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Pieter van West · Sophien Kamoun
John W. van't Klooster · Francine Govers

Ric1, a *Phytophthora infestans* gene with homology to stress-induced genes

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Abstract From a set of *Phytophthora infestans* cDNA clones that were randomly selected from a potato-*P. infestans* interaction cDNA library, a relatively high proportion (5 out of 22) appeared to be derived from the same gene. The gene was designated *ric1*. *P. infestans* contains two copies of *ric1* which share 98% homology at the nucleotide-sequence level and 100% at the amino-acid level. The nucleotide sequence predicts an open reading frame of 171 bp encoding a 57 amino-acid hydrophobic-peptide with two potential membrane-spanning domains. The predicted peptide shows high homology to a peptide encoded by plant genes whose expression is specifically induced during stress conditions. Southern-blot analysis of genomic DNA of several *Phytophthora* species indicated that most species contain *ric1* homologues. During the life cycle of *P. infestans*, *ric1* was expressed in all developmental stages but the level of expression varied. Sporangia and germinating cysts appeared to contain only very little *ric1* mRNA whereas in the mycelium and during in planta growth higher levels were detected. Subjecting the mycelium to osmotic stress or to a high pH resulted in increased *ric1* expression.

P. van West[1] · S. Kamoun[2] · J.W. van't Klooster · F. Govers (✉)
Laboratory of Phytopathology,
Wageningen Agricultural University and Graduate School of Experimental Plant Sciences,
Binnenhaven 9, 6709 PD Wageningen,
The Netherlands
e-mail: francine.govers@medew.fyto.wau.nl
Fax: +31 -317 483 412

Present addresses:
[1] Department of Molecular and Cell Biology,
University of Aberdeen, Institute of Medical Sciences,
Foresterhill, Aberdeen, AB25 2ZD, Scotland, UK
[2] Department of Plant Pathology,
The Ohio State University,
Ohio Agricultural and Development Center,
Madison Avenue, Wooster, OH 444691, USA

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Introduction

Phytophthora infestans (Mont.) de Bary is the causal agent of potato late blight, one of the most devastating diseases of potato (*Solanum tuberosum* L.). This oomycete plant pathogen is able to infect both foliage and tubers and can spread rapidly through host tissue causing destructive necrosis. Little is known about the molecular processes taking place in the *P. infestans*-potato interaction. Identification of genes expressed during this interaction may help in unravelling cellular signalling processes essential for establishing and maintaining a pathogenic interaction. Previously, we identified several so-called in planta induced (*ipi*) genes selected by differential screening of a genomic DNA library of *P. infestans*. A major advantage of this screening was that it yielded exclusively *in planta* induced genes of the pathogen and no host genes. A disadvantage was that the selected genomic clones contained large proportions of non-coding and repetitive DNA and might contain multiple genes on a single vector insert (Pieterse et al. 1993 a). In order to identify additional *P. infestans* genes that are expressed during the interaction with the host we have now screened a so-called interaction cDNA library. The basis for the construction of this cDNA library was poly(A^+) RNA isolated from *P. infestans*-infected potato leaves, 3-days post-inoculation. We selected candidate genes by screening the library with random cDNA probes representing, on the one hand, RNA isolated from non-infected potato leaves and, on the other hand, RNA isolated from the *P. infestans* mycelium. The cDNA clones hybridising to the *P. infestans* probe were considered for further analysis whereas those hybridising to the potato probe were excluded. Due to the origin of the *P. infestans* probe used for the screening, the cDNA clones represent in planta expressed, but not necessarily

in planta induced, genes. Even though the selection was performed in an unbiased manner, 5 of 22 *r*andomly *i*solated *c*DNA clones (*ric*) appeared to be derived from the same gene. Here we describe characterisation of this gene which we have designated *ric1*. The deduced amino-acid sequence shows high homology with a peptide encoded by two stress-induced genes from plants. To investigate whether *ric1* is also a stress-induced gene, *P. infestans* was exposed to stress conditions and *ric1*-expression was analysed and compared to *ric1*-expression during various developmental stages, in vitro and in planta. A possible role of the putative RIC1 protein is discussed.

