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A late blight resistant potato plant overexpresses a gene coding for α-galactosidase upon infection by *Phytophthora infestans*

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Abstract

Late blight of potato, caused by *Phytophthora infestans* was studied by using a resistant clone of potato on one side and a susceptible clone on the other side. A gene coding putatively for an α-galactosidase has been isolated by mRNA reverse transcription polymerase chain reaction differential display and was shown to be differentially expressed between the resistant and the susceptible clone. α-Galactosidases catalyse the hydrolysis of α-1,6 linked α-galactose residues from oligosaccharides and it could be shown in the present work that raffinose content decreases at 30 h after infection by *P. infestans* in the resistant clone.

Additional key words: raffinose, RT-PCR, *Solanum phureja*, *Solanum tuberosum*.

Introduction

Late blight of potato, caused by the oomycete *Phytophthora infestans* is a serious threat to potato production worldwide. The development of cultivars with durable resistance to late blight is a primary objective of potato breeders and was stimulated by the late blight epidemics in the 1840's. The sources of resistance, however, remained mainly unknown to breeders until the early 20th century when potato breeders started to use *Solanum demissum* and *Solanum stoloniferum* in their programs (Haynes and Christ 1999). Vertical resistance, obtained by introgression of major resistance genes from these cultivars, has been overcome by the pathogen and was shown not to be durable. Another type of resistance, called horizontal resistance, is apparently not controlled by major resistance genes but by minor genes each contributing to a small extent to the general resistance of the plant and is supposedly more durable. Nowadays it seems to be the most appropriate alternative to increased fungicide use. Wild potato species and native cultivars represent possible new sources of resistance. Breeding programs have considered this type of resistance worldwide. *Solanum phureja* and *Solanum andigena* have been described to be partially resistant (Nilsson 1981, Colon *et al*. 1995). The native cultivated potato species *Solanum phureja* is particularly interesting because the resistance is polygenic and no major resistance genes (R-genes) that could potentially mask the quantitative resistance have been detected (Trognitz *et al.* 2001).

In the present work, a population derived from a cross between *Solanum phureja* and a susceptible dihaploid *Solanum tuberosum* subsp. *tuberosum* has been used (Ghislain *et al*. 2001). One resistant hybrid (PD88) and one susceptible hybrid (PD60) of this population have been compared in a differential display study (Evers *et al*. 2003a). Sampling was performed 24 h after infection by *Phytophthora infestans.* Several differentially expressed genes were identified, most of them being expressed in the resistant hybrid and not in the susceptible one. Hereafter, one of them, dd1 (EMBL accession number AJ437585, 325 bp), coding putatively for an α-galactosidase was characterized in detail.

The *dd1* matched to different ESTs including ESTs from potato and tomato (Evers *et al*. 2003a). At the protein level, high similarity (79 %) can be observed with an α-galactosidase from *A. thaliana* (NP_189269) and from rice (BAD73696) (74 %).

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Abbreviations: EST - expressed sequence tag; f.m. - fresh mass; ORF - open reading frame; QTL - quantitative trait locus; RT-PCR - reverse transcription polymerase chain reaction.
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α-Galactosidases are generally present in plants (Stano *et al*. 2001), animals and microbes (Rezessy-Szabó *et al*. 2002). They have been described for many species including *Arabidopsis*, avocado, tomato, petunia, bean, coffee and others. α-galactosidases (α-*D*-galactoside galactohydrolase EC 3.2.1.22) catalyse the hydrolysis of α-1,6 linked α-galactose residues from oligosaccharides such as melibiose, raffinose, stachyose and verbascose (Rezessy-Szabó *et al*. 2002). The implication of sugar content in the defence response of a plant to pathogen infection has been shown previously (Evers *et al*. 2003b). To our knowledge, this enzyme has, however, never been described in a plant-pathogen interaction.

The aim of this work was to characterize the *dd1* gene so as to provide clues concerning its potential role in the defence reaction of potato to late blight infection.

