATCCTTGCTCTTTTTAATAATTATGATTTATTGGTGCGGACA
       -170                  -150                  -130                  -110                   -91
GACATATTTTAACCAATCGCATATTGCCCACAAATTATCAACTAATCGCAGTACGCCGCTTGGCCGTCCACATTCTTCGTGGTCGTACAA
        -80                   -60                   -40                   -20                    -1
ATCAGGGCCGTCAAAGGCGCCGGTCTCATTTTGGATGGGATCATTGTTGGATTTCCCTCGACACCCCCAAGTGAAAACCGAAGCACCACA
         10                    30                    50                    70                    90
ATGGCTGACCAGCTGACCGAAGAGCAGATTGCCGAGTTCAAGGAGGCGTTCTCCCTGTTCGACAAGGACGGCGATGGAACCATTACCACC
M  A  D  Q  L  T  E  E  Q  I  A  E  F  K  E  A  F  S  L  F  D  K  D  G  D  G  T  I  T  T
        100                   120                   140                   160                   180
AAGGAGCTTGGCACGGTCATGCGCTCTCTGGGCCAGAACCCCACGGAGGCCGAGCTGCAGGACATGATCAACGAGGTCGACGCTGACGGA
K  E  L  G  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A  D  G
        190                   210                   230                   250                   270
AACGGCACGATTGACTTCCCCGAATTCCTGACCATGATGGCTCGCAAGATGAAGGACACGGACTCCGAGGAGGAGATCCTGGAGGCCTTC
N  G  T  I  D  F  P  E  F  L  T  M  M  A  R  K  M  K  D  T  D  S  E  E  E  I  L  E  A  F
        280                   300                   320                   340                   360
AAGGTGTTCGACAAGGACGGCAATGGATTCATCTCGGCTGCCGAGCTGCGTCACATCATGACCAACCTGGGCGAGAAGCTTACCGACGAG
K  V  F  D  K  D  G  N  G  F  I  S  A  A  E  L  R  H  I  M  T  N  L  G  E  K  L  T  D  E
        370                   390                   410                   430                   450
GAGGTCGACGAGATGATCCGCGAGGCCGACATCGACGGCGATGGACAGATCAACTACGAAGAGTTCGTCAAGATGATGATGTCCAAGTAA
E  V  D  E  M  I  R  E  A  D  I  D  G  D  G  Q  I  N  Y  E  E  F  V  K  M  M  M  S  K  *
        470                   490                   500                   520                   540
GCGTCGCGTCCCCAACGCTTAGTTCCCGATCTTTTGATCCGAAGTATCTTGTTGCCAACGTTGTAGGCGCGGGCGTTTGCTCCCTCTTCG
        550                   570                   590                   610                   630
TTGTAGCTGGCTGCTCCTATCGATCGTAGCTTAAATAATTTAGAAACAGCGTGAAATTGCTTTTTTTTGCTGATGCTTTTTGCGTGGTCG

TTTAGTAC
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Fig. 2. Nucleotide sequence of the *Phytophthora infestans calA* gene and corresponding amino acid sequence of the encoded calmodulin protein. The transcription start site as determined by primer extension is indicated by a closed arrowhead (▼), the TCATT sequence surrounding the transcription initiation site is underlined with a dotted line. The DNA sequence overlined with the dotted line at positions −51 to −71 represents the sequence motif that is conserved among calmodulin promoter sequences from several organisms. Underlined DNA sequences depict the CAAT and TATA-like motifs. Overlined DNA sequence (AAATAA) in the 3′ flanking region of the calmodulin gene represents a putative polyadenylation signal.

1991), and the *in planta*-induced genes *ipiO2A* and *ipiO2B* (C. Pieterse, unpublished) as well as one *Phytophthora megasperma* gene, i.e., the actin gene (Dudler 1990), have a TCATT sequence in which the single transcription initiation site is located. One other *Phytophthora* gene in which the site of transcription initiation has been determined is the actin gene *actB* of *P. infestans* (Unkles *et al.* 1991). However, this gene contains several transcription initiation sites and none of these are located in a sequence motif TCATT. The significance of the conserved TCATT sequence in transcription initiation of *Phytophthora* genes is not clear.