Materials and methods

Phytophthora strains and culture conditions. A Dutch *P. infestans* strain 88069 was used throughout this study. This isolate was routinely grown in the dark on rye agar medium supplemented with 2% (w/v) sucrose (RS-medium) (Caten and Jinks 1968) at 18 °C. The following *Phytophthora* species and strains were analysed for the presence of *ric1* homologous sequences in their genome. *P. clandestina* (UQ 3085), *P. cactorum* (0436), *P. pseudotsugae* (CBS 444.84, PD 95/9141), *P. idaei* (PD 94/959), *P. tentaculata* (CBS 412.96), *P. parasitica* (USA 1751), *P. palmivora* (PD 93/56), *P. megakarya* (UQ 2822), *P. porri* (HH), *P. infestans* (88069), *P. mirabilis* (CBS 150.88), *P. phaseoli* (CBS 556.88), *P. ilicis* (PD 91/595), *P. megasperma* f. sp. *glycinea* (UQ 60), *P. vignae* (20853), and *P. cinnamomi* (2). Mycelium for isolation of DNA and RNA was obtained by growing the *Phytophthora* strains in liquid RS medium for 5–10 days. Plant assays to obtain infected potato leaves were performed as described by van West et al. (1998).

Selection of P. infestans cDNA clones. Construction of the λZAP cDNA library made from poly(A^+) RNA extracted from *P. infestans*-infected potato leaves (cv Ajax), 3 days after spray inoculation, has been described by Pieterse et al. (1994 b). Recombinant bacteriophages were plated and incubated according to the Stratagene λZAP manual. Of the resulting plaques two replicas were made on Hybond N^+ membranes and bacteriophage DNA was released according to the manufacturer's instructions (Amersham International plc., Little Chalfont, Buckinghamshire, UK). [α-^{32}P]dATP-labelled probes used for hybridisation were synthesised on poly(A^+) RNA isolated from in vitro grown mycelia of *P. infestans* and from poly(A^+) RNA isolated from non-infected potato leaves using the Random Primed DNA Labelling System (Gibco BRL, Gaithesburg, Md., USA). The hybridisation patterns were compared. Twenty two random phages that hybridised to the *P. infestans* probe, but not to the potato probe, were propagated and cDNA clones were obtained by in vivo excision according to the Stratagene λZAP manual.

RNA and DNA manipulations. RNA isolation and Northern-blot analyses were performed as described by van West et al. (1998). Genomic DNA of *Phytophthora* strains and potato (cv Ajax) was isolated as described by Raeder and Broda (1985). Southern-blot hybridisations were performed as described by van West et al. (1998). Heterologous hybridisations were performed at 58 °C and the blots were washed in 2 × SSC and 0.5% SDS. Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, Calif.). Sequence analysis was performed using software DNA strider 1.0 (C. Marck Institute de Recherche Fondamentale, France). Database homology searches were conducted using the BLAST software package as available through the Internet. The amino-acid sequence was characterised using the Baylor College of Medicine, Houston, Tex., internet software package.

DNA probes. DNA templates for probe synthesis were the 671-bp *Eco*RI-*Xho*I insert of the *ric1* cDNA from pPi109, and a 796-bp *Hin*dIII fragment from pSTA31 containing the coding region of the *P. infestans* actin gene, *actA* (Unkels et al. 1991). All DNA templates were gel-purified with the Qiaex II Agarose Gel Extraction kit (Qiagen GmbH., Hilden, Germany). The probes were radiolabelled with [α-^{32}P]dATP by using the Random Primed DNA Labelling System (Gibco BRL). To remove non-incorporated nucleotides, the Qiaquick Nucleotide Removal Kit (Qiagen GmbH.) was used.

