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Expression of the *Phytophthora infestans* *ipiB* and *ipiO* genes in planta and in vitro

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Abstract The *ipiB* and *ipiO* genes of the potato late blight fungus *Phytophthora infestans* (Mont.) de Bary were isolated from a genomic library in a screen for genes induced in planta. Expression of these genes was studied during pathogenesis on various host tissues and different host plants, some of which show specific resistance against *P. infestans* infection. During pathogenesis on leaves and tubers of the fully susceptible potato cultivar (cv.) Ajax and on leaves of the fully susceptible tomato cv. Moneymaker, the *P. infestans* *ipiB* and *ipiO* genes show a transient expression pattern with highest mRNA levels in the early stages of infection. During the interaction with leaves of the partially resistant potato cv. Pimpernel, the expression is also transient but accumulation and disappearance of the mRNAs is delayed. Also in *P. infestans* inoculated onto a race-specific resistant potato cultivar and onto the nonhost *Solanum nigrum*, *ipiB* and *ipiO* mRNA is detectable during the initial stages of infection. Apparently, the expression of the *ipiB* and the *ipiO* genes is activated in compatible, incompatible and nonhost interactions. In encysted zoospores, *ipiB* and *ipiO* mRNA accumulation was not detectable, but during cyst germination and appressorium formation on an artificial surface the genes are highly expressed. Expression studies in mycelium grown in vitro revealed that during nutrient starvation the expression of the *ipiB* and *ipiO* genes is induced. For *ipiO* gene expression, carbon deprivation appeared to be sufficient. The *ipiO* gene promoters contain a sequence mo-

tif that functions as a glucose repression element in yeast and this motif might be involved in the regulation of *ipiO* gene expression.

Key words Filamentous fungi · Pathogenicity genes *Phytophthora infestans* · Potato late blight Oomycetes

Introduction

The filamentous oomycete *Phytophthora infestans* (Mont.) de Bary is the causative agent of the late blight disease on potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.). *P. infestans* is a hemibiotrophic pathogen with a rather narrow host range, all host plants being Solanaceae. On potato, the fungus infects both foliage and tubers and is capable of developing and spreading rapidly through host tissue, causing a destructive necrosis.

The disease cycle starts when sporangia of *P. infestans* come into contact with a moist leaf surface. The sporangia germinate directly or form motile biflagellate zoospores, which germinate after encystment. At the tip of the germ tubes, appressoria are formed and infection tubes emerging from these appressoria penetrate the epidermal cells (Pristou and Gallegly 1954). In a fully compatible interaction, hyphal structures grow from the epidermis into the mesophyll cell layer, occasionally forming haustorium-like feeding structures (Coffey and Wilson 1983). Soon after colonization, sporangiophores emerge from the stomata, forming new inocula, which can infect neighbouring plants. In a race-specific resistant host, the fungus is arrested in growth at a very early stage of infection. This is due to a hypersensitive response (HR) of the host plant, which is characterized by rapid cell death of initially invaded host cells and a limited number of cells surrounding the infection site (Tomiyama 1963). The black nightshade *Solanum nigrum* L., a common weed in western Europe, is considered to be a nonhost for *P. infestans*. After infection of

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S. nigrum, *P. infestans* stops growing just prior to penetration of the palisade mesophyll cell layer and before haustoria are produced (Colon et al. 1993).

Molecular studies of the potato-*P. infestans* interaction have demonstrated that upon infection, the expression of certain genes in the host plant is activated (Choi et al. 1992; Fritzeimer et al. 1987; Hahlbrock et al. 1989; Martini et al. 1993; Matton and Brisson 1989; Schröder et al. 1992; Taylor et al. 1990). Several of these genes encode products that might be involved in the inhibition of pathogen development. Also in the pathogen, interaction with the host plant is accompanied by the activation of specific genes (Pieterse et al. 1991, 1993a, b). Gene products of these so-called in planta-induced genes may be putative pathogenicity factors necessary for establishment and maintenance of basic pathogenicity or for the increase of disease severity. To date hardly anything is known about the molecular basis underlying pathogenicity of *P. infestans*. Potential pathogenicity factors such as cell wall degrading enzymes, which may facilitate pathogen entry or dispersion through the host, have been studied (Bodenmann et al. 1985; Cole 1970; Förster 1988; Jarvis et al. 1981) but their involvement in disease development has never been established convincingly. Successful colonization may require the expression of particular pathogenicity genes. The specificity of *P. infestans* for solanaceous hosts, implies a mechanism for recognition in which an exchange of signals between host and pathogen might be essential. It is feasible that signal molecules originating from the host, or environmental conditions in the host, induce the expression of genes in the pathogen that are necessary for the establishment of a successful interaction. Hence, one approach to gaining more insight into the molecular processes involved in pathogenesis, is based on the characterization of *P. infestans* genes that show induced expression during the interaction with the host plant. Once these genes are isolated, their products can be identified and their role in pathogenicity can be studied.

