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Accumulation of defense related transcripts in sunflower hypocotyls (*Helianthus annuus* L.) infected with *Plasmopara halstedii*

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Abstract

A cDNA clone encoding a sunflower chitinase was obtained using degenerated primers in PCR amplifications and RACE procedures. This clone, a phenylalanine ammonia-lyase (PAL) clone and ubiquitin clone were used to analyse the resistance of sunflower (*Helianthus annuus*) to downy mildew. The differential regulation of amounts of PAL (involved in the general pathway of phenylpropanoid synthesis), chitinase (a pathogenesis-related protein) and ubiquitin (involved in proteolytic pathways) mRNA was studied in hypocotyls during the early stages after an aerial infection of sunflower inbred line RHA274 with zoospores from either race 1 (incompatible, host resistant) or race B (compatible, host susceptible) of *Plasmopara halstedii*. Northern analyses showed that transcript levels of PAL, chitinase and ubiquitin were rapidly and strongly increased after infection in incompatible interactions but not in the compatible ones, suggesting that regulation of these mRNAs is an important component of the resistance mechanisms in sunflower.

Introduction

Downy mildew of sunflower (*Helianthus annuus* L.), caused by *Plasmopara halstedii*, can lead to economic crop losses due to primary infection through the roots (Allard, 1979) and early secondary infection (aerial) which result in systemic infection of plants by the fungus (Regnault and Tourvieille, 1991). This obligate parasite is specific to sunflower. It shows a number of physiological races which can be distinguished by their differential virulence on sunflower genotypes (Gulya et al., 1991). Following artificial infection by immersion of whole seedlings in a fungal suspension, Mouzeyar et al. (1993) have shown that resistance correlates with a hypersensitive-like reaction with cell division and lignification developing around the parasite. This resistance is also associated with an increase in chitinase and glucanase activities (Cachinero et al., 1996). However until now, little is known about the molecular processes underlying this incompatible response.

One of the most efficient mechanisms of induced disease resistance in plants can be the hypersensitive reaction whose induction results from the specific recognition event occurring between a component of the host and a component of the pathogen (Keen, 1990). The hypersensitive reaction results in the rapid appearance at the site of attack of a restricted necrotic lesion clearly delimited from surrounding healthy tissue. This early cell death has often been emphasized as a mechanism inhibiting pathogen development. Concomitant with the onset of the hypersensitive response, there is

transcriptional activation of defense genes encoding enzymes for phytoalexin and lignin biosynthesis, such as phenylalanine ammonia-lyase (PAL) (Mawton et al., 1980) and lytic enzymes, such as chitinase (Sahai and Manocha, 1993).

In the present study, we report a comparison of PAL, chitinase and ubiquitin transcript accumulation in incompatible and compatible interactions between *H. annuus* and *P. halstedii*, using sunflowers infected with two races of *P. halstedii*, either virulent or avirulent.

Materials and methods

Sunflower genotypes and P. halstedii *isolates*

Infection methods were developed on the sunflower inbred line RHA274 (USDA), resistant to downy mildew race 1 but susceptible to race B. The downy mildew isolates of races 1 and B are maintained on the inbred line RHA266 (USDA) and the population HAR5 (USDA) respectively, at INRA Clermont-Ferrand (France).

Infection procedure

Preparation of seeds and growth conditions of the plants were those described by Mouzeyar et al. (1993). Aerial contaminations were prepared as previously described by Albourie et al. (1998). After the first pair of leaves had expanded, a suspension of $5 \cdot 10^4$ zoosporangia per ml of distilled water, was applied on leaves with a spray. The seedlings were then covered with transparent polythene bags for 48 h.

Microscopic observations

Sunflower seedlings were fixed in FAA (formalin–acetic acid–ethanol: 5 : 5 : 90). All the light and fluorescence microscopy techniques were described by Mouzeyar et al. (1993). No stain was used.

Total RNA extraction

To analyse the accumulation of defense-related transcripts, the plants were harvested at different times and the RNA was extracted from hypocotyls according to Mazeyrat et al. (1998).

