Isolation of putative pathogenicity genes of the potato late blight fungus *Phytophthora infestans* by differential hybridization of a genomic library

C. M. J. Pieterse†, M. B. R. Riach‡, T. Bleker, G. C. M. van den Berg-Velthuis and F. Govers*

Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

(*Accepted for publication June 1993*)

Plant pathogens produce pathogenicity factors which enable them to parasitize and colonize their host. A strategy for identifying pathogenicity factors involves the isolation and characterization of genes encoding these factors. Potential pathogenicity genes are genes whose expression is induced during pathogenesis. In order to isolate such genes of the late blight fungus *Phytophthora infestans*, a genomic library of *P. infestans* DNA was differentially hybridized using labelled first strand cDNA probes synthesized on mRNA isolated from *P. infestans* infected potato leaves and on mRNA isolated from the fungus grown on a basic medium in culture. In total, nine distinct *in planta* induced (*ipi*) genes were selected. Expression studies revealed that the mRNA levels of seven of these genes, *ipi*A, *ipi*C, *ipi*D, *ipi*J1, *ipi*J2, *ipi*N and *ipi*Q, increased 5–10 fold during colonization. The two other genes, *ipi*B and *ipi*O, showed a transient expression pattern with the highest mRNA levels in the early stages of infection.

INTRODUCTION

The fungus *Phytophthora infestans* (Mont.) de Bary is the causal agent of late blight of potato (*Solanum tuberosum* L.). It is a hemibiotrophic pathogen which infects leaves and tubers of susceptible cultivars, causing rapidly spreading lesions which then become necrotic resulting eventually in complete destruction of infected tissue. Although not confined to potato, the host range of *P. infestans* is rather narrow since all its hosts belong to the *Solanaceae* with potato and tomato (*Lycopersicon esculentum* Mill.) being the most important economically.

Pathogenesis results from a series of complex interactions between host and pathogen. The few potential pathogenicity factors which have been studied in the potato–*P. infestans* interaction are enzymes that degrade cell walls or other structures,

*To whom correspondence should be addressed.

†Present address: Department of Plant Ecology and Evolutionary Biology, University of Utrecht, P.O. Box 800.84, 3508 TB Utrecht, The Netherlands.

‡Present address: Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK.

Abbreviations used in text: DHC, differentially hybridizing clone; *ipi* genes, *in planta* induced genes; *pig*, *P. infestans* gene.

0885–5765/93/070069+11 $08.00/0 © 1993 Academic Press Limited

and probably facilitate pathogen entry and growth through the tissue [*1*, *7*]. However, it is still not known to what extent these factors contribute to disease development. Moreover, the specificity of *P. infestans* for solanaceous hosts is unlikely to be explained by the action of general cell wall degrading enzymes since specialization implies sophisticated mechanisms for recognition involving the exchange of signals between host and pathogen. In both potato and tomato, pathogen attack has been shown to be accompanied by the induction of expression of a particular set of genes whose products are thought to be involved in defence [*5*, *8*, *11*, *18*]. Conversely, genes in the pathogen may be activated during growth in the host. The products of these *in planta* induced genes may be necessary for the establishment of the parasite or for the development of disease. Identification of genes of a plant pathogen which are induced in the host may therefore lead to the identification of essential pathogenicity factors.

Differential hybridization can be a useful tool for the identification of genes whose expression is specifically induced or increased during pathogenesis. Differential screening of cDNA libraries has been frequently and successfully utilized to isolate developmentally regulated genes, tissue specific genes or genes which are differentially expressed under distinct physiological conditions [*17*, *19*, *20*]. Differential hybridization of a cDNA library of *P. infestans* infected potato tissue will result in the selection of host as well as pathogen-derived cDNAs which cannot readily be distinguished. Therefore our approach the isolation of putative pathogenicity genes is based on differential screening of a genomic DNA library of *P. infestans*. In this way only genes of fungal origin are selected. Here we describe this non-biased strategy for the isolation of putative pathogenicity genes. Using this strategy, several *in planta* induced *P. infestans* genes were isolated and their expression during growth of the fungus in the host was studied. Characterization of the corresponding gene products and their function during pathogenesis will increase the understanding of the molecular and cellular processes involved in the interaction between *P. infestans* and its host plant.