Recently, we described the selection of nine in planta-induced genes by differential hybridization of a genomic library of *P. infestans* (Pieterse et al. 1993a). Two of these, *ubi3R* and *calA*, encode ubiquitin (Pieterse et al. 1991) and calmodulin (Pieterse et al. 1993b), respectively. They are expressed during growth of the fungus in vitro but the expression levels increase and remain continuously fivefold higher during pathogenesis on potato (Pieterse et al. 1991, 1993b). Two other in planta-induced genes, *ipiB* and *ipiO*, appear to encode novel proteins with as yet unknown functions. They both belong to small gene clusters. The *ipiB* gene cluster consists of three genes, *ipiB1*, *ipiB2* and *ipiB3*, encoding three highly homologous glycine-rich proteins of 302 (IPI-B1), 343 (IPI-B2) and 347 (IPI-B3) amino acids (aa), respectively (Pieterse et al. 1994). The IPI-B proteins share up to 47% similarity with several plant glycine-rich proteins that are thought to be cell wall proteins. The *ipiO* gene cluster comprises two nearly identical

genes, *ipiO1* and *ipiO2*, each encoding a 152 aa protein (IPI-O1 and IPI-O2, respectively). The IPI-O proteins do not show homology with sequences present in data libraries (Pieterse et al. 1994).

In this paper we describe the expression of the *P. infestans ipiB* and *ipiO* genes during the interaction of the pathogen with susceptible hosts, a race-specific resistant host, a partially resistant host, and a nonhost. To gain more insight into the regulatory mechanism of the activation of *ipiB* and *ipiO* expression during growth of the fungus in planta, we attempted to induce expression of the genes in vitro. It appears that growth of the fungus under starvation conditions results in the activation of *ipiB* and *ipiO* expression.

Materials and methods

Culturing of *Phytophthora infestans*

Two strains of *P. infestans* were used in this study: isolate 88069 (A1 mating type, race 1.3.4.7) and 88177 (A1 mating type, race 1.3.4.7.10.11). These isolates were grown in the dark at 18°C on rye-agar medium containing 2% (w/v) sucrose (Caten and Jinks 1968). For in vitro expression studies, liquid cultures were initiated from sporangia collected from 2-week-old rye-agar cultures in either Henniger synthetic medium (Henniger 1959) or rich rye-sucrose medium (1×10^5 sporangia/ml; 10 ml per 94 mm petri dish). After incubation at 18°C for 3 days, mycelia were washed twice with large volumes of media as defined in the text, or with milliQ or tap water (milliQ water has been filtered through a Millipore MilliQ purification system). Subsequently mycelia were transferred to 10 ml of milliQ or tap water, or to 10 ml of the defined media and incubated at 18°C for the time periods indicated in text and legends. Mycelia were harvested, frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed.

Composition of Henniger synthetic medium

One litre of Henniger synthetic medium (Henniger 1959) contains 200 mg KH_2PO_4 , 500 mg NaNO_3 , 200 mg CaCl_2 , 150 mg MgCO_3 , 50 mg $(\text{NH}_4)_2\text{SO}_4$, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg tartaric acid, 200 mg succinic acid, 400 mg α -ketoglutaric acid, 300 mg L-glutamic acid, 300 mg DL-alanine, 200 mg L-aspartic acid, 200 mg L-leucine, 200 mg γ -amino-N-butyric acid, 80 mg L-tryptophan, 200 mg L-cysteine, 100 mg L-arginine, 200 μg thiamine and 10 g glucose, pH 5.5.

Production of zoospores, cysts, germinating cysts and appressoria

Zoospores were obtained by adding 10 ml of water to 2-week-old rye-agar cultures (94 mm petri dishes) followed by incubation at 10°C. After 3 h of incubation, typically 10^6 zoospores/ml were released into the water. Cyst formation was initiated by vortexing the zoospore suspension for 2 min. Germinating cysts were obtained by incubating the cysts in water for 2 h at 18°C. Appressoria formation was induced in vitro by allowing encysted zoospores to germinate on Plastibrand polypropylene bags (Brand, cat. no. 759 05, Germany) at 18°C and 100% relative humidity for 2 h (E. Schmelzer, personal communication). Encysted zoospores, germinating cysts and germinated cysts with appressoria were harvested, collected by centrifugation and frozen in liquid nitrogen.

Inoculation of plants

For in planta expression studies, the following plants were used: potato cv. Ajax and tomato cv. Moneymaker, which are both susceptible to the *P. infestans* strains used; potato cv. Pimpernel, which is also susceptible but has a high level of field resistance to *P. infestans*; potato line Black 2424 a(5) (referred to as potato line R8), which contains the R8 gene conferring race-specific resistance to *P. infestans* isolates containing the corresponding avirulence gene (e.g. isolate 88069); the black nightshade *S. nigrum*, which is a nonhost to *P. infestans*. Detached leaves were inoculated by spraying the axial side with a suspension of sporangia collected from 2-week-old rye-agar cultures. A spore suspension of 5×10^5 sporangia/ml was used for inoculation of potato cvs Ajax and Pimpernel and tomato cv. Moneymaker. For inoculation of potato line R8 and *S. nigrum*, a spore suspension of 1×10^7 sporangia/ml was used. Inoculated leaves, inserted in a florist's foam oasis, were incubated at 18°C and 100% relative humidity under cool fluorescent light for 16 h per day. Tubers of potato cv. Ajax were inoculated by spraying 1-cm thick tuber slices with the sporangia suspension. Tuber slices were placed in petri dishes and incubated under the same conditions as inoculated leaves. As controls, leaves and tuber slices were sprayed with water and treated similarly to inoculated leaves and tubers.

