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BRIEF COMMUNICATION

Specific activation of *PR-10* pathogenesis-related genes in apple by an incompatible race of *Venturia inaequalis*

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Abstract

Apple (*Malus* × *domestica* Borkh. cv. Golden Delicious) trees were inoculated with fungal pathogens, corresponding either to an incompatible strain of *Venturia inaequalis* or to non-host pathogens (*Venturia pyrina, Alternaria brassicicola*) in order to characterize the regulation of *PR-10* genes in these different situations in relationship to symptom development. Macro- and microscopic observations of the plant-fungus interactions revealed typical symptoms of resistance with *V. inaequalis* and symptoms of hypersensitivity (HR) on around 5 % of leaves with *V. pyrina.* No HR was microscopically observed with *A. brassicicola*. In the non-host situations, the *PR-10* expression in leaves at the transcriptional level was not (*A. brassicicola*) or very slightly (*V. pyrina*) activated. This strongly suggests that *PR-10* is not a component of the non-host resistance in the interactions studied here. By contrast specific up-regulation of *PR-10* was evident after inoculation with *V. inaequalis*.

Additional key words: *Alternaria brassicicola*, resistance mechanisms, *Malus* × *domestica*, *Venturia pyrina*.

The multigenic PR-10 family pools intracellular acidic proteins (16 - 19 kDa) that were identified and characterized in numerous plant dicots and monocots of agronomic importance (van Loon *et al.* 1994, Pinto *et al.* 2005), including apple (Pühringer *et al.* 2000, Ziadi *et al.* 2001). The biological function of PR-10 proteins is not clearly defined, although functional characteristics have been suggested for some PR-10 proteins. Ribonuclease activity has been associated with PR-10 proteins from different plant species, while the binding of ligands of various nature to PR-10 proteins was shown (Liu and Ekramoddoullah 2006). To our knowledge, no information about the function of PR-10 proteins identified in apple is currently available. Another important feature of *PR-10* genes is their activation following biotic stress due to pathogens of different nature, including numerous phytopathogenic fungi, and this also holds true in apple when using fungal elicitors from *Botrytis cinerea* (Pühringer *et al.* 2000) or during infection by the scab agent *Venturia inaequalis* (Poupard *et al.* 2003). It was hypothesized that PR-10 proteins may have a role in local and systemic plant defence mechanisms and previous studies on different plant-fungus pathosystems in compatible/incompatible situations have led to the assumption that PR-10 proteins could participate in plant resistance in incompatible interactions (Constabel and Brisson 1992, Jwa *et al.* 2001, Mould *et al.* 2003, Steiner-Lange *et al.* 2003, Chen *et al.* 2006), with some exceptions (Truesdell and Dickman 1997).

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Abbreviations: Ct - cycle threshold; HR - hypersensitive response; ITS - internal transcribed spacers; PR - pathogenesis related; SEM - scanning electron microscopy.
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In a previous paper (Poupard *et al.* 2003), we demonstrated a differential expression pattern of a *PR-10* gene subclass (named *APa*) after inoculation of apple leaves with a compatible or an incompatible strain of *V. inaequalis*. Our study involved a gene-for-gene interaction pathosystem (defined in Bénaouf and Parisi 2000) with the apple cv. Golden Delicious (carrying the major resistance gene *Vg*) exhibiting a specific interaction with the avirulent strain 1066 (race 7) of *V. inaequalis* (carrying the avirulence gene *AvrVg*). Up-regulation of *APa* gene products was observed later in the incompatible situation than in the compatible one, but higher transcript and protein levels were observed in the former case. However, the relationship between the induction of *PR-10* genes and the resistance mechanisms in cv. Golden Delicious against *V. inaequalis* requires further investigation. Plants may respond in similar ways to host and non-host pathogens (Thordal-Christensen 2003). Moreover, some non-host resistance situations can involve gene-for-gene interactions similar to those that govern parasite-specific resistance within host species (Heath 2001). Here the expression patterns of two *PR-10* gene subclasses that we characterized previously (Ziadi *et al.* 2001) were analyzed in host and non-host situations with fungal pathogens. In the same way, the macro- and microscopic characterization of the different plant-fungus interactions was achieved. The interaction between Golden Delicious and the avirulent strain 1066 of *V. inaequalis* was compared with two non-host situations, corresponding to Golden Delicious-*Venturia pyrina* or Golden Delicious-*Alternaria brassicicola.* Like *V. inaequalis*, *V. pyrina* is a hemibiotrophic scab pathogen of *Rosaceae* causing symptoms on pear leaves and fruits, whereas *A. brassicicola* is a necrotrophic fungal pathogen responsible for black-spot disease in cultivated plants of the *Brassicaceae* family.