Stress conditions. An agar plug with sporulating mycelium was transferred to a Petri dish containing liquid RS-medium. After 5 days of growth at 18 °C, the mycelium was subjected to various treatments for exactly 2 h. After the treatment, the mycelium was collected for extraction of total RNA. The various treatments were: (I) mycelium washed in water and subsequently starved in water; (II) mycelium washed with and incubated in RS medium of pH 4.5 or pH 9.0 (the pH of RS medium is approximately 6.75); (III) mycelium washed with and incubated in RS medium containing 100 mM, 250 mM or 400 mM of NaCl; (IV) mycelium washed with RS medium and incubated at 4 °C or 37 °C; (V) mycelium washed with and incubated in RS medium containing 60 ppm $Pb_3(C_6H_5O_7)_2$; and as controls, (VI) untreated mycelium, and (VII) mycelium washed with, and incubated in RS medium. All washing steps were performed three times.

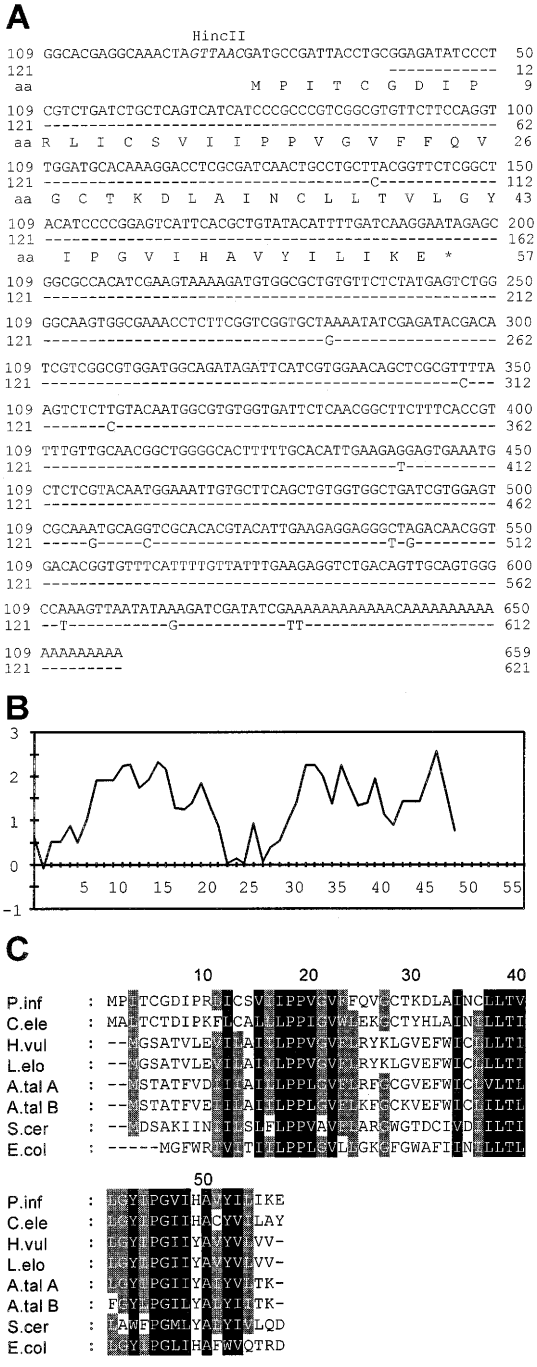
Results

Characterisation of *ric1* cDNA clones

From the set of 22 randomly selected *P. infestans* cDNA clones five (i.e. pPi101, pPi103, pPi108, pPi109 and pPi121) were strongly related as they all cross-hybridised to each other (data not shown). The nucleotide sequence of three of these was determined. Sequence analysis showed that pPi109 contains a continuous open reading frame of 171 bp (Fig. 1 A). The coding sequence starts with an ATG start codon 23-bp downstream from the 5′ end and encodes a putative protein of 57 amino acids. pPi121 is a partial cDNA clone with a 160-bp open reading frame and lacking the N-terminal part of the putative protein (Fig. 1 A). pPi101 is even smaller. At the 5′ end pPi101 is 45-bp shorter than pPi121 but the overlapping sequences of pPi101 and pPi121 are exactly identical. The nucleotide sequence of the coding region in pPi109 differs only at one position when compared with the nucleotide sequence of pPi101 and pPi121, whereas 14 mismatches are found in the 3′ non-coding regions of the cDNA clones. The gene from which the *r*andomly *i*solated *c*DNA clones were derived was designated *ric1*.