RNA isolation and northern blot analysis

RNA was isolated from *P. infestans* encysted zoospores, germinating cysts, mycelium, non-infected plant tissue and infected plant tissue (interaction RNA) using the guanidine hydrochloride RNA extraction method as described by Logemann et al. (1987). For Northern blot analyses, 15 µg of total RNA was electrophoresed on denaturing formaldehyde-agarose gels and blotted onto Hybond-N⁺ (Amersham) by capillary transfer as described by Sambrook et al. (1989). Northern blots were hybridized with DNA probes at 65°C for 20 h in hybridization mix containing 0.5 M sodium phosphate buffer (NaH₂PO₄/Na₂HPO₄, pH 7.2), 7% sodium dodecyl sulfate (SDS) and 1 mM disodium EDTA. Subsequently, blots were washed in $0.5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 65°C and exposed to Kodak X-OMAT AR film.

DNA probes

The *ipiB* probe was derived from a 0.98 kb *SstI-PstI* fragment from the genomic phage clone DHC-B containing the *ipiB2* coding sequence (Pieterse et al. 1994). The 0.63 kb *SstI-XbaI* fragment from the genomic phage clone DHC-O containing the *ipiO1* coding sequence (Pieterse et al. 1994) was used as template to synthesize the *ipiO* probe. The 2.8 kb *PstI* insert from pSTA31 containing the complete *P. infestans actA* gene (Unkles et al. 1991) was used for preparation of the actin probe and the 0.35 kb *EcoRI-XhoI* insert from pPi119, a partial cDNA clone of the *P. infestans* translation elongation factor 1 α (EF-1 α) mRNA (C.M.J. Pieterse, unpublished), was used to synthesize the EF-1 α probe. Probes were labelled with [α -³²P]dATP by random primer labelling (Feinberg and Vogelstein 1983). On Southern blots, all probes have been shown to be specific for *P. infestans*. Under the hybridization conditions used, there is no cross-hybridization with potato DNA sequences (Pieterse et al. 1993b, 1994). On northern blots, however, the *ipiB* probe cross-hybridizes in some cases to RNA isolated from uninfected potato and *S. nigrum* leaves. In the lanes containing interaction RNA, these RNAs can be distinguished from *P. infestans ipiB* mRNA since they differ in size.

Results

Expression of the *ipiB* and *ipiO* genes in compatible interactions

The differential screening procedure, which resulted in the isolation of the *ipiB* and *ipiO* genes, was aimed at the selection of in planta-induced genes of *P. infestans*. To confirm that the expression of the *ipiB* and *ipiO* genes was specifically induced or significantly increased during growth of the pathogen in the plant, and to examine their expression pattern during pathogenesis, northern blot analyses were performed. Initially, the expression was analysed in the interaction from which the cDNA probes for the differential screening were derived, i.e. the interaction between *P. infestans* isolate 88069 and the fully susceptible potato cv. Ajax (Ajax-88069 interaction). On leaves of potato cv. Ajax, first symptoms are visible 24 h after inoculation. They develop from small lesions into completely "water soaked" areas 3 days post-inoculation. In this period the fungus grows and sporulates at the advancing edges of lesions. The centres of the lesions become necrotic and start to decay owing to secondary infections by saprophytic microorganisms. On 1-cm thick tuber slices, infection results in complete colonization of the tissue over a period of 3 days. After 3 days, mycelium appears at the non-inoculated side of the tuber slice.

Total RNA isolated from non-infected tissue, from infected tissue harvested at several time points post-inoculation and from *P. infestans* grown in vitro for 3 days on Henniger synthetic medium was electrophoresed and transferred to membranes. All lanes on the northern blot contains equal amounts of total RNA. RNA extracted from infected tissue (interaction RNA) consists of a mixture of fungal and plant RNA the ratio of which changes during colonization. Signals obtained with probes of differentially regulated genes should therefore be normalized to actual fungal RNA levels. Actin and translation elongation factor 1 α (EF-1 α) mRNA levels have been shown to be good internal standards for the quantification of fungal RNA in the interaction RNA mixture (Mahe et al. 1992; Pieterse et al. 1993a, b). Therefore, the increase in fungal biomass was estimated with probes derived from the constitutively expressed *P. infestans* genes *actA* encoding actin (Unkles et al. 1991) and EF-1 α encoding translation elongation factor 1 α (C.M.J. Pieterse, unpublished). In the Ajax leaves-88069 interaction, actin and EF-1 α transcripts are first detectable 2 days post-inoculation and the amounts increase in time owing to expansion of the fungal biomass (Fig. 1b). In the Ajax tubers-88069 interaction, a similar actin and EF-1 α mRNA accumulation is observed (Fig. 1a). However, 4 days after inoculation the actin and EF-1 α mRNA levels decrease. This is most probably due to the fact that, in these stages, colonization of the tuber tissue is completed. The fungal biomass is no longer expanding and owing to possible death of the