In the promoter region of the *calA* gene, a TATA-like motif (TACAAA) is located 49 nucleotides upstream of the *calA* transcription initiation site at position −95 and a CAAT motif is present at position −167, 121 nucleotides upstream of the transcription initiation site. In the majority of genes identified from higher eukaryotes, the consensus 'core promoter' sequences TATAAA and CAAT are found around 30 bp and 70–90 bp, respectively, upstream of the major transcription initiation site. However, these motifs are not very common in the promoters of filamentous fungi (Gurr *et al.* 1987). Whether the sequence motifs present in the *calA* gene are functional transcription signals is unknown. Just upstream of the transcription initiation site, between positions −71 and −51, there is a 20 nucleotide sequence motif that is conserved in calmodulin genes of several organisms (Zimmer *et al.* 1988). The conservation of this sequence motif, which is also found in some other unrelated eukaryotic genes (Zimmer *et al.* 1988), ranges between 60 and 80% homology. In the calmodulin genes it is always located within 150 nucleotides upstream of the transcription initiation site. The significance of this motif for transcription or regulation of gene expression is unknown. The 3′ untranslated region of the *calA* gene contains a putative polyadenylation signal (AAATAA), 123 nucleotides downstream of the TAA stop codon.

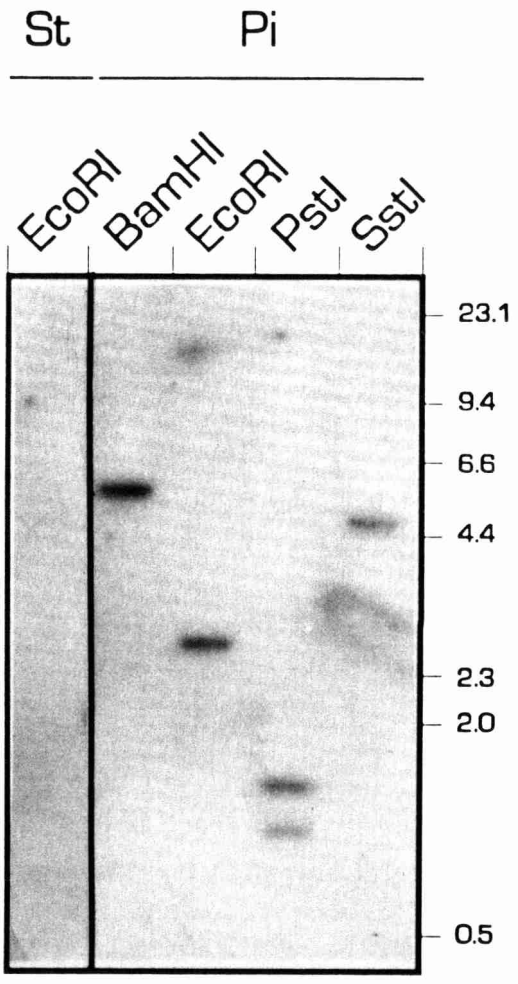

Fig. 3. Southern blot analysis of *Phytophthora infestans* genomic DNA (Pi) digested with *Bam*HI, *Eco*RI, *Pst*I, and *Sst*I and potato genomic DNA (St) digested with *Eco*RI (10 μg/lane). The 342-bp *Pvu*II/*Hin*dIII fragment from the coding region of the *calA* gene was used as probe. The size of the markers is indicated in kilobases.

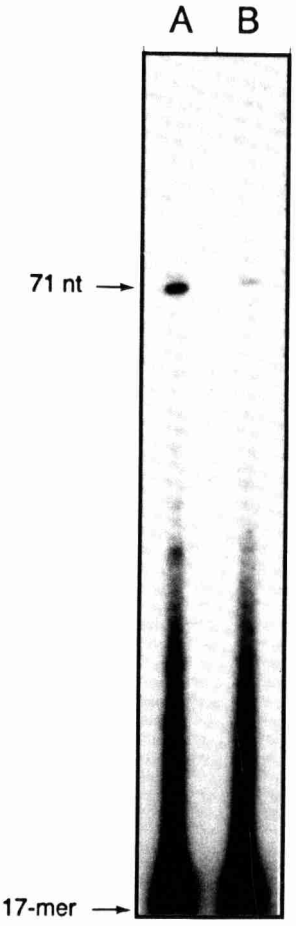

Fig. 4. Primer extension analysis of the *calA* transcript. A [γ-^{32}P]ATP 5′-end-labeled oligonucleotide (5′-GCTCTTCGGTCAGCTGG-3′), complementary to the *calA* sequence at position 9–25, was annealed to 3 μg poly(A)$^+$ RNA and extended with M-MLV reverse transcriptase. Primer extension products were electrophoresed on a denaturing 6% (w/v) polyacrylamide gel next to the products of a dideoxy sequencing reaction using the same oligonucleotide as primer and pPiCM-S as template DNA (not shown). The extension products synthesized on poly(A)$^+$ RNA isolated from the potato-*Phytophthora infestans* interaction (potato cv. Ajax, 3 days postinoculation) are shown in lane A, the extension products synthesized on poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium in lane B.