Occurrence of *ric1* in the *P. infestans* genome and in other *Phytophthora* species

The observation that DNA sequences of pPi109 and pPi121 are not 100%, but 98%, homologous suggests the existence of at least two *ric1* copies in the genome of *P. infestans*. Genomic Southern-blot analysis (Fig. 2 A) revealed the presence of two genomic *Hin*cII fragments



(1.0 kb and 2.3 kb) hybridising to the *ric1* probe, two *Bam*HI fragments (8.5 kb and 11 kb), and two *Pst*I fragments (0.7 kb and 0.75 kb). In the *Eco*RI digest and the *Hin*dIII digest only one hybridising fragment was found (10 and 4.0 kb, respectively). Since there is a *Hin*cII restriction site in the *ric1* cDNA clones, two hybridising fragments can be expected even when *ric1* is a single-copy gene. However, since there are no *Bam*HI and *Pst*I restriction sites present in pPi109, the two hybridising *Bam*HI and *Pst*I fragments support the suggestion that *P. infestans* has two copies of the *ric1* gene. These two copies either are contained within a 4.0-kb

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Fig. 1 A Nucleotide sequence of the *ric1* cDNA clones pPi109 (*109*) and pPi121 (*121*) from *P. infestans* isolate 88069 (GenBank accession No. AJ133023), and the deduced amino-acid sequence of RIC1 (*aa*). The *Hin*cII restriction site is indicated. *Numbers* on the right indicate either nucleotide length or amino-acid length. **B** hydropathy analysis (Kyte and Doolittle 1982) of the RIC1 peptide sequence. **C** alignment of the *P. infestans* RIC1 peptide sequence (*P.inf*) with its homologues CEESP35F from *C. elegans* (*C.ele*) (GenBank accession No. U39649), *blt101* from *H. vulgare* (*H.vul*) (GenBank accession No. Z25537), *ESI3* from *L. elongatum* (*L.elo*) (GenBank accession No. U00966), *RCI2A* and *RCI2B* from *A. thaliana* (*A.tal A* and *A.tal B*) (GenBank accession numbers AAD17302 and AAD17303), a putative protein from *E. coli* (*E.col*) (GenBank accession No. AE000351) and Sc YDR276c from yeast (*S.cer*) (GenBank accession No. U51030). *Black boxes* represent similar or identical amino acids in all peptide sequences. *Grey boxes* represent identical or similar amino acids in 80% of the peptide sequences

*Hin*dIII fragment and a 10-kb *Eco*R1 fragment, or else correspond to two alleles of the *ric1* gene. The *ric1* probe does not hybridise to *Eco*RI-digested genomic DNA isolated from potato (cv Ajax) (Fig. 2 A), thus confirming that the cDNA clones selected from the interaction cDNA library are derived from *P. infestans* and not from potato.

To determine whether other *Phytophthora* species have *ric1* homologues we analysed the genomic DNA of various *Phytophthora* species by Southern-blot hybridisation. Representatives of most taxonomic groups within the genus *Phytophthora* were included in the analysis. As shown in Fig. 2 B most *Phytophthora* species contain one or more genomic fragments that hybridise strongly to *ric1*. The only exception is *Phytophthora porri* where no hybridising fragment is detected, even though this lane contains sufficient and adequately digested *P. porri* DNA (data not shown). These results demonstrate that most *Phytophthora* species contain a *ric1* homologue. Whether or not these homologues are actively transcribed genes remains to be determined.