MATERIALS AND METHODS

Plant and fungal material

P. infestans strain 88069 (A1 mating type, race 1.3.4.7) which was used throughout this study, was maintained on rye-agar medium containing 2% (w/v) sucrose [*2*]. Zoospores were obtained from 2-week-old cultures on 9·4 cm Petri dishes by adding 10 ml of water to the culture and incubating at 10 °C. After a 3 h incubation, typically 10^6 zoospores ml^{-1} were released into the water which was collected and used as inoculum to initiate liquid cultures. To obtain large amounts of mycelium, zoospores were inoculated into rye-sucrose medium or Henniger's synthetic medium [*6*] to give a final concentration of 1×10^5 zoospores ml^{-1} of culture medium. After incubation at 18 °C for 3 days, the mycelium was harvested, frozen in liquid nitrogen and stored at −80 °C until RNA or DNA extractions were performed.

Potato cv. Ajax which contains the R3 gene for late blight resistance but is susceptible to *P. infestans* strain 88069 was used as the host plant. Leaves were inoculated with sporangia collected from 2-week-old rye-agar cultures. The leaves were first inserted into florist's foam oasis and then inoculated by spraying the upper surfaces

with a sporangial suspension (5×10^5 sporangia ml^{-1}) or by spotting 10 μl of the sporangial suspension on the upper surfaces of the leaves. Leaves were incubated at 18 °C and 100% RH under cool fluorescent light (16 h day^{-1}). As a control, leaves sprayed with water were treated similarly to the inoculated leaves.

Differential screening of the P. infestans *genomic library*

A genomic library of *P. infestans* DNA was constructed in λEMBL3 as described previously [*13*]. For differential screening of the genomic library, 8×10^4 individual recombinant bacteriophages were plated with *Escherichia coli* strain MB406 and incubated as described by Sambrook *et al.* [*16*]. Of the resulting plaques, four replicas were made on Hybond-N$^+$ membranes (Amersham) according to the manufacturers instructions. [α-^{32}P]dATP labelled first strand cDNA probes, with a specific activity of 1–2 × 10^8 ct min^{-1} μg^{-1}, were synthesized as described by Sargent [*17*] using M-MLV reverse transcriptase (Gibco-BRL) and oligo(dT) to prime the synthesis reaction. Three templates for cDNA synthesis were used; (i) 1 μg poly(A)$^+$ RNA isolated from 3-day-old mycelium grown on Henniger's synthetic medium (fungal cDNA probe), (ii) 1 μg poly(A)$^+$ RNA isolated from a zone of 1 cm in width at the outer edge of lesions which had developed around the infection site, 3 days after spot-inoculation (interaction cDNA probe), and (iii) 1 μg poly(A)$^+$ RNA isolated from non-infected potato leaves (plant cDNA probe). Duplicate membranes were hybridized with equal amounts (10^7 ct min^{-1}) of the interaction and fungal cDNA probe in a 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) bovine serum albumin (fraction V)), 0·5% (w/v) SDS and 100 μg ml^{-1} calf thymus DNA under high stringency conditions at 65 °C for 16 h. The filters were washed in 2 × SSC, 0·5% SDS at 65 °C and exposed to Kodak X-OMAT S film for 2–7 days using an intensifying screen. As a control, the interaction cDNA probe was removed from the filters which were then rehybridized with the plant cDNA probe (10^7 ct min^{-1}).

Putative positive bacteriophages were selected, plated and screened by a second round of differential hybridization as described above. DNA from the selected phages was isolated using Qiagen columns (Diagen, Düsseldorf, Germany) according to the manufacturers instructions and singly and doubly digested with *Sal*I and *Sst*I. Following electrophoresis on a 0·8% (w/v) agarose gel, the DNA was blotted onto Hybond-N$^+$ membranes by capillary transfer and hybridized with the interaction cDNA probe. Hybridizing DNA fragments were identified and after digestion with the appropriate restriction enzyme(s) and electrophoresis, these fragments were isolated from a 0·8% (w/v) agarose gel. The fragments were labelled by random primer labelling [*4*] and subsequently used as probes for northern blot hybridizations.

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

RNA was isolated from non-inoculated potato leaves, leaves inoculated with *P. infestans* (interaction RNA) and *P. infestans* mycelium grown *in vitro* using the guanidine hydrochloride RNA extraction method as described by Logemann *et al.* [*9*]. On the inoculated leaves, symptoms developed in time from small lesions, 24 h after inoculation, into completely colonized, water soaked leaves, 3 days after inoculation. Poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose as described by

Sambrook *et al.* [16]. For northern blot analyses, 15 μg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N$^+$ membranes by capillary transfer as described in the manufacturers instructions. Hybridization of northern blots was performed at 65 °C in the hybridization mix described above.