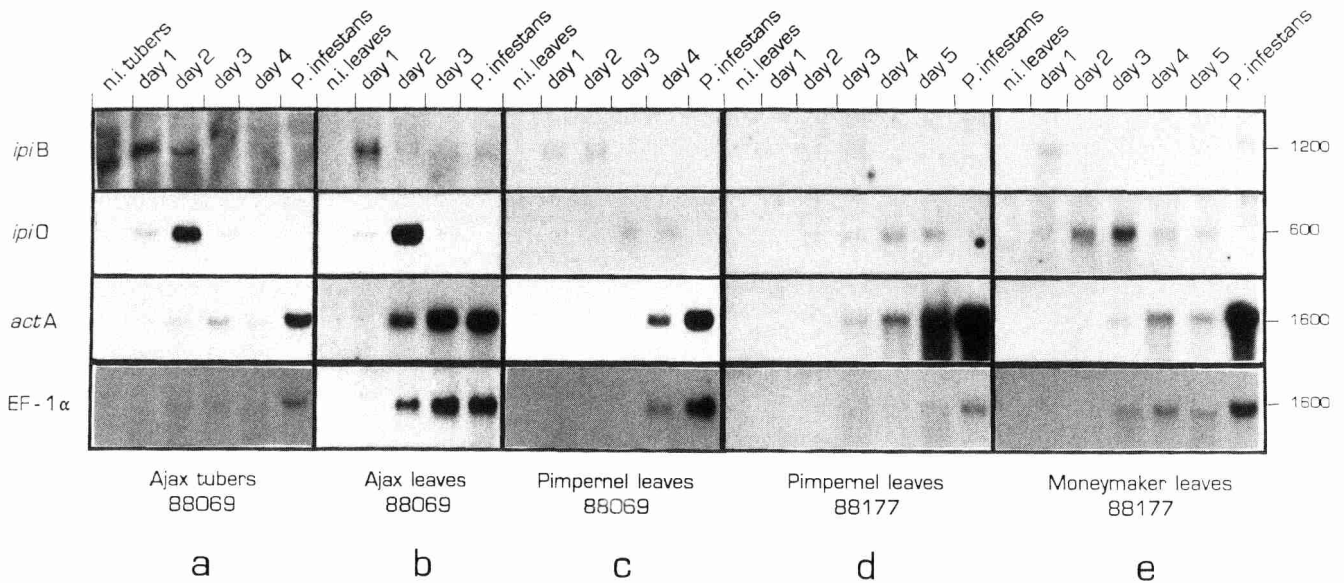


Fig. 1a-e Autoradiographs of northern blots containing total RNA isolated from non-inoculated tuber slices or leaves of the fully susceptible potato cv. Ajax, the partially resistant potato cv. Pimpernel, and the susceptible tomato cv. Moneymaker (*n.i. tubers* and *n.i. leaves*, respectively), from colonized tuber slices or leaves of these host plants at different time points post-inoculation with *Phytophthora infestans* isolate 88069 or 88177 (day 1 - 5), and from 3-day-old *P. infestans* mycelium grown on Henniger synthetic medium (*P. infestans*). Northern blots were hybridized with random primer labelled probes derived from *P. infestans* genes *ipiB2*, *ipiO1*, *actA*, and *EF-1 α* . Transcript lengths are indicated in nucleotides

fungus in old colonized tissue, the relative amounts of actin and *EF-1 α* mRNA decline.

Accumulation of *ipiB* and *ipiO* transcripts was examined by northern blot hybridization using probes derived from *ipiB2* and *ipiO1*. The length of the mRNA hybridizing to the *ipiB* probe is approximately 1200 nucleotides (nt) whereas the *ipiO* probe hybridizes to an mRNA of approximately 600 nt. This is in agreement with the lengths of the open reading frames found in the *ipiB* and *ipiO* genes (Pieterse et al. 1994). By making use of gene-specific oligonucleotides for the *ipiO1* and the *ipiO2* genes, it was determined that both *ipiO* genes are expressed during pathogenesis (data not shown). Therefore, the hybridization signal obtained with the *ipiO* probe represents both *ipiO1* and *ipiO2* mRNA. With regard to the *ipiB* hybridization, it is not known whether expression of *ipiB1*, *ipiB2*, and/or *ipiB3* is responsible for the hybridization signal. Since the *ipiB* genes belong to a multigene family it is also possible that mRNAs derived from other *ipiB*-like genes contribute to the observed *ipiB* mRNA accumulation.

In the 88069 infected Ajax leaves and Ajax tubers, relatively high levels of *ipiB* mRNA are observed at day 1 post-inoculation (Fig. 1a, b). One to 2 days later, the *ipiB* mRNA levels have decreased dramatically to a very low or undetectable level. In lanes containing RNA isolated from non-inoculated tubers an *ipiB* cross-hy-

bridizing band of approximately 1100 nt is visible. Since the *P. infestans ipiB* genes encode glycine-rich proteins (Pieterse et al. 1994), it is likely that this band represents an mRNA encoding a potato glycine-rich protein. In infected leaves as well as in tubers, *ipiO* transcripts are observed in relatively high amounts during the first 2 days post-inoculation. Using densitometric scanning of autoradiographs it was shown that the relative level of *ipiO* mRNA remains essentially constant during the first 2 days after inoculation (Pieterse et al. 1993a). In advanced stages of colonization of both tissues, 3 days post-inoculation, *ipiO* mRNA accumulation declines rapidly. In the fungus grown in vitro on Henniger synthetic medium, *ipiB* and *ipiO* transcripts are hardly detectable. These data demonstrate that during pathogenesis on both leaves and tubers of a susceptible host the *ipiB* and *ipiO* genes are transiently expressed, with the highest expression levels being reached during the early stages of infection.