The apple *Malus* × *domestica* (Borkh.) clone X 972 of cv. Golden Delicious grafted on MM106 and originating from the INRA collection at Angers, France (Unité d'Amélioration des Espèces Fruitières et Ornementales), was used. The grafted potted trees were grown in the greenhouse (photoperiod of 12 h, temperature 20 ± 5 °C, air humidity 60 - 80 %) for 6 weeks until leaves on actively growing shoots developed. For infection with *V. inaequalis* (Cooke) Wint, the strain 1066 of race 7 (isolated from *Malus floribunda* 821 in 1993, origin: Beaucouzé, France) avirulent for the cv. Golden delicious was used. For infection with *V. pyrina* Aderh. or *A. brassicicola* (Schw.) Wilts, strain P101 (isolated from cv. Passe-Crassane of *Pyrus communis* L. in 2000, origin: Beaucouzé, France) and strain 43 (isolated from *Raphanus sativus* L. in 1999, origin: Lot et Garonne, France) were used, respectively. For *V. inaequalis* and *V. pyrina* inoculum, conidial suspensions were obtained by rinsing dried scabbed leaves with distilled water. A conidial suspension of *A. brassicicola* was obtained by scraping fungal cultures in Petri dishes. All conidial suspensions were adjusted to 2.5×10^5 conidia cm^{-3} and inoculation was performed in a growth chamber under the same conditions as those described by Parisi *et al.* (1993). Fifteen trees were inoculated per treatment (control trees were sprayed with distilled water). For RNA analysis, the two youngest inoculated leaves were collected at different times (48, 96, 144 and 192 h) after inoculation. Three independent experiments were conducted in the growth chamber to ensure that the expression patterns were repeatable. The macroscopic symptoms were scored 13 d after inoculation using the scale of Chevalier *et al.* (1991) on a total of 200 leaves per treatment. For scanning electron microscopy (SEM), leaf discs collected 6 d and 13 d after inoculation were treated according to Chevalier *et al.* (1991) and examined using a *JEOL JSM 6301-F* (Croissy sur Seine, France) scanning electron microscope with a field emission gun. For histological studies, leaf samples collected 6 d and 13 d after inoculation were fixed, dehydrated (Chevalier *et al.* 1991) and embedded in *Technovit 7100* resin (Heraeus Kulzer, Wehrheim/Ts., Germany) according to Kroes *et al.* (1998). Sections of 1.5 µm cut with a *Leica* (Wetzlar, Germany) *RM 2165* microtome were stained with toluidine blue and examined using an *Olympus* (Rungis, France) *BH2 RFC* microscope.

Extraction of total RNA and Northern blot hybridization were carried out according to Ziadi *et al.* (2001). The ^{32}P labeled RNA probe corresponds to a 221 bp fragment specific to gene members of the *APa* or the *APb* subclass (Ziadi *et al.* 2001). The standardization of the experiments using the expression of *18S* RNA and the analysis of RNA blots were performed as described in Roux *et al.* (2006). Reverse transcription of mRNA was carried out using oligo-d(T) and random priming. Real-time PCRs were performed according to the manufacturer's instructions (*Applied Biosystems*, Courtaboeuf, France). The primer sequences were: *AP1a*-sense (5'-CATGGGTGTTTTCACATACGAATC-3') with *AP1a*-anti-sense (5'-TTCACTGCTTGTGGTGCA ATC-3'), *AP2b*-sense (5'-ACCCCAGGCAATCAAG CAT-3') with *AP2b*-anti-sense (5'-CTTGTGCTTCAC GTAGCCGTAT-3'), *AP3b*-sense (5'-CCCCAGGCAATC AAGCAA-3') with *AP3b*-anti-sense (5'-CTGTGCTTC ACGTAGCCGTACT-3'), *AP4a*-sense (5'-CATCCCCCC TGCTAGGTTGTA-3') with *AP4a*-anti-sense (5'-CAC TGCCTGTGGTGCAATTTTA-3'), and *ITS*-sense (5'-CGTCGTCGTCTTCGATAAGTCA-3') with *ITS*-anti-sense (5'-GGATTCTGCAATTCACACCAAGT-3'). Each experiment was repeated three times with two different RNA samples. The results were normalized using Cycle threshold (Ct) values obtained for ribosomal *ITS* RNA amplifications run on the same plate. The relative quantitation analysis was performed using the comparative ΔΔCt-method as described by Winner *et al.* (1999). The amount of target in assays relative to the control is represented as $2^{-\Delta\Delta Ct}$.