RESULTS

Differential screening and selection of differentially hybridizing clones

The *P. infestans* genomic library was differentially screened to select genomic clones containing genes whose expression is induced or significantly increased during pathogenesis. The interaction cDNA probe was synthesized on mRNA which was isolated from infected potato leaves. The tissue from which interaction mRNA was isolated should have contained a substantial amount of fungal biomass, otherwise the proportion of labelled cDNA that hybridized to phage DNA of the *P. infestans* genomic library would be too low to be detected. In a very early stage of the interaction, the proportion of fungal biomass will be very low and so we used a zone of 1 cm in width from the outer edges of 3-day-old developing lesions for the isolation of mRNA and subsequent synthesis of the labelled interaction cDNA probe. Microscopical observations indicated that in this zone the fungal biomass was substantial. In addition, the interaction was in the biotrophic stage so that conditions for fungal growth and development were optimal and host cells were still alive. The other cDNA probe for the differential screening, the fungal cDNA probe, was derived from mRNA which was isolated from 3-day-old *P. infestans* mycelium which was grown on the completely synthetic Henniger medium since media based on plant extracts could contain compounds which might influence the expression of pathogenicity genes.

The haploid genome size of *P. infestans* is approximately $2{\cdot}7 \times 10^8$ bp as calculated from microfluorometric determinations of nuclear DNA content [21] and so in order to attain a 99% probability of having any DNA sequence represented in the library, membranes containing DNA of 8×10^4 recombinant bacteriophages with an average insert length of 17 kb were hybridized with both the interaction and fungal cDNA probe. The screening resulted in the selection of 20 recombinant bacteriophages which hybridized relatively strongly to the interaction cDNA probe and relatively weakly or not at all to the fungal cDNA probes. To check whether these differentially hybridizing clones (DHCs) contain sequences which are highly homologous to plant cDNAs present only in the interaction cDNA probe and not in the fungal cDNA probe, the interaction cDNA probe was removed and the filters were rehybridized with the plant cDNA probe. Under the stringent hybridization conditions used, none of the 20 selected DHCs hybridized with the plant cDNA probe. In fact, only a very limited number of recombinant bacteriophages hybridized to the plant cDNA probe ($< 0{\cdot}1\%$) indicating that predominantly fungal derived cDNAs in the interaction cDNA probe hybridized to the *P. infestans* genomic library. In order to screen for false positives, fixed amounts of DNA of the 20 selected DHCs were dot blotted onto membranes and hybridized with the interaction and fungal cDNA probes respectively. Half the DHCs, i.e. DHC-A (previously named DHC-1), DHC-B, DHC-C, DHC-D, DHC-J, DHC-K, DHC-L, DHC-N, DHC-O and DHC-Q, still hybridized differentially with the cDNA probes (data not shown). These genomic clones were analysed further.

Localization of putative in planta *induced* P. infestans *genes on the DHCs and their expression during pathogenesis*

In order to determine the location of the coding regions of the *in planta* induced genes on the DHCs, DNA of these clones was singly and doubly digested with *Sal*I and *Sst*I. Subsequently, Southern blots containing the digested DHC fragments were hybridized with the interaction cDNA probe. In Table 1, the DHCs and the lengths of the hybridizing restriction fragments are listed. From each DHC, only one or at the most two restriction fragments hybridized indicating that the genes which gave rise to differential hybridization, the putative *in planta* induced genes, are located on these fragments. To confirm that these fragments contain genes whose expression is induced or significantly increased during pathogenesis, the fragments indicated in Table 1 were used as probes in northern blot analyses. On the northern blots, labelled DNA fragments from all 10 DHCs hybridized to mRNA transcripts (Fig. 1) indicating that transcriptionally active genes are located on these DNA fragments.