To determine whether the transient expression patterns of the *ipiB* and *ipiO* genes observed in *P. infestans* isolate 88069 during the interaction with the fully susceptible potato cv. Ajax are also found in compatible interactions with other *P. infestans* isolates and other host plants or cultivars, *ipiB* and *ipiO* expression was analysed in leaves of the partially resistant potato cv. Pimpernel inoculated with *P. infestans* isolate 88069 (Pimpernel-88069 interaction) and 88177 (Pimpernel-88177 interaction), and in leaves of the susceptible tomato cv. Moneymaker infected with isolate 88177 (Moneymaker-88177 interaction). Pimpernel has a high level of race-nonspecific resistance or field resistance to *P. infestans*, which results in lower infection efficiencies, slower tissue colonization and reduced sporulation (Umaerus 1970). On leaves of potato cv. Pimpernel and tomato cv. Moneymaker, symptom development was similar to that observed on leaves of potato cv. Ajax but was delayed for approximately 1 to 2 days. During the Pimpernel-*P. infestans* interactions, the actin and *EF-1 α*

transcripts are first detectable 3 to 4 days post-inoculation (Fig. 1c, d). This supports the observation that colonization of leaves of the partially resistant potato cv. Pimpernel is significantly slower than colonization of the fully susceptible potato cv. Ajax. In the Moneymaker-88177 interaction, actin and EF-1 α mRNA levels initially accumulate, and then decline towards the end of the time course (Fig. 1e). This indicates that, as in the case of the Ajax tubers-88069 interaction, the relative fungal biomass decreases at the end of the infection process.

Although the overall levels of *ipiB* and *ipiO* mRNA are lower in the Pimpernel-88069, the Pimpernel-88177 and the Moneymaker-88177 interactions, the transient expression patterns of the *ipiB* and *ipiO* genes are comparable to those found in the Ajax-88069 interactions. However, in comparison with the fully susceptible interactions Ajax-88069 and Moneymaker-88177 (Fig. 1a, b, e), *ipiB* and *ipiO* mRNA accumulation is slower in the partially resistant Pimpernel leaves (Fig. 1c, d). Also the decrease in expression of *ipiB* and *ipiO* starts later. Moreover, the decrease in *ipiO* mRNA is much less dramatic than in the fully susceptible cvs Ajax and Moneymaker in which *ipiO* mRNA disappears almost completely.

Expression of the *ipiB* and *ipiO* genes in an incompatible and a nonhost interaction

In susceptible and resistant potato cultivars, the frequency of penetration of epidermal cells by infection hyphae of *P. infestans* is generally the same (Gees and Hohl 1988). However, in incompatible interactions with resistant cultivars carrying race-specific R-genes, fungal growth is confined to the infection site owing to the HR of invaded host cells. In the nonhost *S. nigrum*, infection hyphae continue to grow from the epidermal cells into the spongy mesophyll. Growth of the fungus is arrested in this cell layer before haustoria are produced (Colon et al. 1993).

To study the expression of the *ipiB* and *ipiO* genes during an incompatible interaction and a nonhost interaction, leaves of potato line R8 and *S. nigrum* were inoculated with *P. infestans* isolate 88069. Infected R8 leaves were harvested 0, 1, and 24 h after inoculation. At 24 h post-inoculation, the invaded host cells were dead and further colonization was prohibited. Infected *S. nigrum* leaves were harvested 24 and 48 h post-inoculation. Small necrotic lesions were visible 48 h post-inoculation. Disease symptoms did not develop further indicating that fungal growth was arrested in an early stage.

Northern blots containing RNA isolated from non-inoculated and inoculated leaves were hybridized with the *ipiB*, *ipiO*, actin and EF-1 α probes, respectively. Although leaves of potato line R8 and *S. nigrum* were heavily inoculated, little or no mRNA of the constitutively expressed actin and EF-1 α genes can be detected in lanes containing RNA isolated from inoculated

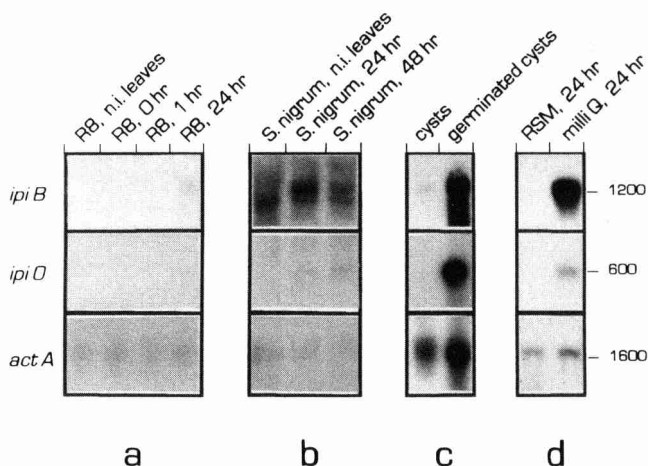


Fig. 2a-d Autoradiographs of northern blots containing total RNA isolated from **a** non-inoculated leaves of potato line R8 (R8, *n.i. leaves*), inoculated R8 leaves immediately after inoculation with *P. infestans* isolate 88069 (R8, 0 hr), 1 h post-inoculation (R8, 1 hr) and 24 h post-inoculation (R8, 24 hr), **b** non-inoculated leaves of *Solanum nigrum* (*S. nigrum, n.i. leaves*) and *S. nigrum* leaves inoculated with *P. infestans* isolate 88069, 24 h (*S. nigrum, 24 hr*) and 48 h post-inoculation (*S. nigrum, 48 hr*), **c** encysted zoospores of *P. infestans* isolate 88069 (*cysts*) and cysts that were allowed to germinate for 2 h in water (*germinated cysts*), and **d** 4-day-old *P. infestans* mycelium (isolate 88069) grown for 3 days on rye-sucrose medium followed by a 24 h incubation on fresh rye-sucrose medium (RSM, 24 hr) or milliQ water (*milliQ, 24 hr*). Northern blots were hybridized with probes derived from *P. infestans* genes *ipiB2*, *ipiO1* and *actA*. Transcript lengths are indicated in nucleotides