First we investigated the development of the different fungal species on apple leaves both macro- and microscopically. Considering the interaction between the apple and *V. inaequalis*, the visual assessment of symptoms performed 13 d after inoculation confirmed

that strain 1066 was incompatible, with 18.8 % of leaves showing resistance symptoms of class 2 (10.4 %, chlorosis and/or necrosis without sporulation) or of class 3a (8.4 %, chlorosis and/or necrosis with slight sporulation) according to the scale of Chevalier *et al.* 1991. When apple tree was inoculated with non-host pathogens, no symptoms (class 0) or symptoms of hypersensitivity (class 1, pin-point pits) were observed, with percentages of leaves showing pin-points of 1.2 and 4.7 % with *A. brassicicola* and *V. pyrina* (Fig. 1*A*), respectively. On the basis of histological and SEM observations, an intense necrotic resistance reaction of leaf cells was observed at 6 d post-inoculation with *V. inaequalis.*At 13 d post-inoculation, a large network of hyphae without conidiophores and showing a necrotic aspect was evident (results not shown). At 6 d after inoculation of apple with *V. pyrina*, the germination of conidia, sometimes with the presence of branching germ tubes, and the formation of appressoria were observed (Fig. 1*B*). No subcuticular stroma was present. At day 6 post-inoculation the upper epidermis and palisade parenchyma cells fully collapsed, whereas the spongy parenchyma and lower epidermis did not undergo any modification (Fig. 1*C*). At the same time, on apple inoculated by *A. brassicicola*, some leaf epidermal cells under the germinated conidia were superficially modified, but did not collapse (Fig. 1*D*). No HR could be microscopically observed.

In the following step, considering the different plant-fungus interactions, the expression of *PR-10* genes in apple leaves was studied by Northern blot analysis corresponding to transcripts of the *APa* subclass or the *APb* subclass. Data of *APa* and *APb* expression were normalized to the expression of *18S* RNA (Figs. 2*A,B*). At 48 and 96 h post-inoculation, no induction of gene expression was observed in the different experimental conditions. At 144 h and 192 h post-inoculation, the expression of *APa* and *APb* transcripts was activated in the presence of *V. inaequalis* (about 4-fold and 3-fold compared to the control plants for *APa* and *APb* transcripts, respectively). Lower relative levels of expression were obtained with *APb* than with *APa* mRNAs. Considering the non-host situations, *i.e.* inoculation with *V. pyrina* or *A. brassicicola*, the contents of *APa* or *APb* transcripts were quite similar to those observed in the non-infected control plants. When analyzing RNA samples from two other experiments performed in the same conditions, similar results than those presented above were obtained. In order to more accurately study the gene expression in the host- and non-host situations, we independently analyzed the levels of expression of four *PR-10* transcripts characterized in a previous paper (Ziadi *et al.* 2001), two belonging to the *APa* subclass, *AP1a* (*Ypr10*Md.b*) and *AP4a* (*Ypr10*Md.d*), and two belonging to the *APb* subclass, *AP2b* (*Ypr10*Md.a*) and *AP3b* (*Ypr10*Md.c*). The expression results obtained by real-time PCR in plant samples collected 192 h after inoculation are presented in Fig. 2*C*. The results are expressed as factors of gene induction in leaves inoculated with a fungal strain compared with the control. After inoculation with *V. inaequalis,* high relative levels of *AP1a*, *AP4a* and

