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The Expression of Genes Controlling the Mechanisms of Defence of Sunflowers against Downy Mildew (*Plasmopara halstedii*)

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Abstract

The resistance reaction of sunflowers to downy mildew is characterised by a hypersensitive reaction with necroses in the hypocotyl, lignification and an increase in total peroxidasic activity. The aim of this study was to identify some of the genes involved and the variation in time of their activity. Effects of both primary downy mildew infections were observed, after 10 min. contact between germinating sunflower seeds and *P.halstedii* zoospores. A search was made for difference in activity of genes coding for ubiquitin, high and low molecular weight Heat Shock Proteins (HSP17.6, HSP 17.9, HSP 70) and calmodulin according to sunflower resistance or susceptibility. Northern blot analyses did not show any differences in gene activity according to sunflower downy mildew reaction. A cDNA, obtained by amplification of Phenylalanine Ammonia Lyase was also used. The differential display technique should make it possible to identify other genes involved in sunflower defence mechanisms against downy mildew.

Introduction

Sunflower downy mildew is caused by the obligate parasite *Plasmopara halstedii*. Resistance is controlled by major genes, noted *Pl*. Histological and biochemical studies have shown that, in the case of incompatible reactions (resistance), sunflower plants show hypersensitivity and localised necroses. These reactions are associated with cellular lignification and a considerable increase in total peroxidase activitiy (Mouzeyar *et al.*, 1994). However, whilst the histochemical reactions are now well known, few molecular studies have been made. The aims of this study were to identify some of the genes involved in resistance mechanisms, to characterise expression kinetics and then to define the periods during the infection process when resistant plants show different reactions compared with plants that are susceptible, or treated only with pure water.

The downy mildew infection technique described by Cohen and Sackston (1973), generally used for determining resistance or susceptibiilty, requires 4h contact between sunflower radicles and the *P.halstedii* zoosporangia suspension. To study early induction of gene expression following contamination, the first step was to develop an infection method with reduced contact time between sunflower radicles and *P.halstedii* zoosporangia suspensions.

Molecular characterisation of disease interactions was undertaken by studies of the expression of genes that are known to be involved in plant responses to stress. In particular, hypersensitive reactions are associated with cellular lignification, so a study was undertaken of the expression of

PAL (Phenyl Alanine Lyase), as this is the first enzyme intervening in the biosynthesis of phenyl propanoids which lead to the formation of phytoalexins and lignins. More recently, a search for genes that are specifically induced during infection, using the technique of Differential Display Reverse Transcription PCR (DDRTPCR) (Liang and Pardee, 1992) has been undertaken.

Materiels and methods

Sunflower genotypes and *P.halstedii* isolates :
Infection methods were developed on the sunflower population Peredovik (susceptible to all downy mildew races), and the inbred lines RHA266 (resistant to downy mildew, race 1 only) and RHA274 (resistant to races 1 and 2, but susceptible to race B). For the molecular studies, the sunflower genotype was an experimental INRA hybrid EL64, resistant to downy mildew race 1, but susceptible to race B. Disinfected seed were germinated for 48h at 24°C. The downy mildew isolates of races 1 and B are maintained at INRA, Clermont-Ferrand or at G.E.V.E.S., Angers.

Infection procedure :
A modifed form of the protocol of Cohen and Sackston (1973) was used. The germinated seed were soaked in a suspension of zoosporangia that had been prepared two hours earlier, for 10 min. They were then rinsed twice in distilled water and placed on damp filter paper in a growth chamber (at 18°C, 60% humidity, 16h photoperiod, light intensity 200µE/m²/s). The radicles were harvested 0, 10 or 40 min., or 1, 6, 12, 24 or 48h after infection.

Total RNA extraction, transfer and hybridization techniques :
The extraction procedure was that described by Bogord *et al.* (1983) and optimised for sunflower RNA : precipitation time in ethanol was 5h and, to obtain better purification, a precipitation in 3M sodium acetate was also carried out. Ten to twenty µg of total RNA were separated by electrophoresis on 1.5% denaturing agarose gel at 50mA for 3 to 4h. The RNA were then transferred to Nylon Hybond H+ membranes (Amersham) in the presence of 4X SSPE pH 7.7. The RNA were fixed to the membrane by a few seconds irradiation with U.V. light (Spectrolinker).

The cDNA probes used for the hybridisations (Ubiquitin, Touch1 (TCH1 = calmodulin), Heat Shock Protein (HSP).17.6, HSP 17.9, HSP70) were labelled with ^{32}P-dCTP with the Pharmacia kit "Ready-to-go". Prehybridisation was carried out for 3h. at 42°C, followed by hybridization for one night at 72°C. Finally, the membranes were rinsed twice for 15 min. in 2X SSPE at 42°C and then placed in cassettes in the presence of MP hyperfilm (Amersham) at -80°C.