PCR amplification of chitinase cDNA

To remove DNA contamination 2 µg of total RNA were treated with RNase-free Dnase I amp. Grad (Gibco BRL, Life Technologies, France) by incubation with 2 µl 10× buffer, 2 u DNase and 14 µl H_2O at room temperature for 15 min. The reaction was stopped by addition of 2 µl EDTA 25 mM and heating at 65 °C for 10 min. For first-strand cDNA synthesis, 0.2 µg of DNase-treated RNA were mixed with 2.5 µM of a 15-mer oligo-dT primer 5′ AAT TCG CGG CCG CTT TTT TTT TTT TTT T 3′ (Oligo-Express France), 9.8 µl of H_2O and heated at 65 °C for 5 min. For reverse transcription 1× Reverse transcriptase buffer, 0.01 M DTT, 20 u of RNasin (Promega, France), 20 µm dNTPs and 100 u of MMLV Reverse transcriptase (Gibco BRL, Life Technologies, France) were mixed and incubated at 42 °C for 60 min. The reaction was stopped by incubation at 95 °C for 5 min.

The primers used for homology cloning of the sunflower chitinase were 5′ TTC TTK GCY CAA ACT TCY CAY GAA AC 3′ (C1) as forward primer and 5′ CCA TTG ATD ATR TTK GTG ATH AMA CCA 3′ (C2) as reverse primer. For PCR amplifications 5 µl of cDNA, 1 u Taq DNA pol, 200 µM dNTP and 0.1 µM of each primers were used in 25 µl final volume reaction. The PCR program consisted of 1 cycle at 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min 30 s with a final 10 min elongation at 72 °C. The amplified products were ligated into pCRII vector (TA cloning kit, Invitrogen) and transformed into *E. coli* strain JM 109 and sequenced.

The sequence permitted the choice of gene-specific primer 5′ CTG ACA CTG CAG CTG GTC GTG TCT CGG G 3′ (C4) and 5′ GTG GAT GGG ACA CTG CAC CAG ATG GGC G 3′ (C3) for cloning the 5′ end and 3′ end of the cDNA using Marathon cDNA amplification kit (Clontech, France). The PCR programme consisted of 5 cycles at 94 °C for 5 s, 72 °C for 3 min, 35 cycles at 94 °C for 5 s, 70 °C for 3 min. The amplified products were cloned into pGEM-T Easy Vector (Promega, France) and sequenced.

Sequence analysis

Sequencing of both strands was performed with dideoxynucleotide chain termination reactions by Genome Express (France). The BLAST program (Altschul et al., 1990) was used for homology search

with data banks. The CLUSTAL programme (Higgins et al., 1992) was used for alignment of the deduced amino-acid sequences of chitinase gene.

Northern blot analysis

For Northern blot preparation, 10 μg of total RNA were separated under denaturing conditions on a 1.5% agarose gel containing formaldehyde and transferred onto Hybond N^+ membranes (Amersham International, UK) following standard techniques (Maniatis et al., 1982). The cDNA fragments used for hybridizations were labeled with [α-^{32}P]dCTP with the Pharmacia 'Ready to go' labeling kit. Probes were sunflower ubiquitin (Binet et al., 1989), PAL (Mazeyrat et al., 1998a) and chitinase.

Results

Microscopic observations

Susceptible plants. In cotyledons, 48 h after secondary infection, fungal structures (mycelium and haustoria) were found throughout the cotyledons (Figure 1A). The cells containing haustoria had a normal appearence and UV-fluorescence was not observed at this stage. Fifteen days after infection, invasion appeared systemic.

Resistant plants. Forty eight hours after infection, an intense fluorescence was observed at the infection sites (Figure 1B). Seventy two hours after infection, resistant plants presented a hypersensitive reaction with characteristic fluorescent necroses and the adjacent cells fluoresced (Figure 1C). At this stage, fungal structures could not be observed. Twenty days after infection, the fungus was still not found in the whole plant.

Chitinase cDNA amplification

With the combination of degenerated oligonucleotides used in PCR amplification, a 420 bp fragment was cloned. This 420 bp fragment, designated Hachit1, represents a central part of sunflower chitinase cDNA. RACE procedures were applied yielding a full-length cDNA in two steps. Amplification of a 671 bp fragment representing the 3′ end of sunflower chitinase cDNA was obtained using a sequence-specific primer based on sequence data from Hachit1 following the