Like most oomycetous genes studied so far, but in contrast to the majority of the known calmodulin genes, the *P. infestans calA* gene has no introns. The few other intron-less calmodulin genes are the calmodulin genes of *Achlya klebsiana* (LeJohn 1989), which is another Oomycete, of *Saccharomyces cerevisiae* (Davis *et al.* 1986) and of *Trypanosoma cruzi* (Chung and Swindle 1990). Most calmodulin genes contain up to five introns, some of which occur at more or less conserved positions in the gene (see references mentioned in the legend of Fig. 5).

Comparison of *P. infestans* calmodulin with calmodulins of other organisms.

The predicted amino acid sequence of the *calA* gene product is highly homologous to calmodulins from other eukaryotic organisms (Fig. 5). The highest identity is found with the calmodulin sequence of the Oomycete *Achlya klebsiana* (96.6%). There are only five amino acid substitutions, which are all located outside the four EF-hand calcium-binding domains. The isoleucine residue at position 109 is unique for *P. infestans* calmodulin. *P. infestans* calmodulin is remarkably similar, 90% identity or more, to calmodulins of several vertebrates, invertebrates, and plants. Also the similarity with calmodulins of lower eukaryotes such as *Trypanosoma cruzi* (91.9%) and *Chlamydomonas reinhardtii* (86.6%), is relatively high. Interestingly, calmodulins of higher fungi such as *Aspergillus nidulans* and especially the yeasts are less homologous to *P. infestans* calmodulin (less than 85% identity). Here, the amino acid substitutions are randomly distributed throughout the sequence. The sequence comparisons suggest that evolutionarily, *P. infestans* is less closely related to higher fungi than to some other eukaryotes. This supports the hypothesis that oomycetous fungi evolved from ancestors other than higher fungi (Barr 1981; Cavalier-Smith 1987; Karlovsky and Prell 1991).

Expression of the *P. infestans* calmodulin gene during pathogenesis on potato.

The *P. infestans calA* gene was isolated from a genomic library by a procedure that was aimed at the selection of *in planta*-induced genes of this fungus. Subsequently, northern blot analyses were performed to confirm that the *calA* gene is indeed a gene that shows induced expression during pathogenesis. When studying gene expression in a plant-fungal interaction by means of northern blot analysis, it is important to take into account the changing ratios of fungal and plant RNA due to the increase of fungal biomass during colonization of the leaves. By using a constitutively expressed fungal gene as probe for the hybridization of northern blots, the increase in the proportion of fungal RNA in the interaction RNA mixtures can

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                    10        20        30        40        50        60        70        80
                     .  ★ ★ ★ ★ ★ ★.  ★  .         .         .  ★ ★.★ ★ ★  ★ .         .
P. infestans        ——MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEE
A. klebsiana        ——.....G.....V.....
Man                 ——.....
Chicken             ——.....
Eel                 ——.....K.....
D. melanogaster     ——.....
Potato              ——..E.....C.....S.A...Q.....NL.....
C. reinhardtii      MAANTE.....A.....S.....ML.....E..H.D.
T. cruzi            ——.....SN...S.....Q..S.....L.....Q.S.....
A. nidulans         ——...S.....VS.Y.....Q.....S.S.....N.....
C. albicans         ——..EK.S.Q.....S..K.....S.S..T.....VNSD.S.....A.
S. pombe            —MTTRN..D.....R.....R.Q..N..SN...V.....S..A.....T.....N...
S. cerevisiae       ——.SSN.....A.....NN.S.SSS..A.....LS.S...VN.LM..I.V...HQ.E.S...AL.S.QL.SN...Q.