Production of sunflower PAL cDNA :
Inverse transcription was obtained by mixing 10µg total RNA with 5µg oligo dT12 at 70°C for 10 min., then adding 1mM dNTPs, 20u RNAsine, 20u reverse transcriptase AMV and incubating for 60 min. at 42°C. Part of the cDNA obtained was amplified by PCR with degenerate PAL primers. Amplification was carried out in an reaction volume of 50µl containing 1µM primer, 1µM dNPTs and 2u Taq polimerase, with 1 cycle at 92°C for 8 minutes, followed by 30 cycles of 1.5 min. at 92°C, 1.5 min. at 52°C, 30 sec. at 72°C and a final extension at 72°C for 15 min. The product of PCR was cloned in a T vector (TA cloning kit, Invitrogen).

DDRTPCR :

The technique was carried out according to the procedure first described by Liang and Pardee (1992). The PCR product (1/3 of the total) were placed on a 5% non-denaturing acrylamide gel. Electrophoresis was carried out in the presence of TBE1X buffer at 300V. The gel was then transferred onto Whatman 3MM paper, dried for 1h at 80°C and exposed to BiomaxMR film (Kodak). Comparison of the electrophoretic profiles, obtained with the same pairs of primers, made it possible to define bands that were specific to resistance or susceptibility. The parts of the gel containing PCR fragments of interest were cut out and rehydrated. The DNA was re-dissolved at 100°C for 15 min., precipitated for 1 night at -20°C in presence of 0.5mg/ml glycogen (Boehringer) and 0.3M sodium acetate in 2.5 volume of pure ethanol, then centrifuged and dissolved in 10µl water. The fragments obtained were then re-amplified by PCR.

Results

Downy mildew infection technique :

Microscopical observation of zoosporangia suspensions showed that intact zoosporangia were visible 2h after suspension preparation and that most zoospores were liberated after about 2.5h. Susceptible sunflower seedlings showed 100% infection when they were placed in contact with this suspension for 10 min. Resistance and susceptibility reactions for the 3 control genotypes, Peredovik, RHA266 and RHA274 to races 1 and B were always those expected. This technique was therefore used for the infections before analyses of DNA and RNA.

Cloning of PAL cDNA :

The use of degenerate primers of PAL in RTPCR resulted in amplification of a 700pb cDNA. The amino acid sequence of this cDNA showed a minimum of 88% identity and 95% similarity with those of cDNA clones of parsley PAL (Lois *et al.* 1989), tobacco (Pellingrini *et al.*, 1994) and potato (Joos and Hahlbrook, 1992).

Accumulation of mRNA :

Total RNA extracted from sunflower radicles at different times after infection with either downy mildew race 1 or race B were analysed. In general, the quantity of transcripts was quite large during the first 24h in all types of plants, resistant, susceptible or control. However, 10 min. after infection, there was a reduction in PAL mRNA in resistant plants before an increase in this concentration in both resistant and susceptible plants after 24h (fig. 1 and fig. 2). Other hybridizations, carried out with heterologous (TCH1, HSP70) or homologous (ubiquitin, HSP17.6, HSP 17.9) probes did not provide any evidence for any special role of these genes in resistance reactions.

DDRTPCR :

The limits of the results presented above led us to develop DDRTPCR on sunflower RNA. With only a small number of combinations of primers so far tested, it has already been possible to detect differential PCR fragments. Some may be induced during both compatible and incompatible reactions, whereas others are inactivated by these reactions. Cloning and analysis of expression are in progress.

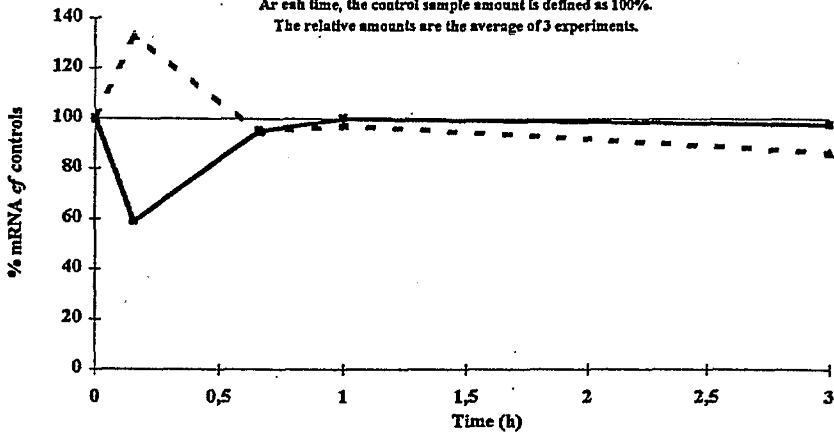
Fig 1 : Time course of PAL mRNA accumulation during interaction by P. kalstedii
Ar eah time, the control sample amount is defined as 100%.
The relative amounts are the average of 3 experiments.
% mRNA of controls
140
120
100
80
60
40
20
0
0
0,5
1
1,5
2
2,5
3
Time (h)