Materials and methods

Plants and treatments: This work was carried out on two hybrids resulting from a cross between the diploid *Solanum phureja* Juz. & Buk. accession CHS-625 and a *S. tuberosum* L. dihaploid clone, called PS-3. Hybrid PD88 was resistant to late blight and hybrid PD60 was susceptible.

These plants were maintained pathogen-free *in vitro*. Shoots were grown in polyethylene jars on a Murashige and Skoog (1962) medium devoid of any growth regulators. Media were supplemented with 30 g dm^{-3} sucrose and adjusted to pH 5.6 with KOH prior to the addition of 'Roland' agar (8 g dm^{-3}) and subsequently autoclaved for 20 min at 121 °C and 118 kPa. Before acclimatization, 1-naphthalene acetic acid was added to the medium at 0.3 mg dm^{-3} for rooting. Plants were acclimatized in pots in a mixture of soil:sand (3:1) and raised in incubation chambers. Both *in vitro* and *ex vitro* cultures were maintained at 20 °C with a 12-h photoperiod and an irradiance of 25 μmol m^{-2} s^{-1}.

The pathogen isolate used, called PCZ127, was of Peruvian (Cusco) origin and has been kindly provided to us by the International Potato Center (Lima). It was a very complex isolate possessing virulence genes 1, 2, 3, 4, 5, 6, 7, 9, 10, and 11. It was of the A1 mating type. *P. infestans* was maintained and multiplied as a pure culture on agar plates or on potato slices.

Sporangia were washed with distilled water from the upper side of a sporulating lesion on agar plates or on a potato slice and passed through a 30 μm mesh filter to remove mycelium and other debris. Spore concentration was determined by counting in a Fuchs-Rosenthal cell (Marienfeld, Lauda-Königshofen, Germany). The sporangial suspension was then incubated at 4 °C for 2 h to promote zoospore release. Inoculations were performed on 2 - 3 months old plants. A concentration of 12 000 sporangia per cm^3 was applied by spray in all the inoculations. Mock inoculations were performed by spraying control plants with water.

DNA extraction and Southern blotting: DNA was extracted using a CTAB procedure based on the protocol of Doyle and Doyle (1987). After electrophoresis in 1 % agarose, the gel was transferred to a tray containing denaturation buffer (0.5 M NaOH, 1.5 M NaCl) and incubated for 30 to 45 min at room temperature under agitation. The gel was rinsed with H_2O and then incubated in neutralization buffer (1 M Tris-HCl, 3 M NaCl, pH 7) for 1 h. Transfer was carried out onto a positively charged nylon membrane according to Weising *et al.* (1995) in 20× SSC by capillarity overnight. After transfer, the membrane was briefly rinsed in 6× SSC and baked in an oven at 80 °C for 2 h.

RNA extraction and Northern blotting: RNA was extracted according to the hot phenol/SDS method as described in Krieg (1996). After electrophoresis, the gel was incubated for 40 min in hydrolysis buffer (50 mM NaOH, 10 mM NaCl) at room temperature with shaking. Then it was incubated twice 20 min at room temperature with shaking in neutralization buffer (0.2 M Tris, pH 7.4, 18× SSC). Transfer of the RNA was carried out on a positively charged membrane by capillarity in 20× SSC according to Sagerström and Sive (1996). After the transfer, the membrane was baked for 2 h at 80 °C.

DNA probe labeling: Radioactive labeling of DNA probes was performed with ^{32}P labeled dATP by using the *Megaprime*™ DNA labeling system (*General Electrics Healthcare*, Roosendaal, The Netherlands) according to the instructions of the manufacturer.

cDNA library construction and screening: A cDNA library constructed from the resistant parent CHS-625 after infection by *P. infestans* was used (kindly provided by Céline Schweitzer, Luxembourg). Briefly, this had been performed by using the *SMART*™ cDNA library construction kit from *Clontech*. For cDNA synthesis, a modified oligo dT primer primed the first-strand synthesis and a special SMART Oligo served as a short, extended template at the 5' end of the mRNA. After digestion with a restriction enzyme (*Sfi* I) and size fractionation, cDNA was ligated into an *Sfi* I-digested λ TriplEx2 vector.