                    90        100       110       120       130       140       149          % identity
                     .  ★ ★ ★ ★ ★ ★  .         .         .  ★ ★ ★ ★ ★ .★  .
P. infestans        ILEAFKVFDKDGNGFISAAELRHIMTNLGEKLTDEEVDEMIREADIDGDGQINYEEFVKMMMSK*————————      100.0
A. klebsiana        .....QG.....M.....*————————      96.6
Man                 .R...R.....Y.....V.....V.....Q..TA.*————————      94.6
Chicken             .R...R.....Y.....V.....V.....Q..TA.*————————      94.6
Eel                 .R...R.....Y.....V.....V.....Q..TA.*————————      93.9
D. melanogaster     .R...R.....V.....V.....T..T..*————————      95.9
Potato              LK.....Q.....V.....V.....R..LA.*————————      89.9
C. reinhardtii      LR.....V.....SE.....V.....V.....R..T.GATDDKDKKGHK*      86.6
T. cruzi            .K...R.....V.....V.....*————————      91.9
A. nidulans         .R.....R.N.....V..SI.....D.....Q....R.D.N...QL..Q.*————————      85.1
C. albicans         .A.....RN.D.K.....LL.SI....S.AD..Q..K...TNN..E.DIQ..TLLLAA.*————————      71.6
S. pombe            VR.....Y.TVE..T.VL.S...R.SQ...AD.....T....V.....SRVIS..*————————      74.5
S. cerevisiae       L.....N.D.L.....K.VL.SI.....A...D.L..VSΔ..S.E..IQQ.AALLΔ..*————————      60.8
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Fig. 5. Comparison of the predicted amino acid sequence of *Phytophthora infestans* calmodulin with the sequences of calmodulins from: *Achlya klebsiana* (LeJohn 1989), man (Fischer *et al.* 1988), chicken (Putkey *et al.* 1983), eel (Lagace *et al.* 1983), *Drosophila melanogaster* (Yamanaka *et al.* 1987), potato (Jena *et al.* 1989), *Chlamydomonas reinhardtii* (Zimmer *et al.* 1988), *Trypanosoma cruzi* (Chung and Swindle 1990), *Aspergillus nidulans* (Rasmussen *et al.* 1990), *Candida albicans* (Saporito and Sypherd 1991), *Schizosaccharomyces pombe* (Takeda and Yamamoto 1987), and *Saccharomyces cerevisiae* (Davis *et al.* 1986). Identical sequences are marked by a dot. The Δsymbol indicates a one-residue gap introduced into the sequence to give optimal alignment with the other proteins. Expected Ca^{2+}-binding residues, located in the four EF-hand Ca^{2+}-binding domains as proposed by Watterson *et al.* (1980), are indicated by a ★.

be determined. Genes encoding actin and translation elongation factor 1α (EF-1α) have been shown to be very useful constitutively expressed marker genes for assessing the expression of differentially regulated genes during plant-pathogen interactions (Mahe *et al.* 1992). In our experiments we used the *P. infestans* actin (*actA*) gene (Unkles *et al.* 1991) as well as the *P. infestans* EF-1α gene (C. Pieterse, unpublished) as constitutively expressed marker genes. The specificity of the *calA*, *actA*, and EF-1α probes for *P. infestans* was shown by hybridization of the probes to Southern blots containing genomic *P. infestans* and potato DNA. Under the high-stringency conditions used, the probes hybridized only to *P. infestans* DNA and not to potato DNA (*calA* probe: see Fig. 3; *actA* and EF-1α probe: data not shown). Also on northern blots the three probes do not hybridize to potato RNA isolated from noninoculated leaves (Fig. 6A).

The expression of the *calA* gene was studied on two different potato cultivars during growth of the fungus *in planta* and during growth of the fungus *in vitro*. Leaves of the partially resistant potato cv. Pimpernel and the moderately susceptible potato cv. Ajax were inoculated with isolate 88069 of *P. infestans*. On potato cv. Ajax, the first symptoms are visible 24 hr after inoculation. They develop from small lesions into completely "water-soaked" areas 3 days postinoculation. In this period the fungus grows and sporulates at the advancing edges of developing lesions. The centers of the lesions become necrotic and start to decay due to secondary infections by saprophytic microorganisms. Compared to potato cv. Ajax, symptom development on potato cv. Pimpernel was delayed for approximately 1 day. Infected leaves of potato cvs. Ajax and Pimpernel were harvested 1, 2, and 3, and 1, 2, 3, and 4 days postinoculation, respectively. Noninoculated control leaves were sprayed with water and similarly treated to the inoculated leaves. *In vitro* grown mycelium was harvested from nonsporulating cultures (3 day old), moderately sporulating cultures (7 day old) and heavily sporulating cultures (14 day old) which were grown on both Henniger synthetic medium and rich rye-sucrose medium.