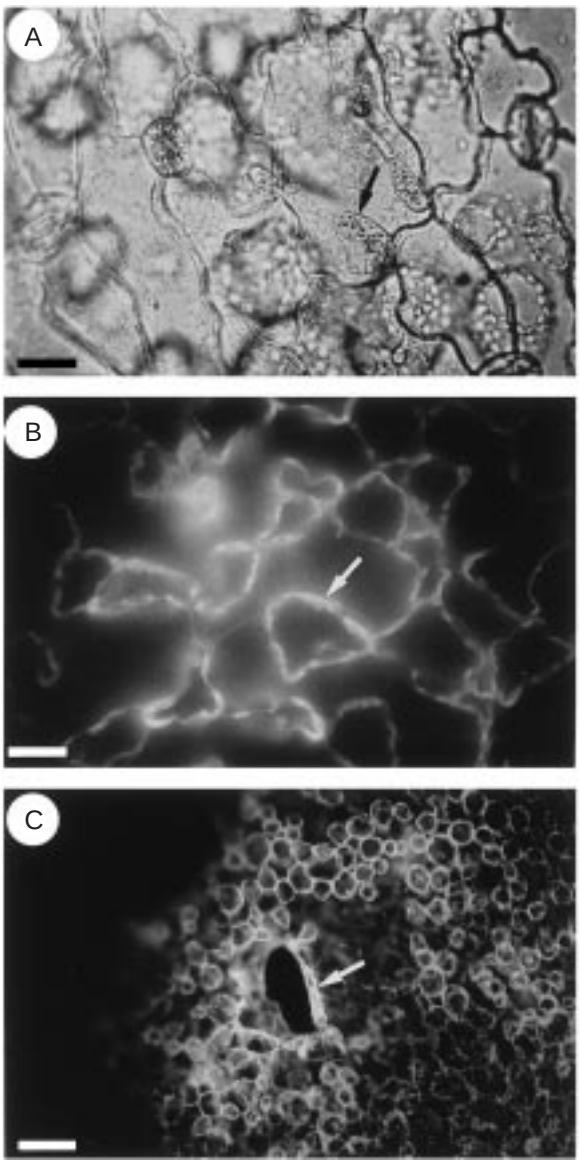

Figure 1. Section of downy mildew infected cotyledons. *Susceptible* (A) 48 h after infection the fungus is found in cotyledons (arrow) (scale bar = 145 μm). *Resistant*: UV fluorescence micrographs of resistant sunflower seedlings. (B) 48 h after infection, the cells which react hypersensitively fluoresce (arrow) (scale bar = 36 μm). (C) 72 h after infection, fluorescence concerned several cells around the necrosis (arrow) (scale bar = 36 μm).

RACE protocol. A 773 bp product, representing the 5′ part of chitinase cDNA was obtained, using a sequence-specific primer based on sequence data from Hachit1 following the RACE protocol. The clones were identified as representing the same gene from

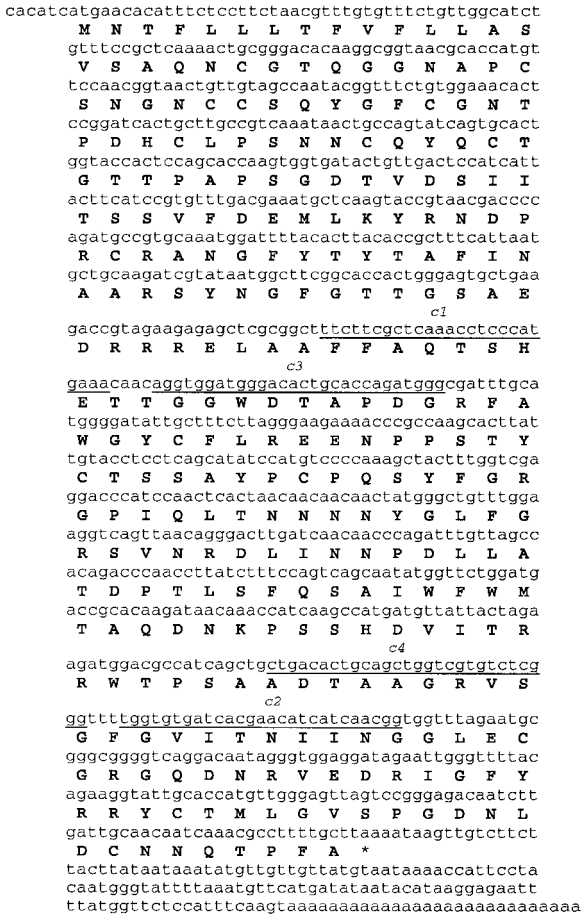

Figure 2. Nucleotide and deduced amino acid sequence of sunflower chitinase (GenBank accession U96640). The four sequences used for primer annealing in PCR amplification are underlined.