The RIC1 peptide

The protein encoded by *ric1* consists of only 57 amino acids. The calculated molecular mass of this peptide is 6137 Da and the pI is 6.51. Hydropathy analysis (Kyte and Doolittle 1982) suggests that RIC1 is highly hydrophobic and lacks a signal sequence (Fig. 1 B). It contains two potential membrane-spanning domains at amino-acid positions 11–27 and 33–51, and four cysteine residues, which could be involved in the formation of disulphide bridges.

Comparison of the RIC1 amino-acid sequence to known sequences available in databases revealed significant similarity with stress-induced proteins from plants (Fig. 1 C). The two identical putative peptides encoded by a cold-stress-induced gene from *Hordeum vulgare*, *blt101* (Goddard et al. 1993), and a salt-stress-induced gene from *Lophopyrum elongatum*, *ESI3* (Gulick et al.

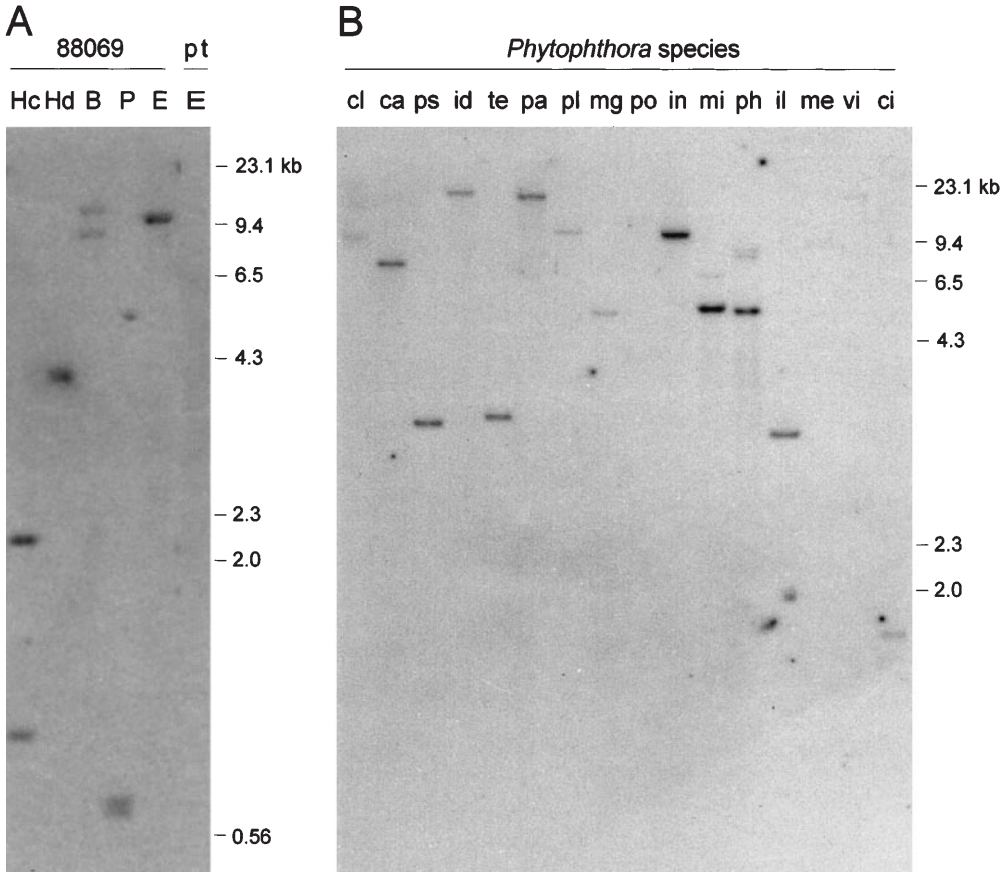

Fig. 2 A Occurrence of *ric1* sequences in the *P. infestans* genome. The Southern blot contains genomic potato DNA of cv Ajax (*pt*) (15 µg) digested with *Eco*RI (*E*) and genomic *P. infestans* DNA of strain 88069 (15 µg/lane) digested with *Hin*cII (*Hc*), *Hin*dIII (*Hd*), *Bam*HI (*B*), *Pst*I (*P*) and *Eco*RI (*E*) that was hybridised with the *ric1* cDNA probe. Molecular-size markers are indicated in kb. **B** occurrence of *ric1* homologues in the genus *Phytophthora*. Southern-blot analysis of *Eco*RI-digested genomic DNA isolated from *P. clandestina* (*cl*), *P. cactorum* (*ca*), *P. pseudotsugae* (*ps*), *P. idaei* (*id*), *P. tentaculata* (*te*), *P. parasitica* (*pa*), *P. palmivora* (*pl*), *P. megakarya* (*mg*), *P. porri* (*po*), *P. infestans* (*in*), *P. mirabilis* (*mi*), *P. phaseoli* (*ph*), *P. ilicis* (*il*), *P. megasperma* f. sp. *glycinea* (*me*), *P. vignae* (*vi*), and *P. cinnamomi* (*ci*). The DNA was hybridised with the *ric1* probe. Molecular-size markers are indicated in kilobases (kb)

1994), share 22 out of the 54 amino acids with RIC1, and an additional 13 amino-acid residues are similar. Also two peptides encoded by cold-induced genes from *Arabidopsis thaliana*, RCI2A and RCI2B (Capel et al. 1997), share high similarity (35 and 34 out of 54 amino acids, respectively). However, the highest homology was found with an Expressed Sequence Tag from