TABLE 1

Differentially hybridizing clones (DHCs) selected from a P. infestans *genomic library by differential screening and the length of internal restriction fragments which hybridize to the interaction cDNA probe*

	Hybridizing restriction fragments		
	Single digests		Double digest
DHCs	*Sal*I (kb)	*Sst*I (kb)	*Sal*I/*Sst*I (kb)
DHC-A	ND	3·2*	ND
DHC-B	10·2	1·8†/1·7†	1·8/1·7
DHC-C	4·9/2·1	5·0†	3·4/1·6
DHC-D	2·7	5·2†	1·1
DHC-J	8·0/5·1	1·7†/1·7†	1·4/1·2
DHC-K	9·0	2·7†	2·7
DHC-L	7·0†	19·0	7·0
DHC-N	ND	4·4*‡	ND
DHC-O	ND	4·5†	ND
DHC-Q	5·5	17·0	5·5†

ND = not determined.

*Gene specific probe derived from this fragment used as probe for northern blot analysis (see Figure 1).

†Fragment used as probe for northern blot analysis.

‡The 4·4 kb *Sst*I-fragment contains two coding regions, one for the *ipi*N gene, the other for the EF-1α gene. The latter was identified when parts of the 4·4 kb *Sst*I-fragment were sequenced and compared to sequence data libraries.

The northern blots contained equal amounts of total RNA whether from non-infected leaves, from infected leaves 1, 2 and 3 days after spray-inoculation or from 3-day-old mycelium grown on Henniger's synthetic medium. RNA extracted from infected leaves (interaction RNA) consists of a mixture of fungal and plant RNA. During colonization of the leaves, the fungal biomass increased and in consequence the ratio of fungal to plant RNA increased. When studying the induction of gene expression

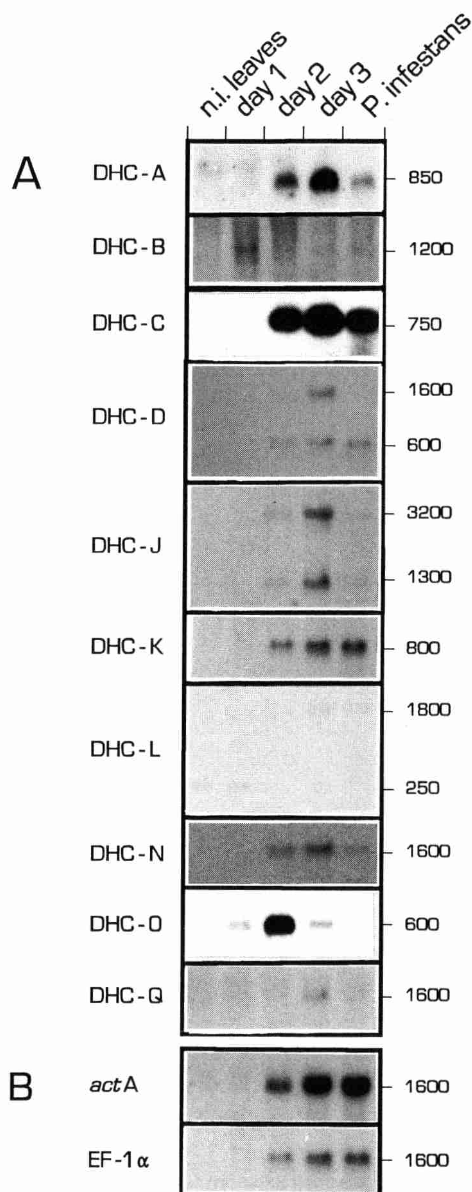

FIG. 1. Autoradiographs of northern blots containing total RNA isolated from non-inoculated potato leaves, from *P. infestans* infected at different times after inoculation, and from *P. infestans* mycelium grown *in vitro*. n.i. leaves, RNA isolated from control, non-inoculated leaves; day 1, 2 and 3, RNA isolated from infected leaves 1, 2, and 3 days after inoculation; *P. infestans*, RNA isolated from 3-day-old mycelium grown on Henniger's synthetic medium. Northern blots were hybridized with the following probes: (A) DHC-A: *Sst*I/*Nco*I-insert from pPIN1 (CMJP, unpublished) containing the first 754 bp of a gene specific 5′ non-coding region of the *ubi*3R gene [*13*]; DHC-B: 1·8 kb *Sst*I-fragment from DHC-B (hybridization with the 1·7 kb *Sst*I-fragment from DHC-B gave an identical pattern); DHC-C, DHC-D, DHC-J, DHC-K, DHC-L, DHC-O, DHC-Q: restriction fragments indicated in Table 1; DHC-N: 1·1 kb *Pst*I-fragment located within the 4·4 kb *Sst*I-fragment from DHC-N at a distance of 0·75 kb downstream from the stop codon