For the plating out of the library, 0.5 cm^3 XL1 blue cells (in 10 mM $MgSO_4$) were incubated at 37 °C during 15 min with dilutions of the library yielding approximately 20 000 clones per plate. 0.5 cm^3 of these infected cells were transferred to 7 - 8 cm^3 of 45 °C top agarose and poured on plates. Incubation was at 37 °C overnight. Transfer was performed on positively charged nylon membranes. The membranes were put on top of the agarose and let stand for 1 - 2 min. The filters were then

incubated successively for 3 min each time (phage side up) in denaturation buffer, in neutralization buffer and finally in 2× SSC. Between each incubation step, membranes were allowed to dry for 3 min. Finally, they were heated for 2 h at 80 °C.

A primary screening was performed on 10 plates (150 mm) containing approximately 20 000 clones each followed by secondary screening at lower density. Hybridization was carried out using probes radioactively labeled through a random priming procedure (*Megaprime*™ DNA labeling system).

Sequencing was performed through a commercial company (*Eurogentec*, Liège, Belgium). Homology search was done by using the *BLAST* and *TBLASTx* network services at the *EMBL* Nucleotide Sequence Databases.

Cloning of *dd1* genomic sequence: Genomic DNA amplification was performed based on the cDNA sequence. Upstream primer 01 (5'GGA-ACC-AAT-CAT-AGC-GAA-GG 3') and downstream primer 02 (5' ATG-AGA-GGG-GAC-CTG-ACC-AT 3') were used in the following PCR reaction: 1 min at 94 °C, 25 cycles (30 s at 94 °C, 45 s at 53 °C, 2 min at 72 °C) followed by 2 min at 72 °C.

Raffinose analysis: For raffinose determination, samples were collected and frozen in liquid nitrogen. 250 mg of leaf tissue were homogenized in 1 cm^3 95 % (v/v) ethanol. After centrifugation at 17 000 *g* during 10 min, the supernatant was collected in an Eppendorf tube and the pellet was washed with 0.5 cm^3 95 % (v/v) ethanol. 0.5 cm^3 of the pooled supernatants were dried in a vacuum centrifuge. The dried residue was dissolved in 1 cm^3 of double-distilled H_2O and filtered on an *ACRODISC13* nylon filter (0.45 μm pore size) (*PALL Life Sciences*, St-Germain-en-Laye, France). Analyses were performed on an ion exchange liquid chromatography system (*Dionex*, Wommelgem, Belgium). Sugars were separated on a *CarboPac PA10* (*Dionex*) guard column (4 × 50 mm) and a column (4 × 250 mm) at a flow rate of 1 cm^3 min^{-1} using 16 mM NaOH as eluent. Detection was by triple-pulsed amperometry with a gold working electrode according to Wilson *et al*. (1995). Calibration was performed using custom-made external standard solution at the beginning of every batch. Every 10 injections, a standard solution was used to confirm the calibration of the system.

Statistical analyses: Results from sugar analysis are mean values of at least 5 independent measurements. *ANOVA* analysis of variance and Student-Newman-Keuls tests were realized for multiple comparisons of means.

Results

***Accumulation of dd1* transcript:** *dd1*, a 325 bp long cDNA fragment, has been obtained by mRNA RT-PCR differential display. To further characterize the expression of *dd1*, Northern blot analyses were performed. The expression pattern of the resistant hybrid PD88 was compared to the one of the susceptible hybrid PD60 by sampling at four different time points (0, 1, 2 and 3 days post infection). *dd1* was expressed in the resistant hybrid one day after infection by *P. infestans* whereas it was detected neither for the uninfected plants nor the susceptible hybrid (Fig. 1).