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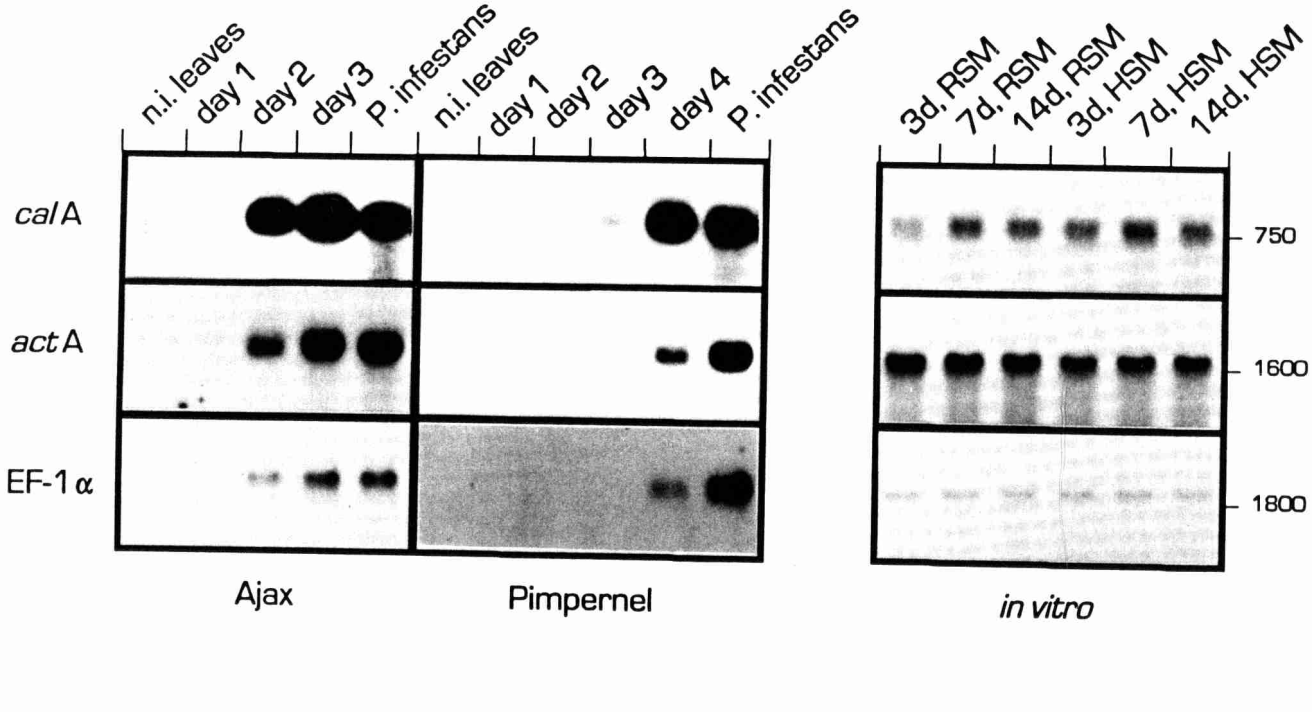