the overlapping region. Combination of these two sunflower cDNA sequences gave a 1090 bp cDNA exhibiting an open reading frame coding for a peptide of 309 amino acids (Figure 2) corresponding to a predicted molecular mass of 33 000 Da with calculated isolelectric point of 5.4. The combined cDNA clones obtained revealed a high degree of amino acids sequence identity with previously described endochitinases from pea (Vad et al., 1993), tobacco (Shinshi et al., 1984) and potato (Gaymor, 1988) as shown in Figure 3.

Kinetics of mRNA accumulation after infection

RNA samples from sunflower hypocotyls isolated at different time points following infection by *P. halstedii* were blotted and hybridized with the PAL (Figure 4A), chitinase (Figure 4B) and ubiquitin (Figure 4C) clones. Expression of these genes was examined in hypocotyl tissue collected 6, 12, 24, 48 and 72 h after infection either with the avirulent race 1 or virulent race B of *P. halstedii*. Loading of equal amounts of RNA was checked with an 18S rRNA probe (Figure 4D). To determine if spraying modulates gene transcript levels, control seedlings were treated with water.

A low level of PAL transcripts representing an approximately 2.3 kb mRNA was detected in sunflower hypocotyls of control plants and also, in the compatible interaction. Inoculation with the avirulent race 1 resulted in the rapid accumulation of PAL mRNA with a maximum amount of mRNA 24 and 48 h after infection.

Chitinase mRNA accumulation was detectable 6 h after infection in the incompatible combination with a maximum level at 24 and 48 h. Accumulation of chitinase mRNA was also detected 48 h after infection in the compatible combination, but at this time point, the incompatible combination exhibited the highest levels of transcript accumulation.

Northern blot analyses of ubiquitin mRNA from sunflower hypocotyls harvested at different time points after infection revealed two different sizes of mRNA with about 1600 and 1300 bases, but the first one was generally most abundant. However, in the incompatible combination, 24 and 48 h after infection, the 1.3 kb ubiquitin transcript was strongly accumulated. In contrast, these 1.3 kb transcripts remained poorly expressed, as for the control, in the compatible combination.

Discussion

Microscopic observations indicate that resistance reactions lead to inhibition, isolation and death of the fungus. Cells in contact with fungus develop a hypersensitive reaction with fluorescent compounds. These cells collapse and their walls form a solid mass around the fungus. These results agree with those of Mouzeyar et al. (1993) who found hypersensitive-like reactions after primary infection. However, these reactions appear more rapidly after secondary infection. Thus, it appears that in a resistant response, invading hyphae come into contact with an antimicrobial environment soon after infection (penetration). Until now, little is known about the molecular processes underlying this incompatible response in sunflower.

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sunflower :  --------MNTFLLLTFVFLLASVSAQNCGTQGGNAPCSNGNCCSQYGFCGNT
pea       :  ---MKRTLKVS.FI.CLLP.FLGSK.EQ..S.A.G.V.P..L...KF....S.
tobacco   :  ----------------SL..L.A..EQ..S.A.G.R.AS.L...KF.W....
potato    :  MRRHKEVNFVAY..FSLLV.VSAAL.....S...GKA.AS.Q...KF.W....

sunflower :  PDHCLPSNNCQYQCTG-----TTPAPS---GDTVDSIITSSVFDEMLKYRNDP
pea       :  DPY.G--DG..S..KS-SPTP.I.T..TGG.-D.GRLVP..L..Q.......G
tobacco   :  N.Y.G.-G...S..P.G----P..PGG----GDLG...S..M..Q...H...N
potato    :  N.Y.GS-G...S..P.GG---PG.G.G----GDLG.A.SN.M..Q...H..EN

sunflower :  RCRANGFYTYTAFINAARSYNGFGTTGSAEDRRRELAAFFAQTSHETTGGWDT
pea       :  ..AGH.....D...A....F.......DDNTKKK.....L...........P.
tobacco   :  A.QGK...S.N........FP....S.DTTA.K..I..............A.
potato    :  S.QGKN..S.N........FP....S.DINA.K..I..............AS