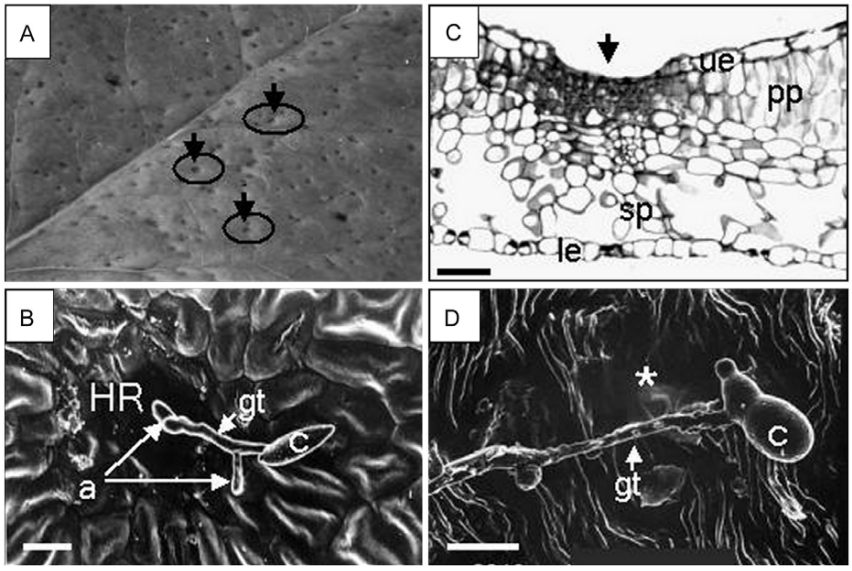

Fig. 1. *A* - Hypersensitive response (pin-points indicated by *arrows*) observed on Golden Delicious leaves at 13 d post-inoculation with strain P101 of *V. pyrina*. *B* - Scanning electron micrograph showing a germinating conidium of *V. pyrina* strain P 101 on an apple leaf at 6 d post-inoculation. The underlying epidermal leaf cells fully collapsed, leading to a hypersensitive response (HR); *bar* = 10 µm; a - appressorium, c - conidium, gt - germ tube. *C* - Histological aspect of a pin-point (*arrow*) in an apple leaf at 6 d post-inoculation with *V. pyrina* strain P 101. The upper epidermis collapsed and the palisade parenchyma was strongly necrotic; *bar* = 25 µm; le - lower epidermis, pp - palisade parenchyma, sp - spongy parenchyma, ue - upper epidermis. *D* - Scanning electron micrograph of a germinating *A. brassicicola* conidium (strain 43) on an apple leaf at 6 d post-inoculation. The underlying epidermal cell surface did not collapse and was only slightly modified (*asterisk*); *bar* = 10 µm; c - conidium, gt - germ tube.

AP3b mRNAs were obtained (15- to 30-fold that of control), whereas the relative level of *AP2b* mRNA was slightly increased (less than 3-fold that of control). The expression of *AP1a*, *AP4a*, *AP2b* and *AP3b* was activated at a low level (about 2-fold that of control) in leaves inoculated with *V. pyrina* and was not induced in leaves inoculated with *A. brassicicola*.