Gene *dd1* was only expressed on the first day of the infection, therefore the next step was to focus this time course expression by sampling at closer time intervals around the first day (18, 24, 30, 48 and 72 h). It could be confirmed that the expression of *dd1* was maximal at 24 h post infection. A slight expression could be shown in water-treated plants at the same sampling time.

As the expression pattern of some defence-related genes has been reported to correlate with stress-related genes, investigations on the stress inductivity of *dd1* were performed. Neither *in vitro* culture, which implements a variety of stresses for plants including for example mechanical injury, wounding, high relative humidity in the flask and high osmotic potential (Kevers and Gaspar 1986), nor a treatment with BTH, an inducer of systemic acquired resistance and pathogenesis-related genes, induced *dd1* at the level detected by a one-day inoculation with *P. infestans* (data not shown).

Evaluation of *dd1* gene copy number and polymorphism: To investigate the number of genes corresponding to the *dd1* cDNA in the potato genome, genomic DNA from the parents of the PD population and from two hybrids (PD88R and PD60S) was analysed by

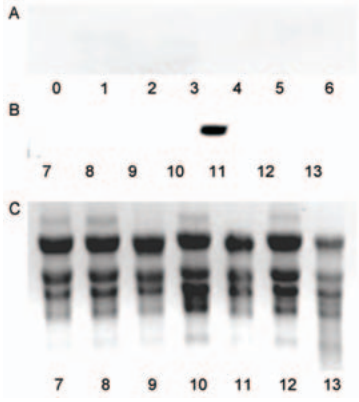

Fig. 1. Northern blot of leaf RNA hybridized with a *dd1* ^{32}P-labelled probe. *A* - *lanes* 0 - 3: RNA from the susceptible hybrid, mock-inoculated, sampled on days 0, 1, 2 and 3; *lanes* 4 - 6: RNA from the susceptible hybrid, infected, sampled from days 1, 2 and 3. *B* - *lanes* 7 - 10: RNA from the resistant hybrid, mock-inoculated, sampled on days 0, 1, 2 and 3; *lanes* 11 - 13: RNA from the resistant hybrid, infected, sampled on days 1, 2 and 3. *C* - Uniform loading of the samples was checked after electrophoresis through ethidium bromide fluorescence under UV light.

hybridisation on Southern blots. Hybridisation signals were obtained with DNA from resistant and susceptible plants. The *dd1* cDNA probe hybridised to 3 - 4 bands suggesting that *dd1* belongs to a small gene family in potato (Fig. 2). No polymorphism was observed between the four clones using EcoRI, XhoI and HindIII as restriction enzymes.

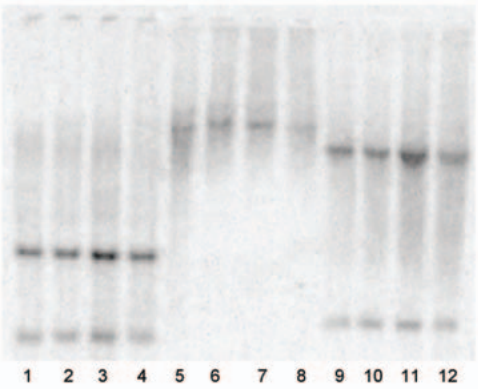

Fig. 2. Southern blot hybridized with a dd1 ^{32}P-labelled probe. *Lanes* 1, 5, 9: DNA from the resistant parent CHS-625; *lanes* 2, 6, 10: DNA from the resistant hybrid PD88R; *lanes* 3, 7, 11: DNA from the susceptible hybrid PD60S; *lanes* 4, 8, 12: DNA from the susceptible parent PS3. *Lanes* 1-4: EcoRI digested DNA; *lanes* 5-8: XhoI digested DNA; *lanes* 9-12: HindIII digested DNA.