the nematode *Caenorhabditis elegans*. Thirty one amino acids out of 57 are identical and 14 are similar. Also yeast and *Escherichia coli* appear to have genes encoding proteins homologous to RIC1. Interestingly, these homologous proteins all have similar sizes, ranging from 51 to 57 amino acids, and the highest homology is found in the putative membrane-spanning domains.

High identity was also found in the putative membrane-spanning domains of another class of proteins. However, these putative proteins are much larger. The amino-acid sequences range from 79 residues in the case of a protein from a cyanobacterium, *Synechocystis* sp. (Genbank accession No. D64005), to 133 residues of a yeast homologue (Genbank accession No. P14359) (data not shown).

Expression of *ric1*

Detailed expression studies were performed to determine in which stages of the life cycle of *P. infestans* the *ric1* gene is active. Northern blots containing total mRNA isolated from in vitro grown material such as zoospores, cysts, germinated cysts, sporangia, young mycelia and old mycelia, and from *P. infestans* spot-inoculated potato leaves were prepared as described previously (van West et al. 1998) (Fig. 3 A). Hybridisation of the RNA with the *ric1* probe showed that *ric1* is expressed at all stages. However, in sporangia and germinating cysts little *ric1* mRNA was detected when compared to the other stages. The highest level was observed in in vitro-grown old mycelium. Hybridisation with a probe derived from the constitutive expressed actin gene *actA* (Unkles et al. 1991) was performed to check whether equal amounts of RNA were loaded on the gel.

Expression analysis of *ric1* during in planta growth of *P. infestans* on detached potato leaves showed that *ric1* mRNA is first detectable 3 days after inoculation (Fig. 3 A). When comparing this to the steady increase in the relative amount of actin mRNA in the total RNA samples over time, a continuous increase in the relative amount of *ric1* mRNA is observed. The relative amount of actin mRNA is considered as a measure of the *P. infestans* biomass in the infected leaf. Since *ric1* mRNA follows the pattern of actin mRNA, we conclude that *ric1* gene expression is not specifically up- or down-regulated during in planta growth.