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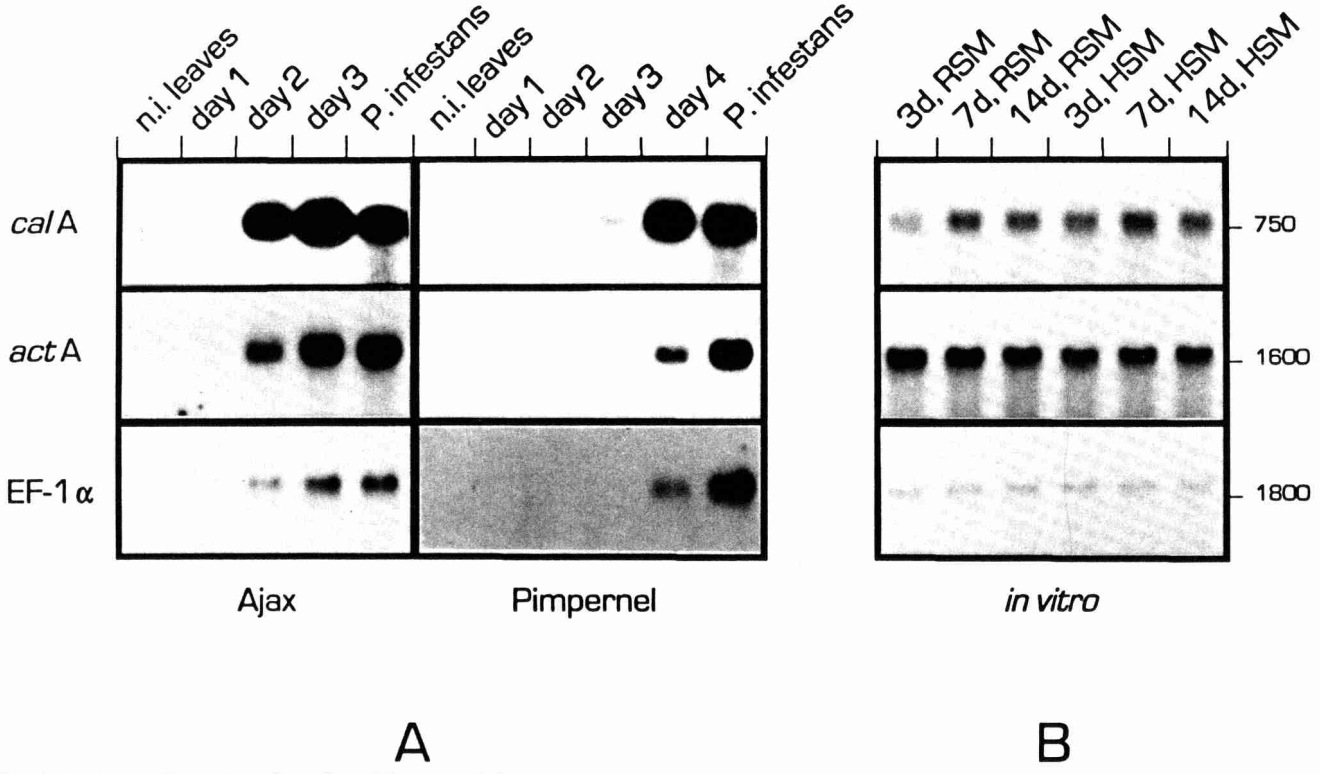

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mm petri dishes) for 3 hr at 10° C with a layer of 10 ml of water on top of the mycelium. Liquid cultures were initiated from a zoospore suspension in either Henniger synthetic medium (Henniger 1959) or rich rye-sucrose medium (1×10^5 zoospores per milliliter) and, after incubation at 18° C for 3, 7, and 14 days, mycelium was harvested. For inoculation of plants, sporangia were collected from 2-wk-old rye-agar cultures and concentrated to 5×10^5 sporangia per milliliter. Leaves were inoculated by spraying the axial side of the leaves with the sporangial suspension. After inoculation, leaves were inserted in florist foam oasis and incubated at 18° C and 100% relative humidity under cool fluorescent light (16 hr/day). The partially resistant potato cultivar Pimpernel and the moderately susceptible potato cv. Ajax were used as host plants.

Differential screening, subcloning, and sequencing.

A genomic library of *P. infestans* DNA was constructed in λEMBL3 and screened as described previously (Pieterse *et al.* 1991). For the differential screening, [α-^{32}P]dATP labeled first-strand cDNA probes with a specific activity of $1\text{–}2 \times 10^8$ cpm/μg were synthesized on 1) poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium and 2) poly(A)$^+$ RNA isolated from infected leaflets of potato cv. Ajax (3 days post-inoculation) as described by Sargent (1987). Hybridization of filters containing recombinant bacteriophage DNA of the *P. infestans* genomic library was performed at 65° C in hybridization mix containing 5× SSC (750 mM NaCl, 75 mM sodium citrate), 5× Denhardt's solution (0.1% [w/v] Ficoll, 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] BSA [fraction V]), 0.5% (w/v) sodium dodecyl sulphate (SDS), and 100 μg/ml calf thymus DNA. After hybridization, the filters were washed in 2× SSC, 0.5% (w/v) SDS at 65° C, and exposed to Kodak X-AR film.