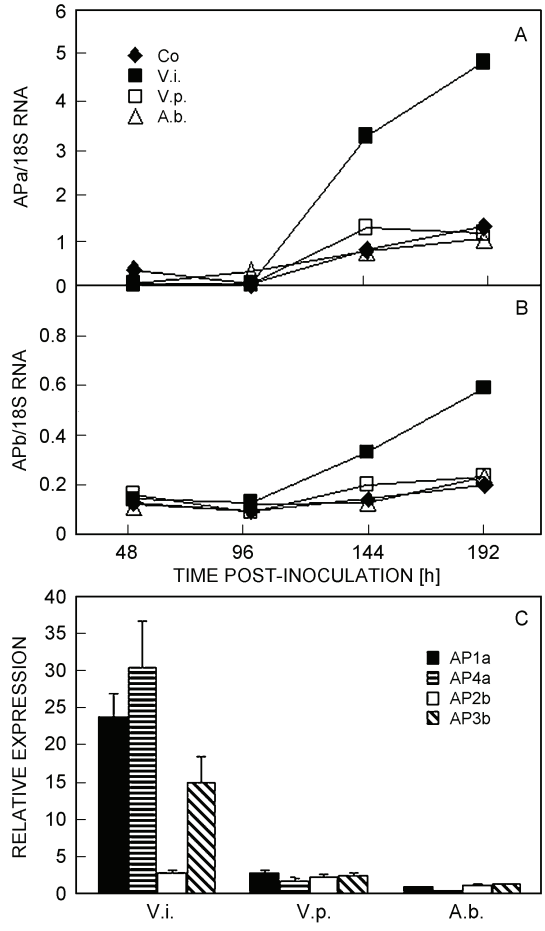

Fig 2. *A, B* - Relative levels of expression of *APa* and *APb* genes determined after Northern blotting analysis of *APa*, *APb* and *18S* RNA genes in apple leaves of cv. Golden Delicious after inoculation with host and non-host fungal strains. The autoradiographic signal intensities for *APa*, *APb* and *18S* RNA were quantified and the ratios of *APa* versus *18S* RNA (*A*) and *APb* versus *18S* RNA (*B*) were determined. V.i. - *V. inaequalis* incompatible strain, V.p. - *V. pyrina*, A.b. - *A. brassicicola*, Co - control leaves sprayed with water. *C* - Relative quantification of *AP1a*, *AP4a*, *AP2b* and *AP3b* transcripts using real-time PCR. Samples from leaves collected 192 h after inoculation with host and non-host fungal strains. The measured transcript quantities were normalized in each sample using the Ct obtained for the *ITS* amplification run on the same plate. The relative quantification analysis was performed using the comparative Ct method. The values (relative expression level) represent the number of times the genes are expressed in inoculated leaves compared with that of leaves treated with water. Values represent the mean ± SD of three repetitions. V.i. - *V. inaequalis* incompatible strain, V.p. - *V. pyrina*, A.b. - *A. brassicicola.*

The aim of this study was to better characterize the regulation of *PR-10* genes in the apple cv. Golden Delicious after a host- or non-host fungal pathogen attack. Our results clearly demonstrated that two *PR-10* gene subclasses, and especially the *APa* subclass, were specifically activated during interaction with an incompatible strain of *V. inaequalis*. Conversely, no *PR-10* gene induction or very low level of gene activation was shown after inoculation with *V. pyrina*, in spite of some hypersensitive reactions on apple leaves observed both at a macroscopic and microscopic level. Expression of *PR-10* gene was not induced in the apple-*A. brassicicola* interaction. Mysore and Ryu (2004) reported the occurrence of two main types of non-host resistance, a most common type showing no visible symptoms, and a second type leading to non-host HR with cell death. From our results, it seems likely that the non-host resistance against *V. pyrina* could belong to this latter type. By contrast, this is unlikely in the case of *A. brassicicola*, as only 1 % of leaves exhibited macroscopic reactions classed as pin points and no HR could be microscopically observed. In the two non-host situations, the fungal development was restricted to the germination of conidia and in the case of *V. pyrina*, the formation of appressoria was observed. It is not known if the fungal penetration occurs in the apple - *V. pyrina* interaction, but if it is the case, the fungus development is certainly quickly stopped because no subcuticular stroma was observed using SEM. In the apple - *A. brassicicola* situation, the lack of leaf penetration by the fungus is highly probable, as a very limited plant response was observed. The non-establishment of a host-pathogen typical relationship can explain the non-induction of *PR-10* genes in non-host interactions studied here.

By contrast to the current work, most previous studies that considered non-host interactions between plants and microorganisms have documented the up-regulation of *PR-10* genes in such situations, especially when considering phytopathogenic bacteria (Swoboda *et al.* 1995, Robert *et al.* 2001). In non-host interactions including fungal pathogens, contrasting results were obtained: results similar to those shown here with apple were obtained in birch leaves inoculated with the birch pathogen *Taphrina betulina* or in the non-host interaction with *Fusarium solani* (Swoboda *et al.* 1995). In sorghum, *PR-10* gene products accumulated faster after plant inoculation with *Cochliobolus heterostrophus*, which is non-pathogenic to sorghum, by comparison with an incompatible interaction with the sorghum pathogen *Colletotrichum sublineolum* (Lo *et al.* 1999). In pea, up-regulation of a *PR-10* gene was shown both in a compatible (*Fusarium solani* f. sp. *pisi*) and a non-host interaction (*Fusarium solani* f. sp. *phaseoli*) (Fristensky *et al.* 1985). These various results concerning *PR-10* expression are in agreement with the diversity of molecular responses suggested by the variability of non-host resistance mechanisms (Thordal-Christensen 2003, Mysore and Ryu 2004) and indicate that, at least in sorghum, *PR-10* genes could contribute to early defence

responses that are triggered to stop the development of a non-host fungus. In other plant-fungus interactions, as reported above in birch or apple, *PR-10* genes may not be part of the non-host resistance or may not be a main component of it. In conclusion, the present study highlighted the specific induction of *PR-10* genes in the apple-*V. inaequalis* pathosystem by comparison to non-host situations involving fungal pathogens. Besides the expression studies described in this paper, investigations to define the exact biological function of apple *PR-10* genes are highly needed to gain further insight into their potential role in plant defences.

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