leaves, indicating that the proportion of fungal RNA in the interaction RNA mixtures is very low (Fig. 2a, b; EF-1 α hybridization is not shown but is similar to the actin hybridization). Nevertheless, in infected R8 leaves *ipiB* and *ipiO* mRNA is detectable 24 h post-inoculation (faintly visible after reproduction of autoradiograph). In lanes containing RNA isolated from R8 leaves immediately after inoculation (Fig. 2a; R8, 0 h), no hybridization signals can be detected. This indicates that expression of the *ipiB* and *ipiO* genes is activated within 24 h after inoculation. At 24 and 48 h of inoculation of the nonhost *S. nigrum* (*S. nigrum*-88069 interaction), accumulation of *ipiB* and *ipiO* mRNA is observed. In addition to the 1200 nt *ipiB* mRNA, extra bands are visible that cross-hybridize with the *ipiB* probe. These extra bands are also visible in lanes containing RNA isolated from non-inoculated *S. nigrum* leaves. It must therefore be concluded that they represent *S. nigrum* mRNAs that are most probably derived from homologous genes encoding glycine-rich proteins. These results demonstrate that the *ipiB* and *ipiO* genes are expressed in the initial stages of incompatible and nonhost interactions.

Expression of the *ipiB* and *ipiO* genes prior to host cell penetration

In early stages of compatible, incompatible and nonhost interactions *ipiB* and *ipiO* mRNA accumulation is ob-

served. Immediately after inoculation of the leaves no *ipiB* and *ipiO* mRNA is detectable (Fig 2a; R8, 0 h). However, this does not exclude the possibility that the genes are expressed but that the relative proportion of fungal RNA in the interaction RNA mixture is too low to detect *ipiB* and *ipiO* mRNA. To assess whether the *ipiB* and *ipiO* genes are transcriptionally activated in stages prior to host cell penetration, their expression was studied in encysted zoospores and in germinating cysts by northern blot analyses. Hybridization with the actin probe (Fig. 2c) and the EF-1 α probe (not shown) showed that the RNA amounts in the two lanes are not equal. Hybridization with the *ipiB* and *ipiO* probes revealed that, despite unequal loading, *ipiB* and *ipiO* mRNAs accumulate to high levels in germinating cysts whereas in encysted zoospores, *ipiB* and *ipiO* mRNA is hardly detectable (Fig. 2c). Expression of the *ipiB* and *ipiO* genes was further analysed in germinated cysts with appressoria formed at the tip of the germ tubes. Appressorium formation can be induced in vitro by spreading zoospores on an artificial surface of polypropylene (E. Schmelzer, personal communication). Under the conditions used, approximately 50% of the germ tubes formed an appressorium. Owing to the small amount of material that can be obtained with this method, RNA yields are relatively low. Hybridization of a northern blot containing the total amount of RNA isolated from appressoria revealed the presence of *ipiB* and *ipiO* mRNA in this RNA sample (data not shown), whereas actin and EF-1 α mRNA could not be detected. It must be concluded that the *ipiB* and *ipiO* genes are expressed prior to host penetration as soon as the encysted zoospores germinate. Expression continues when the fungus forms appressoria and starts to penetrate the host. Apparently, the very first activation of *ipiB* and *ipiO* gene expression does not require contact with host tissue. Unfortunately, our data do not allow us to draw conclusions on changes in expression levels during appressorium formation and host penetration. Hence, it is not clear whether contact with the host enhances expression.

Induction of *ipiB* and *ipiO* gene expression by nutrient deprivation

Germinated cysts, in which the *ipiB* and *ipiO* genes are expressed at high levels, were obtained by incubating encysted zoospores in water for 3 h. Under these conditions the fungus is in fact exposed to starvation stress. To gain further insight into the influence of growth conditions on transcriptional regulation of the *ipiB* and *ipiO* genes, we attempted to induce *ipiB* and *ipiO* gene expression by nutrient deprivation of mycelium grown in vitro. Rich rye-sucrose medium (RSM) was inoculated with spores. After 3 days the mycelia were transferred to either milliQ water or fresh RSM and were allowed to grow for another day. Northern blots containing RNA isolated from these mycelia were hybridized with the