sunflower :  APDGRFAWGYCFLREENPPSTYCT-SSAYPCP--QSYFGRGPIQLTNNNNYGL
pea       :  ....PY......VS.Q.TQEV..S-PKDW..APGKK.Y........H.Y....
tobacco   :  ....PY.....W...QGS.GD...P.GQW..APGRK........ISH.Y...P
potato    :  ....PY.........RGN.GD..PP..QW..APGRK........ISH.Y...P

sunflower :  FGRSVNRDLINNPDLLATDPTLSFQSAIWFWMTAQDNKPSSHDVITRRWTPSA
pea       :  A.QAIKE.........S.N..V..KT.......P.A..........G......
tobacco   :  C..AIGV..L.....V....VI..K..L.....P.SP...C....IG..Q..S
potato    :  C..AIGV..L.....V....VI..KT.L.....P.SP...C....IG..N..S

sunflower :  ADTAAGRVSGFGVITNIINGGLECGRGQDNRVEDRIGFYRRYCTMLGVSPGDN
pea       :  ..SS....P.Y..........I...H......D..V...K...QIF..D..G.
tobacco   :  ..R..N.LP..................T.S..Q..........SI........
potato    :  ..R..N.LP..................T....Q..........SI...T....

sunflower :  LDCNNQTPFA--------
pea       :  ......RS..--------
tobacco   :  ...G..RS.GNGLLVDTM
potato    :  ...V..RW.GNALLVDTL
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Figure 3. Comparison of predicted amino acid sequence of sunflower chitinase with the homologous proteins from pea (Vad et al., 1993), tobacco (Shinshi et al., 1984), potato (Gaymor, 1988). Dots represent amino acids that are identical to the protein predicted from the sunflower chitinase cDNA and hyphens represent gaps introduced to optimize sequence alignment.

In these studies, we isolated a complete cDNA of a chitinase. An amino acid sequence deduced by sequence analysis of this cDNA is highly homologous with endochitinase reported previously (Vad et al., 1993; Shinshi et al., 1984; Gaymor, 1988) as shown in Figure 3.

Following aerial infection of sunflower with zoospores of *P. halstedii*, an induction was observed in the quantity of PAL, chitinase and ubiquitin mRNA. In the incompatible interaction, a large increase in amounts of PAL, chitinase and ubiquitin mRNA was observed 24 and 48 h after infection, whereas, in the compatible combination, levels of mRNA apparently reflect the pattern observed for control or exhibit a lower level of accumulation. It can therefore be assumed that regulation of the amount of these mRNAs is an important component of the control of resistance mechanisms.

The strong induction of PAL in sunflower hypocotyl after infection with *P. halstedii* suggests that the enzyme is transcriptionally regulated. It can be assumed that regulation of mRNA is an important component of the control of the phytoalexin defense response in sunflower (Gutierrez et al., 1995). A similar conclusion has been reached in other plant-parasite systems (Habereder et al., 1989; Kervinen et al., 1997). Transgenic tobacco plants with suppressed PAL enzyme activity by anti-sense gene constructs have generally shown increased disease susceptibility after infection by the virulent fungal pathogen *Cercospora nicotianae*, thus providing direct evidence that PAL is an important component in disease resistance (Maher et al., 1994).

Accumulation of chitinase mRNA was also detected in the incompatible combination. Cachinero et al. (1996), have shown an increase of chitinase activity in