The location of the *in planta*-induced genes on the DNA inserts present in the selected genomic λ clones was determined by differential hybridization of Southern blots containing digested DNA of the selected λ clones. A single differentially hybridizing 5.0-kb *Sst*I fragment from one of the selected λ clones, i.e. DHC-C, was subcloned in the vector pTZ19U resulting in the plasmid named pPiCM-S. Of pPiCM-S, a detailed restriction map was made and several subclones were constructed. All DNA manipulations were performed essentially according to Sambrook *et al.* (1989). To generate overlapping deletion clones, pPiCM-S was partially digested with *Sau*3AI and electrophoresed on a 0.7% (w/v) agarose gel along with linearized pPiCM-S as marker. Singly cut, linearized DNA was isolated from the gel and digested to completion with *Bam*HI. DNA fragments were then circularized by ligation and transformed to *E. coli* cells. DNA sequencing of overlapping subclones and deletion clones was performed on double-stranded DNA by the dideoxy chain termination method (Sanger *et al.* 1977) using the Multiwell Microtitre Plate Sequencing System (Amersham) and [α-^{35}S]dATP as a label. For the analysis of sequence data and screening of sequence data libraries, the Sequencing Analysis Software Package, version 6.0, of the Genetics Computer Group (GCG) of the University of Wisconsin was used (Devereux *et al.* 1984).

Isolation of RNA and poly(A)$^+$ RNA and northern blot analyses.

RNA was isolated from *P. infestans* mycelium, noninoculated potato leaves, and colonized potato leaflets (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). Poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989). For northern blot analysis, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N$^+$ membranes (Amersham) by capillary transfer as described by the manufacturer. Northern blots were hybridized at 65° C in the hybridization mix as described above.

Isolation of genomic DNA and Southern blot analyses.

Genomic DNA of *P. infestans* and potato was isolated from *in vitro* grown mycelium and from leaves of potato cv. Ajax as has been described previously (Pieterse *et al.* 1991). Genomic DNA was digested with various restriction enzymes and size-separated on a 0.7% (w/v) agarose gel. Following electrophoresis, the DNA was transferred to Hybond-N$^+$ membrane (Amersham) by capillary transfer. Hybridization of Southern blots was performed at 65° C in the hybridization mix as described above.

DNA probes.

The calmodulin probe was derived from the coding region of the *P. infestans calA* gene and consists of a 342-bp *Pvu*II/*Hin*dIII-fragment from pPiCM-S. The 2.8-kb *Pst*I insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles *et al.* 1991) was used as actin probe and the 0.35-kb *Eco*RI/*Xho*I insert from pPi119 which is a partial cDNA clone of the *P. infestans* translation elongation factor 1α (EF-1α) mRNA (C. Pieterse, unpublished), was used as EF-1α probe. DNA probes were labeled by the random primer labeling technique (Feinberg and Vogelstein 1983).

Primer extension of poly(A)$^+$ RNA.

The oligonucleotide (5′-GCTCTTCGGTCAGCTGG-3′) complementary to the sequence at position 9–25 relative to the ATG start codon of the *calA* gene was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Five nanograms of labeled primer was annealed to 3 μg of poly(A)$^+$ RNA and extended using M-MLV reverse transcriptase as described by the manufacturer (Gibco BRL). The primer extension products were analyzed by electrophoresis on a 6% (w/v) polyacrylamide gel and visualized by autoradiography.

ACKNOWLEDGMENTS

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mm petri dishes) for 3 hr at 10° C with a layer of 10 ml of water on top of the mycelium. Liquid cultures were initiated from a zoospore suspension in either Henniger synthetic medium (Henniger 1959) or rich rye-sucrose medium (1×10^5 zoospores per milliliter) and, after incubation at 18° C for 3, 7, and 14 days, mycelium was harvested. For inoculation of plants, sporangia were collected from 2-wk-old rye-agar cultures and concentrated to 5×10^5 sporangia per milliliter. Leaves were inoculated by spraying the axial side of the leaves with the sporangial suspension. After inoculation, leaves were inserted in florist foam oasis and incubated at 18° C and 100% relative humidity under cool fluorescent light (16 hr/day). The partially resistant potato cultivar Pimpernel and the moderately susceptible potato cv. Ajax were used as host plants.

Differential screening, subcloning, and sequencing.