Cloning of a partial *dd1* genomic sequence: In order to obtain a genomic sequence corresponding to the *dd1* cDNA, PCR amplification on genomic DNA was performed using primers chosen on the *dd1* cDNA fragment from differential display. DNA from the two parents of the population and from the hybrids PD60 and PD88 was used.

One main amplification product of approximately 1000 bp was obtained for the four plant lines. The 1 kb PCR fragment was cloned and sequenced for the four plants (both parents and two hybrids) (Fig. 3).The gene appears to have at least 3 introns with typical border sequences (GT in the 5' position and AG in the 3' position).

A very high homology between the DNA fragments from the four clones can be observed. The sequences from the two parents were homologous to 97 %. The sequence from the resistant parent was 99 % homologous to the susceptible hybrid and the sequence from the susceptible parent was 99 % homologous to the resistant hybrid.

Mapping of *dd1*: The map location of the *dd1* gene was determined in order to test its possible association with a known QTL for horizontal resistance to late blight similarly to Trognitz *et al.* (2002). Analysis of trait-marker association was performed on a series of four phenotypic data sets of field resistance to late blight collected during 3 years (1997, 1998, 1999) and at 3 locations (Comas (Peru), Huancayo (Peru) and Quito (Ecuador)).

The QTL for late blight resistance on chromosome V is quite reproducible between the four field data (Ghislain *et al.* 2001). However, the *dd1* sequence mapped in a region of chromosome V shown not to be associated with this QTL.

Cloning of a full-length *dd1* cDNA: To confirm the high homology of *dd1* to α-galactosidase and to obtain a longer cDNA probe, a cDNA library from the *S. phureja* clone CHS-625 infected by *P. infestans* was screened with the *dd1* cDNA fragment. One positive clone was obtained after secondary screening. Sequencing of this clone revealed a cDNA fragment of 2251 bp. A large open reading frame is present in this cDNA with a coding sequence of 1080 bp starting with the initiation codon at position 543 and ending at position 1622, 629 bp before the polyA tail.

Highly significant similarities were found with a pathogen-induced catalase-like mRNA from *S. brevidens* and with different ESTs. At the protein level, highest matches corresponded to α-galactosidases from *Arabidopsis thaliana,* tomato, rice and coffee (Table 1).

Table 1. Similarities of the full-length *dd1* cDNA with sequences in databases. Expected values of best match given in parentheses.

Matching sequence from data base	Origin of matching sequence and accession numbers	DNA [%] (match)	Protein [%] (identities)	Protein [%] (positives)
mRNA sequence	*Lycopersicum esculentum* BT013622	95 (0)		
Pathogen-induced catalase-like mRNA	*Solanum brevidens* AY064207	97 (e^{-159})		
EST (mixed tissues)	*S. tuberosum* BQ512590	98 (0)		
EST (flower)	*L. esculentum* AW737528	98 (0)		
EST (flower)	*L. esculentum* BI933123	95 (0)		
α-galactosidase	*Arabidopsis thaliana* NP_189269		62 (e^{-137})	79
α-galactosidase	*Oryza sativa* BAD73696		61 (e^{-121})	74
α-galactosidase	*L. esculentum* AAF04591		28 ($3\ e^{-12}$)	49
α-galactosidase	*Coffea arabica* CAI47559		29 ($3\ e^{-11}$)	50