in plant-fungus interactions by means of northern blot analysis, the mRNA level of a constitutively expressed fungal gene can be used as an internal standard since the increase in signal obtained with a probe of such a marker gene should reflect the relative increase in the proportion of fungal RNA in the interaction RNA mixture. Genes encoding actin and the translation elongation factor 1α (EF-1α) have been shown to be useful marker genes [*10*]. In *P. infestans* the actin gene *act*A (isolated by Unkles *et al.* [*21*]) as well as the EF-1α gene (isolated by CMJP, unpublished) are constitutively expressed during colonization of potato leaves. Furthermore, the expression of the two genes remains at the same level in mycelium both during non-sporulating and sporulating growth of *P. infestans* on Henniger's synthetic medium and rye-sucrose medium, [*15*]. The *act*A gene has also been shown to be constitutively expressed in sporangia, zoospores, and germinating cysts of *P. infestans* (S. E. Unkles, personal communication). In our experiments we used the *act*A and the EF-1α mRNA levels as internal markers to determine the proportion of fungal RNA in the interaction RNA mixture. The specificity of the *act*A and EF-1α probes was analysed on Southern blots containing potato and *P. infestans* DNA and on northern blots containing RNA isolated from healthy potato leaves and from *P. infestans* grown *in vitro*. Under the hybridization conditions used, the *act*A as well as the EF-1α probe hybridized only to *P. infestans* derived nucleic acids and not to potato RNA or DNA (Fig. 1; Southern blot data not shown).

In order to determine whether the transcripts visualized by northern blot hybridization (Fig. 1) were derived from genes induced *in planta*, the relative amounts of the various mRNA transcripts during pathogenesis were quantified and compared to the mRNA levels detectable during growth of the fungus *in vitro*. To do this, autoradiographs of the northern blots were subjected to densitometric scanning using the Cybertech Image Processing Software, version 1.20 (Cybertech, Berlin). Quantification of the hybridization signals obtained with probes of the marker genes *act*A and EF-1α showed that the proportion of fungal RNA in the interaction RNA mixture isolated from infected leaves increased from 5% 1 day after inoculation to 70% by 3 days after inoculation. In order to determine the expression levels of the putative *in planta* induced genes during pathogenesis, the mRNA levels were normalized to actual fungal RNA levels and the real increase in mRNA levels during growth of the fungus *in planta* was calculated (Table 2).

The densitometric scanning of autoradiographs of the northern blots showed that eight of the ten selected DHCs (DHC-A, DHC-B, DHC-C, DHC-D, DHC-J, DHC-N, DHC-O and DHC-Q) contain one or two genes of which the mRNA levels are significantly higher during pathogenesis compared to the mRNA levels during growth of the fungus *in vitro*. DHC-A, DHC-B, DHC-C, DHC-D, DHC-N, DHC-O and DHC-Q each contain one *in planta* induced gene and these genes were designated *ipi*A, *ipi*B, *ipi*C, *ipi*D, *ipi*N, *ipi*O and *ipi*Q, respectively. From DHC-B, two non-overlapping *Sst*I-fragments of 1·7 and 1·8 kb in length were used as probes. Both fragments hybridized to mRNA transcripts of 1200 nt with similar hybridization patterns

of the EF-1α gene. (B) *act*A: 2·8 kb *Pst*I-insert from pSTA31 containing the complete *P. infestans* *act*A gene [*22*]; EF-1α: 0·35 kb *Eco*RI/*Xho*I-insert from pPi119 which is a partial cDNA clone of the *P. infestans* EF-1α mRNA (CMJP, unpublished). Transcript lengths are indicated in nt. The figure shown in panel DHC-A has been published previously by Pieterse *et al.* [*13*].