A genomic library of *P. infestans* DNA was constructed in λEMBL3 and screened as described previously (Pieterse *et al.* 1991). For the differential screening, [α-^{32}P]dATP labeled first-strand cDNA probes with a specific activity of $1–2 \times 10^8$ cpm/μg were synthesized on 1) poly(A)$^+$ RNA isolated from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium and 2) poly(A)$^+$ RNA isolated from infected leaflets of potato cv. Ajax (3 days post-inoculation) as described by Sargent (1987). Hybridization of filters containing recombinant bacteriophage DNA of the *P. infestans* genomic library was performed at 65° C in hybridization mix containing 5× SSC (750 mM NaCl, 75 mM sodium citrate), 5× Denhardt's solution (0.1% [w/v] Ficoll, 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] BSA [fraction V]), 0.5% (w/v) sodium dodecyl sulphate (SDS), and 100 μg/ml calf thymus DNA. After hybridization, the filters were washed in 2× SSC, 0.5% (w/v) SDS at 65° C, and exposed to Kodak X-AR film.

The location of the *in planta*-induced genes on the DNA inserts present in the selected genomic λ clones was determined by differential hybridization of Southern blots containing digested DNA of the selected λ clones. A single differentially hybridizing 5.0-kb *Sst*I fragment from one of the selected λ clones, i.e. DHC-C, was subcloned in the vector pTZ19U resulting in the plasmid named pPiCM-S. Of pPiCM-S, a detailed restriction map was made and several subclones were constructed. All DNA manipulations were performed essentially according to Sambrook *et al.* (1989). To generate overlapping deletion clones, pPiCM-S was partially digested with *Sau*3AI and electrophoresed on a 0.7% (w/v) agarose gel along with linearized pPiCM-S as marker. Singly cut, linearized DNA was isolated from the gel and digested to completion with *Bam*HI. DNA fragments were then circularized by ligation and transformed to *E. coli* cells. DNA sequencing of overlapping subclones and deletion clones was performed on double-stranded DNA by the dideoxy chain termination method (Sanger *et al.* 1977) using the Multiwell Microtitre Plate Sequencing System (Amersham) and [α-^{35}S]dATP as a label. For the analysis of sequence data and screening of sequence data libraries, the Sequencing Analysis Software Package, version 6.0, of the Genetics Computer Group (GCG) of the University of Wisconsin was used (Devereux *et al.* 1984).

Isolation of RNA and poly(A)$^+$ RNA and northern blot analyses.

RNA was isolated from *P. infestans* mycelium, noninoculated potato leaves, and colonized potato leaflets (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* (1987). Poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989). For northern blot analysis, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N$^+$ membranes (Amersham) by capillary transfer as described by the manufacturer. Northern blots were hybridized at 65° C in the hybridization mix as described above.

Isolation of genomic DNA and Southern blot analyses.

Genomic DNA of *P. infestans* and potato was isolated from *in vitro* grown mycelium and from leaves of potato cv. Ajax as has been described previously (Pieterse *et al.* 1991). Genomic DNA was digested with various restriction enzymes and size-separated on a 0.7% (w/v) agarose gel. Following electrophoresis, the DNA was transferred to Hybond-N$^+$ membrane (Amersham) by capillary transfer. Hybridization of Southern blots was performed at 65° C in the hybridization mix as described above.

DNA probes.

The calmodulin probe was derived from the coding region of the *P. infestans calA* gene and consists of a 342-bp *Pvu*II/*Hin*dIII-fragment from pPiCM-S. The 2.8-kb *Pst*I insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles *et al.* 1991) was used as actin probe and the 0.35-kb *Eco*RI/*Xho*I insert from pPi119 which is a partial cDNA clone of the *P. infestans* translation elongation factor 1α (EF-1α) mRNA (C. Pieterse, unpublished), was used as EF-1α probe. DNA probes were labeled by the random primer labeling technique (Feinberg and Vogelstein 1983).

Primer extension of poly(A)$^+$ RNA.

The oligonucleotide (5′-GCTCTTCGGTCAGCTGG-3′) complementary to the sequence at position 9–25 relative to the ATG start codon of the *calA* gene was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Five nanograms of labeled primer was annealed to 3 μg of poly(A)$^+$ RNA and extended using M-MLV reverse transcriptase as described by the manufacturer (Gibco BRL). The primer extension products were analyzed by electrophoresis on a 6% (w/v) polyacrylamide gel and visualized by autoradiography.

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