To determine whether *ric1* gene expression is specifically induced during stress conditions, as has been

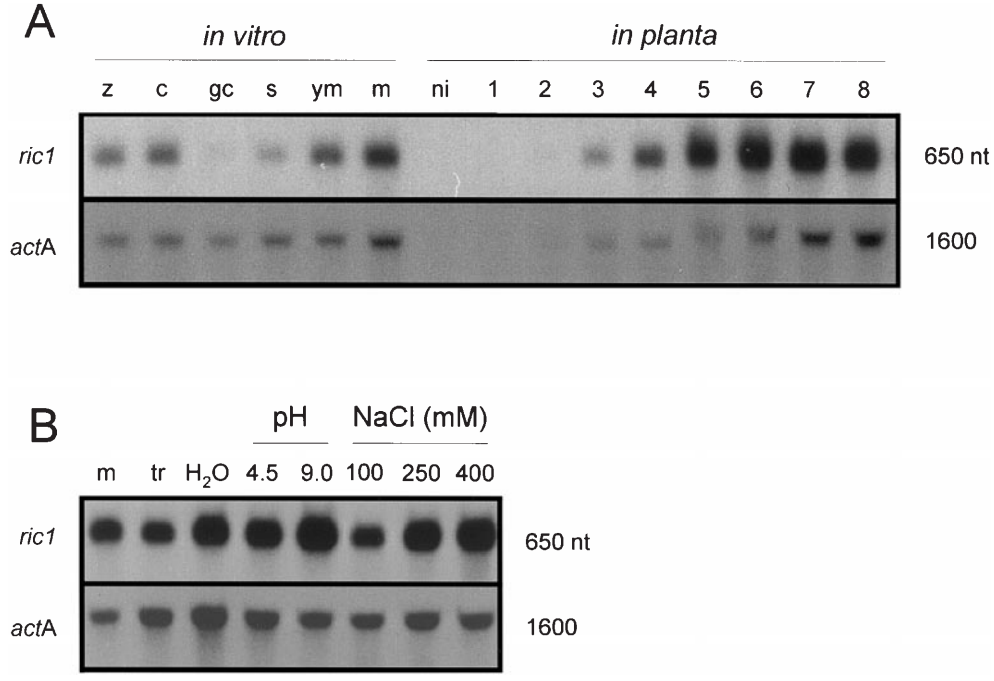

Fig. 3 A Expression of *ric1* during growth and differentiation of *P. infestans* in vitro and in planta. Autoradiograph of a Northern blot containing total RNA (10 µg) isolated from *P. infestans* 88069 zoospores (*z*), cysts (*c*), cysts germinated in water for 2.5 h (*gc*), sporangia (*s*), young mycelium (*ym*), mycelium (*m*), from potato leaves (cv Bintje) 1–8 days after spot inoculation with *P. infestans* 88069 (*1–8*) and from non-inoculated potato leaves (*ni*), and hybridised with *ric1* and *actA* probes. Transcript lengths in nucleotides (*nt*) are indicated on the right. **B** expression of *ric1* in in vitro grown mycelium of *P. infestans* during various treatments. Autoradiograph of a Northern blot containing total RNA (10 µg) isolated from mycelium that was subjected to various stress treatments and hybridised with *ric1* and *actA* probes. The various treatments were: transfer of medium (*tr*), starvation in water (H_2O); acidic conditions, pH 4.5 (*4.5*); and basic conditions, pH 9 (*9.0*); salt 100 mM NaCl (*100*), 250 mM NaCl (*250*) and 400 mM NaCl (*400*); non-treated mycelium (*m*). Transcript lengths in nucleotides (*nt*) are indicated on the right

described for its plant homologues *blt101* and *ESI3* (Goddard et al. 1993; Gulick et al. 1994), we subjected *P. infestans* cultures to various treatments that might be experienced as stress conditions. The treatments were applied for 2 h and, subsequently, total RNA was isolated and the presence of *ric1* mRNA analysed by Northern-blot hybridisations (Fig. 3 B). Physical force, like washing of a mycelial mat and transfer to a new Petri dish containing fresh medium, did not result in an increase of *ric1* mRNA levels. Also, transfer to water (starvation), or to medium containing heavy metal ions (Pb^{2+}), did not seem to effect *ric1* gene expression (data not shown); nor did cold treatment (4 °C) or heat shock (37 °C) (data not shown). An approximately two-fold increase in *ric1* mRNA levels (based on densitometric scanning of the signals) was noted in mycelia transferred to basic medium (pH 9), whereas hardly any increase could be observed after transfer to acidic medium (pH 4.5). An increase in *ric1* mRNA levels was also noted in mycelia exposed to high salt concentrations. In medium with a sodium-chloride concentration of 400 mM, a 60% increase was observed within only 2 h. These results demonstrate that *ric1* gene expression is up-regulated when a *P. infestans* mycelium encounters stress conditions induced by high pH or salt.