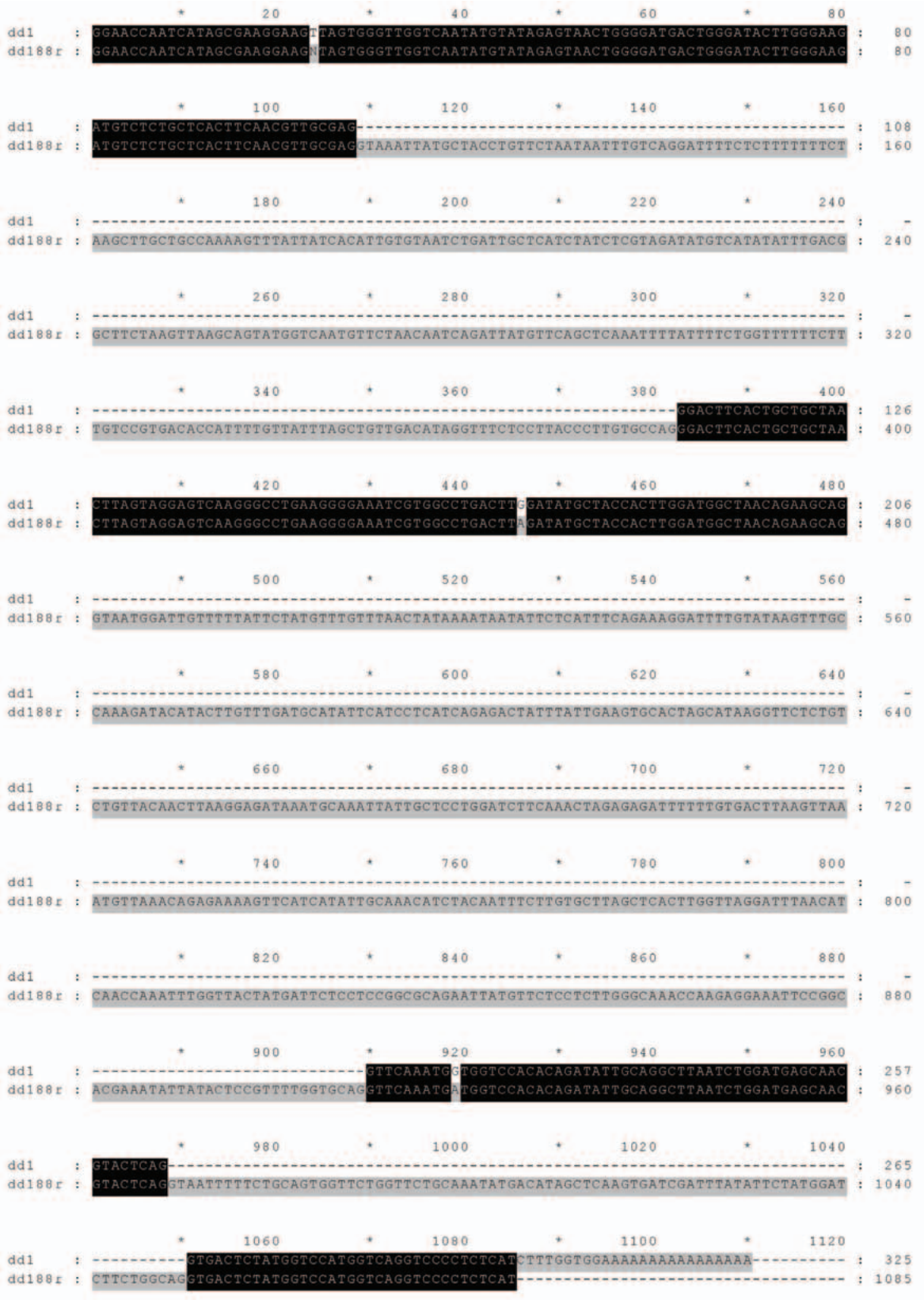

Fig. 3. Sequence alignment of the 1085 bp genomic DNA amplification product from the resistant hybrid (dd188r) with the *dd1* cDNA (325 bp, obtained after differential display). Identities are indicated in black.

This protein, with a calculated isoelectric point of 6.6 and a molecular mass of 40.1 kDa started with a series of hydrophilic amino acids. The calculated molecular mass (40.1 kDa) was close to the one of a soybean α-galactosidase (40 kDa) and of two French bean α-galactosidase isoenzymes with molecular masses of 38.3 and 39.6 kDa (Guimaraes *et al*. 2001).