TABLE 2

mRNA levels of P. infestans ipi *and* pig *genes produced during colonization of potato leaves normalized on the mRNA levels produced during growth* in vitro

Gene	Location	Transcript length (nt)	Normalized levels*				
			n.i.†	day 1†	day 2†	day 3†	*P. infestans*†
*ipi*A (*ubi*3R)	DHC-A	850	—	—	10	10	1
*ipi*B	DHC-B	1200	—	> 100	1	1	1
*ipi*C (*cal*A)	DHC-C	750	—	—	5	4	1
*ipi*D	DHC-D	1600	—	—	10	10	1
*pig*D	DHC-D	600	—	—	1	1	1
*ipi*J1	DHC-J	3200	—	—	10	8	1
*ipi*J2	DHC-J	1300	—	—	9	9	1
*pig*K	DHC-K	800	—	—	1	1	1
*pig*L	DHC-L	1800	—	—	—	1	1
		250	#	#	—	—	—
*ipi*N	DHC-N	1600	—	—	5	5	1
*ipi*O	DHC-O	600	—	35‡	35‡	1‡	—
*ipi*Q	DHC-Q	1600	—	—	5	9	1

*mRNA levels were measured by densitometric scanning of autoradiographs and normalized to actual fungal RNA levels using the signals obtained with probes derived from the constitutively expressed marker genes *act*A and EF-1α. Densitometric scanning of the *act*A and EF-1α signals shown in Fig. 1B revealed that interaction RNAs isolated at day 1, day 2 and day 3 after inoculation contained 5, 25 and 70% fungal RNA respectively. All values obtained by densitometric scanning of the hybridization signals shown in Fig. 1A were adjusted to the equivalent of 100% fungal RNA and subsequently compared to those values obtained with RNA isolated from *P. infestans* grown *in vitro*. Numbers indicate the increase in actual mRNA levels in comparison to the mRNA levels in *P. infestans* grown *in vitro* which is set at 1.

†see legend to Figure 1.

‡*ipi*O mRNA is not detectable in *P. infestans* grown in culture and so signals obtained in lanes containing interaction RNA are compared with each other.

— no mRNA detectable.

mRNA of plant origin.

(Fig. 1). Presumably, the *ipi*B gene spans the two fragments although it can not be excluded that the two fragments contain two or more genes of a gene cluster. DHC-J contains two distinct *in planta* induced genes which were termed *ipi*J1 (corresponding to the 3200 nt transcript) and *ipi*J2 (corresponding to the 1300 nt transcript). On northern blots, the mixture of the two co-migrating 1·7 kb *Sst*I-fragments from DHC-J hybridizes to the *ipi*J1 and *ipi*J2 transcripts (Fig. 1). Apparently, the *ipi*J1 and *ipi*J2 transcripts are derived from two distinct genes both of which are at least partially, located on one of the 1·7 kb *Sst*I-fragments. This is supported by the fact that two *Sal*I-fragments (8·0 and 5·1 kb) and two *Sal*I/*Sst*I-fragments (1·4 and 1·2 kb) from DHC-J hybridize to the interaction cDNA probe.

Two of the nine selected *in planta* induced genes, *ipi*B and *ipi*O, show a transient expression pattern during the infection process. The highest *ipi*B mRNA level is detectable 1 day after inoculation and is more than 100-fold higher than the level produced during growth of the fungus *in vitro*. Two and 3 days after inoculation, the *ipi*B mRNA level is much lower (Table 2). *Ipi*O is highly expressed up to 2 days after inoculation, but by the third day the *ipi*O mRNA level is only 3% of that on days 1

and 2 after inoculation. The *ipi*O mRNA transcript was not detectable in the fungus grown in culture. The transient expression pattern of the *ipi*B and *ipi*O genes suggests a role for their gene products in the early stages of infection. In comparison to mRNA levels found during growth of the fungus *in vitro*, mRNA levels of the other seven *ipi* genes were consistently higher during pathogenesis (Table 2). It may be that high levels of their gene products are required during the pathogenic interaction. Expression of the *ipi* genes in *P. infestans* was also studied during growth on rye-sucrose medium and Henniger's synthetic medium. The mRNA levels of all nine *ipi* genes were the same on both media (data not shown), indicating that the large differences in chemical composition between these two media did not affect the expression level of the genes.