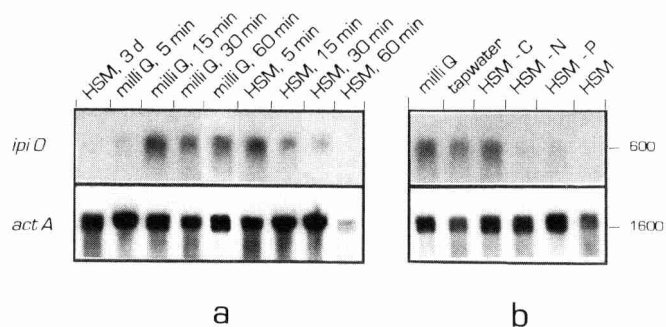


Fig. 3a, b Autoradiographs of northern blots containing total RNA isolated from **a** 3-day-old *P. infestans* mycelium (isolate 88069) grown on Henniger synthetic medium (HSM, 3 d), 3-day-old mycelium grown on HSM followed by a 5, 15, 30 and 60 min incubation on milliQ water (*milliQ*, 5, 15, 30, and 60 min), and 3-day-old mycelium grown on HSM followed by a 60 min incubation on milliQ and a subsequent incubation of 5, 15, 30 and 60 min on fresh HSM (*HSM*, 5, 15, 30, and 60 min) and **b** 3-day-old *P. infestans* mycelium (isolate 88069) grown on Henniger synthetic medium followed by a 1 h incubation on milliQ water (*milliQ*), tap water (*tap water*), fresh Henniger synthetic medium (*HSM*), and HSM deprived of carbon sources (*HSM -C*), nitrogen sources (*HSM -N*) or phosphate sources (*HSM -P*). Northern blots were hybridized with probes derived from *P. infestans* genes *ipiO2* and *actA*. Transcript lengths are indicated in nucleotides

ipiB, *ipiO* and actin probes. In RNA isolated from mycelium that had been grown for an additional 24 h on fresh RSM, *ipiB* and *ipiO* transcripts were hardly detectable (Fig. 2d). However, 24 h after transfer of the mycelium to milliQ water, both *ipiB* and *ipiO* transcripts had accumulated to high levels, indicating that *ipiB* and *ipiO* gene expression is induced during nutrient deprivation.

Transcriptional activation of the *ipiO* genes by nutrient deprivation was studied in more detail. The accumulation of *ipiO* mRNA was assessed in mycelium that had been grown in defined Henniger synthetic medium (HSM) for 3 days and subsequently transferred to milliQ water. Mycelium was harvested 5, 15, 30 and 60 min after transfer to milliQ water. After 60 min on milliQ water, the mycelium was transferred back to fresh HSM and harvested after 5, 15, 30 and 60 min. As early as 5 min after transfer to milliQ water, *ipiO* mRNA can be detected and it reaches a maximum level within 15 min (Fig. 3a). When the mycelium is subsequently transferred from milliQ water to HSM, the accumulated *ipiO* mRNA disappears completely within 60 min.

To investigate whether the induction of *ipiO* gene expression in vitro is caused by general starvation conditions or whether limitation of specific components in the medium is the inducing factor, the expression of *ipiO* was examined in 3-day-old mycelia that had been grown on HSM, and transferred to milliQ water, tap water or HSM deprived of nitrogen, carbon or phosphate sources. After 1 h of incubation on milliQ water or tap water, high levels of *ipiO* mRNA can be detected (Fig. 3b). Similar amounts of *ipiO* mRNA can be detected in mycelium that was transferred to HSM deprived of

carbon sources, whereas in mycelia transferred to HSM without nitrogen or phosphate sources, accumulation of *ipiO* mRNA does not occur. These data suggest that expression of the *ipiO* genes is induced in vitro under conditions of carbon deprivation although it cannot be excluded that a change in osmolarity causes the induction of *ipiO* gene expression.

The specific starvation conditions for induction of *ipiB* gene expression are currently being investigated. Preliminary results indicate that neither carbon, nitrogen nor phosphate deprivation activates *ipiB* gene expression in vitro.

Discussion

Expression of the *ipiB* and *ipiO* genes of *P. infestans* was studied during pathogenesis on different host tissues and different host plants with various types of resistance against *P. infestans*. During pathogenesis on leaves and tubers of the fully susceptible potato cv. Ajax and on leaves of the fully susceptible tomato cv. Moneymaker, the *ipiB* and *ipiO* genes show a transient expression pattern in the two *P. infestans* isolates tested. The highest mRNA levels are observed in early stages of infection suggesting a role for the IPI-B and IPI-O proteins in the onset of the interaction. On leaves of the partially resistant potato cv. Pimpernel, the expression patterns of *ipiB* and *ipiO* genes are also transient but the accumulation and disappearance of the mRNAs is strongly delayed. Hybridization of the northern blots with the actin and EF-1 α probes shows that the increase in fungal biomass within leaf tissue of the partially resistant potato cv. Pimpernel is lower than in tissue of fully susceptible plants. This indicates that *ipiB* and *ipiO* gene expression is correlated with the rate of tissue colonization. This can also be observed when comparing the *ipiB* and *ipiO* mRNA accumulation patterns in infected Ajax and Moneymaker leaves. On the latter host, disease development is delayed for approximately 1 day relative to its course on potato cv. Ajax. The decline in *ipiB* and *ipiO* mRNA levels also starts 1 day later, indicating that expression of the *ipiB* and *ipiO* genes is activated as long as new uninfected tissue is available. As soon as host tissue is completely colonized, the expression of the *ipiB* and *ipiO* genes ceases.

In encysted zoospores, *ipiB* and *ipiO* mRNA accumulation cannot be detected. However, during cyst germination and the formation of appressoria, both genes are highly expressed, which demonstrates that the expression of the *ipiB* and *ipiO* genes is already induced at stages prior to host penetration and that primary induction of *ipiB* and *ipiO* gene expression is probably not directly dependent on host factors. Also in the initial stages of the incompatible interactions between the race-specific resistant potato line R8 and the nonhost *S. nigrum*, the *ipiB* and *ipiO* genes are transcriptionally active. Apparently, the regulatory conditions for *ipiB* gene expression, as well as those for *ipiO* gene expres-

sion, are similar during pathogenesis on hosts with distinct resistance properties. The outcome of the resistance reaction determines the speed of colonization and thus the duration of conditions that support *ipiB* and *ipiO* gene expression. During pathogenesis, the timing of *ipiB* gene activation and repression differs from that of the *ipiO* genes. In addition, *ipiO* gene expression is specifically induced by carbon deprivation whereas this seems not to be the case for the *ipiB* genes. These observations indicate that the regulatory mechanisms involved in *ipiB* and *ipiO* gene expression are different.