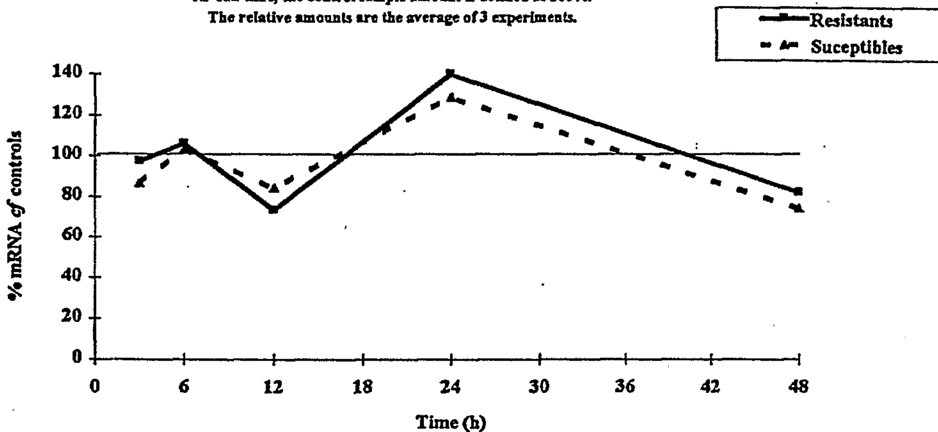
Fig 2 : Time course of PAL mRNA accumulation during interaction by P. kalstedii.
Ar eah time, the control sample amount is defined as 100%.
The relative amounts are the average of 3 experiments.
Resistants
Suceptibles
% mRNA of controls
140
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Time (h)

Discussion

The preliminary study made it possible to reduce the time of contact with the *P.halstedii* zoosporangia suspension required for infection of germinated sunflower seeds. This was necessary to permit study of genes intervening in the first stages of resistance/susceptibility reactions. Accumulation of mRNA of some defence genes in other species has been found to be very rapid. For example, in *Phaseolus* beans, 5 minutes after a treatment with an elicitor, there was accumulation of mRNA from PAL, CHS (Chalcone synthase), and CHI (chalcone isomerase) genes (Hedrick *et al.*, 1988). The present study showed that after the 2h necessary for liberation of zoospores from zoosporangia, a contact time of 10 minutes was sufficient to give infection. This indicates that zoosporangia can penetrate radicles less than 10 minutes after their liberation.

Using degenerate primers, by RTPCR, a 700bp fragment of cDNA clone coding PAL was obtained. Its amino acid sequence shows close identity with those of PAL clones from many plant species. Two potential sites of glycosylation are present on sunflower cDNA, suggesting that sunflower PAL is glycosylated in the same way as potato PAL, during its maturation in the Golgi bodies (Shaw *et al.*, 1990).

Hybridizations carried out with the PAL cDNA probe demonstrated a 2.5kb transcript. Ten minutes after downy mildew infection, the level of PAL mRNA was greater in susceptible plants than in those with efficient resistance genes. This is unusual, since generally, there are greater, or earlier, increases in RNA in resistant plants than in susceptible ones during the infection process. For example, infection of soya-bean roots with *Phytophthora megasperma* caused an increase in PAL RNA and an increase in their transcriptions. For the resistant soya-bean plants, this increase began 1 to 2h after inoculation, whereas, in susceptible plants, the reaction occurred only after 9h (Habereder *et al.*, 1989). During primary infection of sunflowers by *P.halstedii*, lignin synthesis was observed only 5 days after infection in resistant plants (Mouzeyar *et al.*, 1993), so it is possible that, in the present case, PAL mRNA is involved at a later stage than in soya-bean. Osakabe *et al.* (1995) cloned different PAL genes. These authors measured the mRNA content of 4 PAL genes in stems of *Populus kitakamiensis* and showed that the level of transcription of *palg2b* was greater than that for other PAL genes. Thus, it appears that different forms of PAL may exist, with differential activity.

The different hybridisations carried out have made it possible to distinguish certain times after downy mildew infection when there is a slight difference of expression between resistant and susceptible plants. These times were therefore selected for application of DDRTPCR. The aim of the procedure was to isolate genes specifically expressed during defence reactions of sunflower seedlings to downy mildew infection. The DDRTPCR method makes it possible to compare, quite rapidly, different physiological conditions, in particular the profiles of resistant and susceptible plants. The use of this technique led to definition of the conditions in which the profiles obtained are reproducible. However, non-denaturing gels must be used because it has been shown that the use of sequencing gels could create profiles in which the bands were doubled (Bauer *et al.*, 1993).

This technique has made it possible, from only a few combinations of primers, to isolate differential PCR fragments. The next step will be to clone these fragments and then it should be possible to obtain complete cDNA clones by rapid amplification of cDNA ends. The expression of these genes will be studied to determine whether they are induced or inhibited when defence mechanisms are activated. The identification of genes involved in these mechanisms should help to obtain a better understanding of the nature of resistance of a plant in response to infection.

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