In addition to the *ipi*D transcript of 1600 nt, the 5·2 kb *Sst*I-fragment from DHC-D hybridizes to another transcript of 600 nt (Fig. 1) but the mRNA level of the corresponding *P. infestans* gene (*pig*), designated *pig*D, does not change during pathogenesis. In all three digests of DHC-D DNA, a single restriction fragment hybridizes to the interaction cDNA probe. The hybridizing fragment in the *Sal*I/*Sst*I digest is 1·1 kb in length which makes it unlikely that both *ipi*D and *pig*D are located in this clone. Most likely, *ipi*D and *pig*D are homologous genes and either *ipi*D or *pig*D is located on the 1·1 kb *Sal*I/*Sst*I-fragment. DHC-K and DHC-L each contain a single transcriptionally active gene of which the expression level is not increased during pathogenesis. The genes are named *pig*K and *pig*L, respectively. In addition to the 1800 nt *pig*L transcript, the 7·0 kb *Sal*I-fragment of DHC-L hybridizes to a 250 nt mRNA. In RNA isolated from non-infected leaves the same 250 nt mRNA transcript is present indicating that this transcript is of plant origin. Apparently, there is a substantial sequence homology between the 7·0 kb *Sal*I-fragment from DHC-L and the 250 nt plant mRNA. The reason why DHC-L as well as DHC-K hybridized differentially to the fungal and the interaction cDNA probe in the initial screening is not clear.

DISCUSSION

In this study we have shown that differential screening of a genomic library can be used to select *P. infestans* genes whose expression is significantly increased during growth in potato relative to their expression during growth of the fungus in a synthetic medium. Differential hybridization of a genomic library has not often been utilized for the selection of differentially expressed genes. In contrast to cDNA libraries, genomic libraries have large vector inserts and high amounts of non-coding and repetitive DNA. In addition, selected clones may have multiple genes on a single vector insert. In combination with the low specificity of the cDNA probes it is more difficult to isolate the genes of interest. In our case the use of a genomic library of *P. infestans* DNA was preferable since this approach yields fungal genes exclusively. The use of a cDNA library representing mRNA from potato infected with *P. infestans*, will yield host- as well as pathogen-derived cDNAs and these cannot be distinguished directly.

In the first differential hybridization, twenty DHCs were selected. Ten DHCs still showed a differential hybridization pattern in a dot blot experiment and were studied in more detail by means of northern blot analysis. In total nine genes whose expression is induced *in planta* were identified. The number of positive genomic clones obtained with this procedure does not reflect the real number of *P. infestans* genes induced in the

host. When a genomic clone contains a constitutively expressed gene in the proximity of a differentially regulated one, the latter may not be detectable in the differential screening procedure since the signal of the constitutively expressed gene might overshadow that of the differentially regulated gene. Even if only one transcriptionally active gene is located on a genomic clone, the gene could escape identification if its level of expression was too low for detection by this procedure. It is also unlikely that this procedure allows identification of all possible pathogenicity genes since only those pathogenicity genes whose expression is specifically induced or significantly increased during growth of the pathogen in the host would be identified.

After the isolation of the *ipi* genes, the next step is the determination of the nucleotide sequence of the coding regions of the isolated genes. So far, the nucleotide sequence of *ipi*A, *ipi*B, *ipi*C and *ipi*O have been determined. *Ipi*A, which has been renamed *ubi*3R, appeared to encode polyubiquitin [*13*], a protein which plays a key role in several cellular processes such as selective degradation of intracellular proteins, maintenance of chromatin structure, regulation of gene expression and modification of cell surface receptors [*12*]. *Ipi*C, now renamed *cal*A, appeared to code for calmodulin [*15*], a calcium binding protein which is known to play an essential role in basic cellular processes such as signal transduction, ion transport and cytoskeleton function [*3*]. The nucleotide sequence of the transiently expressed genes *ipi*B and *ipi*O have no significant similarity with sequences present in any data library [*14*]. Further studies to examine the regulation *ipi*B and *ipi*O gene expression and the function of their gene products during pathogenesis are in progress.

The differential screening is a non-biased approach which should enable the identification of *P. infestans* genes whose role in pathogenicity cannot be predicted by other methods. The study of differentially expressed genes of *P. infestans* during pathogenesis could therefore lead to the identification of important pathogenicity genes. Due to the choice of cDNA probes in the differential screening it is possible that some of the selected *P. infestans ipi* genes are involved in metabolic adjustments of the pathogen in response to the change in nutrient environment which occurs during infection. Growth conditions *in vitro* are artificial, hence alterations in metabolism may occur when the fungus is transferred from a synthetic medium to the host plant. Nevertheless, changes in the nutrient environment encountered as the parasite invades the host may be an important trigger for the induction of genes involved in pathogenesis, and so, genes in this category may be of interest as well. At present, the molecular and cellular processes which are involved in the interaction of *P. infestans* with its host are poorly understood. Molecular cloning and characterization of *in planta* induced *P. infestans* genes will lead to a better understanding of these processes. Knowledge of the regulation and expression of these putative pathogenicity genes could allow the development of specific control methods for potato late blight.