Germination of encysted zoospores and sporangia of *P. infestans* occurs naturally on leaf surfaces in a moist environment where they are deprived of nutrients. In initial stages of the interaction, the fungus uses its own food reserves. Once these reserves are exhausted, the host provides all the nutrients essential for growth and development of the fungus (Hohl 1991). To obtain these nutrients, *P. infestans* has to colonize the host tissue. Hence, the pathogen must activate the machinery required for growth and development in the host environment. Starvation stress might therefore be a potential trigger for the expression of pathogenicity genes. From the in vitro experiments it is evident that starvation stress is the inducing environmental condition for transcriptional activation of the *ipiO* genes. This may also be true for the *ipiB* genes although this has to be studied in more detail. Several other in planta induced genes of plant pathogenic microorganisms show induced expression upon starvation stress, e.g. the avirulence gene *avr9* of the tomato leaf mould fungus *Cladosporium fulvum*, which is induced by nitrogen deprivation (van den Ackerveken et al. 1994) and the *MGR1* gene of the rice blast fungus *Magnaporthe grisea*, which is transcriptionally activated during nitrogen and glucose starvation (Talbot et al. 1993). It is tempting to speculate that starvation conditions or changes in nutrient conditions encountered upon infection are general stimuli for the induction of pathogenicity genes. Subsequent production of pathogenicity factors can facilitate development of the fungus, resulting in the establishment of a compatible interaction in which the fungus obtains nutrients from the host plant. When fungal spores germinate on a resistant host or a nonhost, the initial stimuli for the activation of pathogenicity genes will be the same as on a susceptible host. However, the resistance response of the resistant host prevents further growth of the fungus. The fact that in *S. nigrum*, *ipiB* and *ipiO* mRNA is detectable at 48 h post-inoculation suggests that the fungus is still deprived of nutrients at that stage. Apparently, this nonhost interaction fails to develop into a biotrophic stage.

Carbon deprivation appears to be a specific stimulus for transcriptional activation of the *ipiO* genes. Interestingly, the *ipiO1* and *ipiO2* genes (Pieterse et al. 1994) have a sequence motif in their 5' flanking regions that is highly homologous to a glucose repression element present in the promoters of the glucose repressed genes *GAL1*, *GAL4*, and *SUC2* (Nehlin and Ronne 1990;

Gene	DNA region surrounding glucose repression element
<i>SUC2</i> , element A	-482 AGTAATA AAAATGCGGGG AAT -502
<i>SUC2</i> , element B	-451 TTAGGAA ATTATCCGGGG GCG -431
<i>GAL1</i>	-200 TTAGCCT TATTTCTGGGG TAA -180
<i>GAL4</i>	-96 GAAGCTG AAAATCTGGGG AAG -76
<i>ipiO1</i>	-441 TTTCCCG TAAATCTGGGG GCA -421
<i>ipiO2</i>	-440 TTTCCCG TAAATCTGGGG CAT -420

Fig. 4 Alignment of glucose repression elements in the *GAL1*, *GAL4*, and *SUC2* gene of *Saccharomyces cerevisiae* (Nehlin and Ronne 1990; Nehlin et al. 1991) with a homologous sequence in the promoter regions of the *ipiO1* and *ipiO2* genes of *P. infestans* (Pieterse et al. 1993b). Nucleotides matching the consensus sequence (A/T)(A/T)(A/T)(A/T)T(C/G)(C/T)GGGG as proposed by Nehlin et al. (1991) are indicated by an asterisk. Numbers indicate positions relative to the ATG start codon

Nehlin et al. 1991) of *S. cerevisiae* (Fig. 4). A C₂H₂ zinc-finger DNA binding protein encoded by the *MIG1* gene of *S. cerevisiae* has been shown to be involved in repression of gene expression by binding to the glucose repression element in the promoter regions of these three genes under glucose rich conditions. Whether this sequence motif in the 5' flanking regions of the *ipiO* genes indeed functions as a glucose repression element in *P. infestans* needs to be investigated.

Whether or not the *ipiB* and *ipiO* gene products are essential pathogenicity factors is still unknown. The most direct way to determine this is by disrupting the gene of interest and analysing the effect on the pathogenic properties of *P. infestans*. However, this approach requires a highly efficient DNA transformation system and although the procedure for obtaining stable transformants of *P. infestans* is established (Judelson et al. 1991), the efficiency of transformation is still low. Moreover, *P. infestans* is a heterothallic and diploid organism, and some isolates are even tetraploid, which makes it difficult to disrupt target genes in this organism. Therefore, a better approach might involve reducing or blocking the synthesis of *ipiB* and *ipiO* gene products by means of anti-sense RNA. The anti-sense technique has been tested in *P. infestans* using sense and anti-sense GUS constructs and has proven to be effective in inhibiting the accumulation of GUS by up to 98% (Judelson et al. 1993).

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