According to the method of Von Heijne (1986) for the recognition of signal sequence it does not seem to have an N-terminal signal peptide. The method of Reinhardt and Hubbard (1998) suggested a cytoplasmic localization.

Raffinose content: α-galactosidases degrade oligoaccharides of the raffinose family. Thus, HPLC determination of raffinose and galactose was performed in leaves and stems of the non-infected and *P. infestans* infected hybrid PD88 during a time-course experiment. Galactose was undetectable in stems and leaves. Raffinose could not be detected in the stems. In the leaves, however, raffinose content was stable until 30 h after infection and significantly decreased afterwards (Fig. 4). 72 h after the infection, raffinose was undetectable in the leaves. In the control plants, an early decrease in raffinose content was observed, followed by stabilization at 40 nmol g^{-1}.

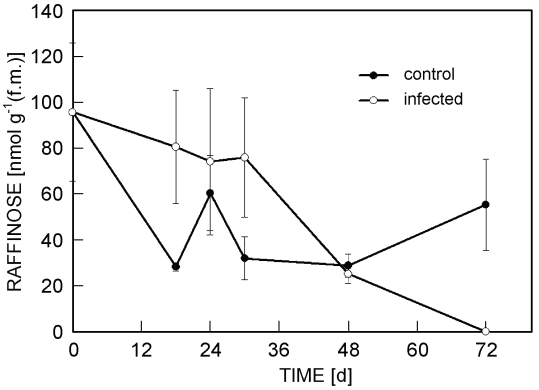

Fig. 4. Mean raffinose content in leaves of non-infected and infected hybrid PD88R. Results are means of 5 independent measurements and indicated with standard deviation. Infection by *P. infestans* was performed on day 0.

Discussion

A gene coding putatively for an α-galactosidase (or melibiase) was characterized in the context of the potato-*P. infestans* interaction. Its differential expression in a resistant *S. tuberosum* × *S. phureja* hybrid upon infection by *P. infestans* was confirmed through a series of analyses performed on independent RNA samples (mRNA RT-PCR differential display, dot blotting, Northern blotting). Mapping of the *dd1* gene showed that it was located on chromosome V but apparently outside of a QTL for late blight resistance. Similar results were observed for other genes known to be candidate genes for the resistance of potato to late blight.

To our knowledge, this is the first report describing an α-galactosidase from potato on one side, and suggesting a potential role for this enzyme in the plant-pathogen interaction. No expression was observed after exposure to other stresses like those of *in vitro* culture conditions. Treatment with BTH, an inducer of systemic acquired resistance, did not activate this gene either. α-galactosidase is part of the galactose, the glycerolipid and the sphingoglycerolipid metabolism and hydrolyses sugars from the raffinose family. In the present work, raffinose content was shown to decrease after infection of a resistant hybrid with *P. infestans*. These observations point to a putative implication of this enzyme in the defence response of potato. A potential role for α-galactosidase in the stress response in plants has been described in petunia in relation to cold stress (http://abstracts.aspb.org/aspp2001/public/P33/0436.html). α-galactosidases are also suggested to be associated with the stress response to dessication (Pukacka and Wojkiewicz 2002). It is well known that common responses of plants to various types of stress exist; thus, it seems that the expression of α-galactosidase in the present work would not be specific for the potato late blight interaction, but would rather be a common response to a stress condition. However, it was not expressed in BTH-treated and in *in vitro* cultured plants. Other types of abiotic stress like cold or salt stress should be studied for the expression of α-galactosidase to confirm that it is part of a general adaptation reaction.

The metabolic pathway in which α-galactosidase is implemented belongs to the functional class of the energy metabolism in which biosynthesis and degradation of polysaccharides occur. The diminution of raffinose content as well as important changes in other sugar contents upon infection by *P. infestans* (Evers *et al*. 2003b) point to an implication of sugar and sugar metabolism in plant-pathogen interactions.

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