Discussion

In this study we describe the isolation and characterisation of the *P. infestans ric1* gene. Based on the presented cDNA sequences and the detailed genomic Southern-blot studies, we assume that *P. infestans* has two copies of *ric1*, which are both expressed. The 171-bp open reading frame encodes a small protein of 57 amino acids with a predicted molecular mass of 6137 Da. The predicted RIC1 protein is a hydrophobic peptide with two putative membrane-spanning domains. This suggests that RIC1 is a membrane component.

Previously we reported the cloning of so-called in planta induced (*ipi*) genes, using a differential screening procedure of a genomic library of *P. infestans* (Pieterse et al. 1993 a). Two of these *ipi* genes, *ubi3R* and *calA*, encode polyubiquitin and calmodulin, respectively (Pieterse et al. 1991, 1993 b). Both *ubi3R* and *calA* are expressed during in vitro growth of mycelia, but expression increases significantly during the interaction with host plants when *P. infestans* grows in planta. One other *ipi* gene, *ipi*O, is not expressed in vitro in mycelium but is highly expressed in planta during the (pre-)infection stages and in the tip of invading hyphae during colonisation of the leaf

(Pieterse et al. 1994 a; van West et al. 1998). The selection procedure that was followed for the isolation of the *ipi* genes was specifically designed to identify *P. infestans* genes whose expression is considerably induced during growth on the host. Here, however, we employed an unbiased random selection procedure of expressed genes. Since the cDNA library was derived from infected potato leaves, the genes represent in planta expressed genes. Twenty two cDNA clones were analysed of which five seemed to originate from the same gene, *ric1*. Expression studies showed that in vitro, *ric1* is predominantly expressed in mycelial stages of the life cycle of *P. infestans*. Low expression was observed in sporangia and germinating cysts. The *ric1* expression increased in young mycelium and reached its highest level in in vitro grown old mycelium. In planta expression studies demonstrated that *ric1* gene expression is not specifically increased during growth of *P. infestans* in potato leaves. Hence *ric1* is not an in planta induced gene.

Based on cross-hybridisation studies we conclude that most *Phytophthora* species contain *ric1* homologues. However, *ric1* is conserved not only within the genus *Phytophthora*. Other plants, *C. elegans*, *Saccharomyces cerevisiae* and some prokaryotes contain highly similar homologues. Based on the amino-acid alignments, we conclude that RIC1 is closely related to a putative peptide present in plants. Four genes from three different plants species, *blt101*, *ESI3*, *RCI2A* and *RCI2B*, encode similar peptides and expression of these genes is induced upon stress (Goddard et al. 1993; Gulick et al. 1994; Capel et al. 1997). Expression of *ric1* is also induced upon stress. The basal level of *ric1* expression in mycelial growth stages throughout the life cycle of *P. infestans* increases considerably when the mycelium is exposed to high salt or high pH. Whether *P. infestans* encounters these conditions during growth in planta is unknown. However, it is likely that pH changes and osmotic changes occur during colonisation of the leaf due to defence responses or to leakage of degrading leaf cells in the *inter* and *extra* cellular spaces. We hypothesise that RIC1 is a structural protein that is necessary to maintain membrane integrity, especially during unfavourable conditions. Now that targeted gene-silencing in *P. infestans* is feasible (van West et al. 1999), future studies with *ric1*-silenced transformants may elucidate the function of the putative RIC1 peptide.

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