We wish to thank Pierre de Wit for continuous support and helpful comments on the manuscript. We acknowledge financial support from EMBO and AFRC to M. B. R. Riach.

REFERENCES

1. **Bodenmann J, Heiniger U, Hohl HR.** 1985. Extracellular enzymes of *Phytophthora infestans*: endo-cellulase, β-glucosidases, and 1,3-β-glucanases. *Canadian Journal of Microbiology* **31**: 75–82.

2. **Caten CE, Jinks JL.** 1968. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* **46**: 329–347.
3. **Cheung WY.** 1980. Calmodulin—an introduction. In: Cheung WY, ed. *Calcium and Cell Function, Vol. 1. Calmodulin.* New York: Academic Press, 2–12.
4. **Feinberg AP, Vogelstein G.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**: 6–13.
5. **Fritzemeier K-H, Cretin C, Kombrink E, Rohwer F, Taylor J, Scheel D, Hahlbrock K.** 1987. Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiology* **85**: 34–41.
6. **Henniger H.** 1959. Versuche zur kultur verschiedener rassen von *Phytophthora infestans* (Mont) de By. auf künstlichen Nährböden. *Phytopathologische Zeitschrift* **34**: 285–306.
7. **Jarvis MC, Threlfall DR, Friend J.** 1981. Potato cell wall polysaccharides: degradation with enzymes from *Phytophthora infestans*. *Journal of Experimental Botany* **32**: 1309–1319.
8. **Kombrink E, Schröder M, Hahlbrock K.** 1988. Several 'pathogenesis-related' proteins in potato are 1,3-β-glucanases and chitinases. *Proceedings of the National Academy of Science USA* **85**: 782–786.
9. **Logemann J, Schell J, Willmitzer L.** 1987. Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**: 16–20.
10. **Mahe A, Grisvard J, Dron M.** 1992. Fungal- and plant-specific gene markers to follow the bean anthracnose infection process and normalize a bean chitinase mRNA induction. *Molecular Plant-Microbe Interactions* **5**: 242–248.
11. **Matton DP, Brisson N.** 1989. Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* **2**: 325–331.
12. **Monia BP, Ecker DJ, Crooke ST.** 1990. New perspectives on the structure and function of ubiquitin. *Bio/technology* **8**: 209–215.
13. **Pieterse CMJ, Risseeuw EP, Davidse LC.** 1991. An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Molecular Biology* **17**: 799–811.
14. **Pieterse CMJ, van West P, Verbakel HM, Brassé PWHM, van den Berg-Velthuis GCM, Govers F.** 1993. Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*. *Gene*, in press.
15. **Pieterse CMJ, Verbakel HM, Hoek Spaans J, Davidse LC, Govers F.** 1993. Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. *Molecular Plant-Microbe Interactions* **6**: 164–172.
16. **Sambrook J, Fritsch EF, Maniatis T.** 1989. *Molecular Cloning: A Laboratory Manual, 2nd edition.* New York: Cold Spring Harbor Laboratory Press.
17. **Sargent TD.** 1987. Isolation of differentially expressed genes. In: Berger SL, Kimmel AR, eds. *Methods in Enzymology, Vol. 152. Guide to Molecular Cloning Techniques.* San Diego: Academic Press, 423–432.
18. **Taylor JL, Fritzemeier K-H, Häuser I, Kombrink E, Rohwer F, Schröder M, Strittmatter G, Hahlbrock K.** 1990. Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. *Molecular Plant-Microbe Interactions* **3**: 72–77.
19. **Timberlake WE.** 1980. Developmental gene regulation in *Aspergillus nidulans*. *Developmental Biology* **78**: 497–510.
20. **Timberlake WE.** 1986. Isolation of stage- and cell-specific genes from fungi. In: Bailey JA, ed. *NATO ASI Series, Series H; Cell Biology, Vol. 1. Biology and Molecular Biology of Plant-Pathogen Interactions.* Berlin: Springer Verlag, 343–357.
21. **Tooley PW, Therriën CD.** 1987. Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. *Experimental Mycology* **11**: 19–26.
22. **Unkles SE, Moon RP, Hawkins AR, Duncan JM, Kinghorn JR.** 1991. Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* **100**: 105–112.