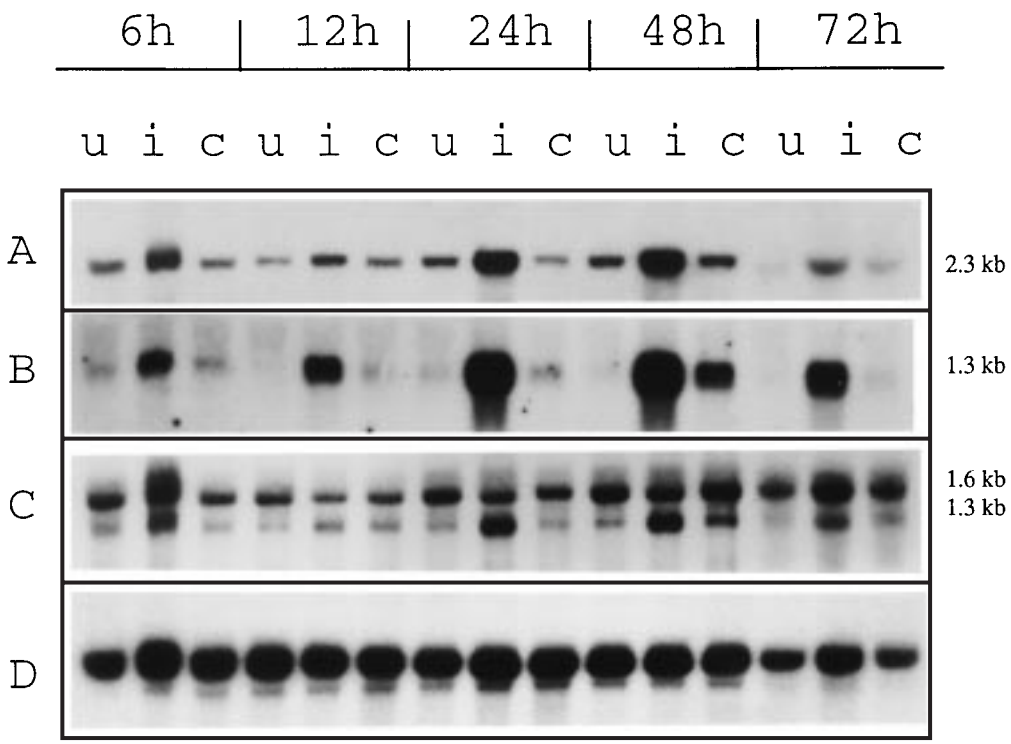

Figure 4. Northern blot hybridization of total RNA from *Helianthus annuus* hypocotyls infected with *Plasmopara halstedii* (i) incompatible interaction with race 1; (c) compatible interaction with race B) and uninfected (u) at the times indicated (hours). 10 μg of total RNA were separated on formaldehyde agarose gels, blotted and hybridized with PAL cDNA (A), chitinase cDNA (B) or ubiquitin cDNA (C). Equal loading of RNA was confirmed by hybridization with a cDNA probe corresponding to the 18S ribosomal RNA (D).

hypocotyls of sunflower infected by *P. halstedii*, earlier in incompatible than in compatible reactions. Bell et al. (1986) have shown, in bean, significant induction of defense genes not only in directly infected sites but also in regions of the hypocotyl at a distance from the infection site, implying some sort of intercellular signaling mechanism. As suggested by Templeton and Lamb (1988), it is possible that components released by chitinase action are involved in a signal transduction pathway.

The ubiquitin mRNA (1.3 kb transcript) pattern is apparently similar to those of PAL and chitinase. Accumulation of this 1.3 kb mRNA was also detected in the incompatible combination. One major function of ubiquitin is to participate in a complex and highly selective protein degradation pathway (Viestra, 1987). Expression of polyubiquitin genes has been reported after elicitor and exogenous H_2O_2 treatment (Levine et al., 1994). Polyubiquitin is required for the recycling of damaged proteins. A dominant negative ubiquitin transgene causes spontaneous hypersensitive lesions (Becker et al., 1993) suggesting that ubiquitin also functions in regulation of programmed cell death (Hammond-Kosack and Jones, 1996).

Different timing of PAL, chitinase and ubiquitin mRNA accumulation is thus observed between compatible and incompatible combinations. In the incompatible interaction the rapid accumulation of the mRNA is specifically induced by an avirulent race of *P. halstedii* and is not a result of nonspecific stress caused by colonization of plant by the fungus. The rapid induction of these mRNAs could be the result of recognition by the plant of a race-specific elicitor produced by the fungus. In contrast, in the compatible interaction, the fungus is not inhibited and massively invades host tissue. The stress induced by fungal growth may cause a slow and delayed induction of mRNA accumulation. Differential expression of defense genes during compatible and incompatible plant–pathogen interactions is frequently observed (Hammond-Kosack and Jones,

1996; Knogge, 1996). Therefore, the mechanisms of plant defense not only include different types of gene expression (Mazeyrat et al., 1998b), but also develop specific sequences of events in time.

The present results suggest that PAL, chitinase and ubiquitin should be good markers of resistance after secondary infection of sunflower by *P. halstedii*. This should help to identify new genes activated in resistance and in the long run, to determine the molecular basis of sunflower defense mechanisms against *P. halstedii*.

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