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In Situ Localization of Rapidly Accumulated Phenylalanine Ammonia-Lyase mRNA Around Penetration Sites of *Phytophthora infestans* in Potato Leaves

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A combination of immunohistochemistry and *in situ* RNA-RNA hybridization enabled the localization of phenylalanine ammonia-lyase mRNA in young potato (*Solanum tuberosum* L. cv. Datura) leaf tissue infected with the late-blight fungus *Phytophthora infestans*. Within a few hours postinoculation, a marked increase in the mRNA concentration, relative to a low but detectable level in uninfected tissue, was observed in a halo of healthy-looking cells around fungal penetration sites. At this early stage of the infection process, fungal penetration sites already appeared as small local lesions caused by hypersensitive cell death. The response was more rapid in incompatible interactions than in compatible ones of a selected potato cultivar, carrying the *P. infestans* resistance gene R1, with appropriate races of the fungus.

Additional keywords: disease resistance, false-color image analysis, phenylpropanoid metabolism.

Phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase catalyze two key reactions of general phenylpropanoid metabolism in higher plants. Increases in the corresponding mRNA levels in pathogen-infected plant tissue may indicate the stimulation of one or more of several defense-related phenylpropanoid pathways (Hahlbrock and Scheel 1987). Fritzemeier *et al.* (1987) have recently reported the rapid accumulation of the two mRNAs upon infection of mature leaves from two selected potato cultivars with corresponding races of *Phytophthora infestans*, causal agent of potato late blight. In a cytological study using the same two potato cultivars, Datura (carrying *P. infestans* resistance gene R1) and Isola (R4), we demonstrated the close spatial association of the invading fungus and hypersensitive cell death in the infected tissue (Cuypers and Hahlbrock 1988).

We were now interested in the precise cellular localization of PAL mRNA accumulation, which had previously been measured in whole leaf extracts and indirectly associated with pathogen defense (Fritzemeier *et al.* 1987). For this purpose, we used the method of *in situ* RNA hybridization for localizing PAL mRNA in cryo-sectioned potato leaves. We chose young leaves of cv. Datura for these studies for the following reasons. First, young leaf tissue proved to be much more amenable to the various treatments involved in the hybridization procedure than the mature leaves used previously. Second, cv. Datura responded more rapidly to inoculation with *P. infestans* spores than cv. Isola (Fritzemeier *et al.* 1987), and young leaves responded more rapidly than mature leaves (Cuypers and Hahlbrock 1988). The site of fungal penetration and hypersensitive cell death was determined, in adjacent leaf sections, by immunohistochemistry and light microscopy by using a previously developed procedure (Cuypers and Hahlbrock 1988).

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MATERIALS AND METHODS

Plant material and inoculation. The potato (*Solanum tuberosum* L.) cv. Datura was grown under controlled conditions in a phytochamber and inoculated with zoospores of *P. infestans* races 1 or 4, as described previously (Cuypers and Hahlbrock 1988). The spore concentration was adjusted to 2–5 $\times$ 10^5 spores ml^{-1}. Droplets of the suspension were applied to the cavities of half-opened leaf buds. These were detached, immediately transferred to a water-containing vial, and incubated at 100% humidity.

Tissue preparation. According to the method described by Schmelzer *et al.* (1988), tissue pieces were taken at the indicated times following inoculation and fixed in buffer containing 2% formaldehyde, 0.2% glutaraldehyde, and 0.1 M sodium phosphate, pH 7.0. The specimens were embedded in Tissue-Tek O.C.T. (Miles) and cryo-sectioned (12 μm). Sections were alternatively taken for immunofluorescence and *in situ* hybridization.

Immunohistochemistry. The fungus was identified by indirect immunofluorescence staining as described previously (Cuypers and Hahlbrock 1988).

Preparation of the RNA probe. The 900-bp *Eco*RI fragment of a potato PAL cDNA (Fritzemeier *et al.* 1987) was subcloned in the Bluescribe vector in both orientations according to conventional techniques (Maniatis *et al.* 1982). The DNA template (500 ng) was transcribed in a mixture of 50 μCi of lyophilized (^{3}H)UTP (1 mCi/ml; 40–50 μCi/mmol), 400 μM each of ATP, CTP, and GTP, 30 mM DTT, 25–40 units of RNAsin, and 10 units of T7 polymerase in transcription buffer (40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl), as recommended by the manufacturer (Vector Cloning Systems). To obtain fragment lengths of 50–200 bp, which can best penetrate the tissue (Brahic and Haase 1978), the RNA was partially hydrolyzed by treatment with equal volumes of 80 mM $NaHCO_3$ and 120 mM Na_2CO_3 for 1 hr at 60° C (Pardue 1985). The RNA fragments had a specific radioactivity of

$7–10 \times 10^7$ dpm/μg.

***In situ* RNA-RNA hybridization.** To ensure a high signal-to-noise ratio, the RNA had to be fixed firmly to cell structures in the tissue and at the same time remain accessible for the probe. Optimization of the hybridization procedure for potato leaf sections was routinely followed by staining with Acridine Orange (Schmelzer *et al.* 1988). For young potato leaf tissue the pretreatments described by Schmelzer *et al.* (1988) and the conditions for RNA-RNA hybridization described by Somssich *et al.* (1988) gave satisfactory results. The tissue sections on each slide were covered with 10 μl of the hybridization solution (50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 500 μg/ml of denatured and sonicated herring sperm DNA, and 500 μg/ml of yeast rRNA), containing 20 ng of RNA transcript (sense or antisense), and a siliconized coverslip. The edges were sealed with rubber cement, diluted 1:1 with petroleum ether. The slides were incubated under high humidity for 16–20 hr at 50° C. The coverslips were then carefully removed, and the slides were washed six times for 1 hr at 50° C in 1× saline sodium citrate (SSC) with 50% formamide, interrupted after the third wash by a brief wash in 1× SSC and incubation for 30–60 min at 37° C with 20 μg/ml of ribonuclease A to remove single-stranded RNA. The slides were washed twice for 20 min in 1× SSC at room temperature and dehydrated with increasing ethanol concentrations.

Microautoradiography. The slides were coated with Kodak NTB-2 film emulsion, exposed, developed, and fixed as described by Schmelzer *et al.* (1988). They were mounted in polyvinyl alcohol and examined under a light microscope (IM 35 and Axiophot, Zeiss, W. Germany). Photomicrographs were taken with Kodak Ektachrome X-200 and Agfapan 25 or Agfaortho 25 films.

RESULTS

Fungal infection and tissue preparation. Essentially the same procedure as described previously for mature potato leaves was used to inoculate and cryo-section young leaves. In agreement with the overall difference in the speed of lesion development (Cuypers and Hahlbrock 1988), early morphological changes upon infection with *P. infestans* were more rapid and more intense in young than in mature leaves of cv. Datura. In the incompatible interaction, sizable infection craters were frequently seen 3 hr following inoculation. Hypersensitive cell death during fungal penetration progressed strictly sequentially from the epidermis through the tissue, probably due to the lack of large intercellular spaces that appear to facilitate random spread of hyphae in mature leaves (Cuypers and Hahlbrock 1988). The craters, resulting in young leaves, usually contained a mixture of plant cell debris and fungal material. The latter was identified by immunofluorescence labeling. In the compatible interaction, tissue damage at these early stages appeared less drastic, but otherwise similar to the

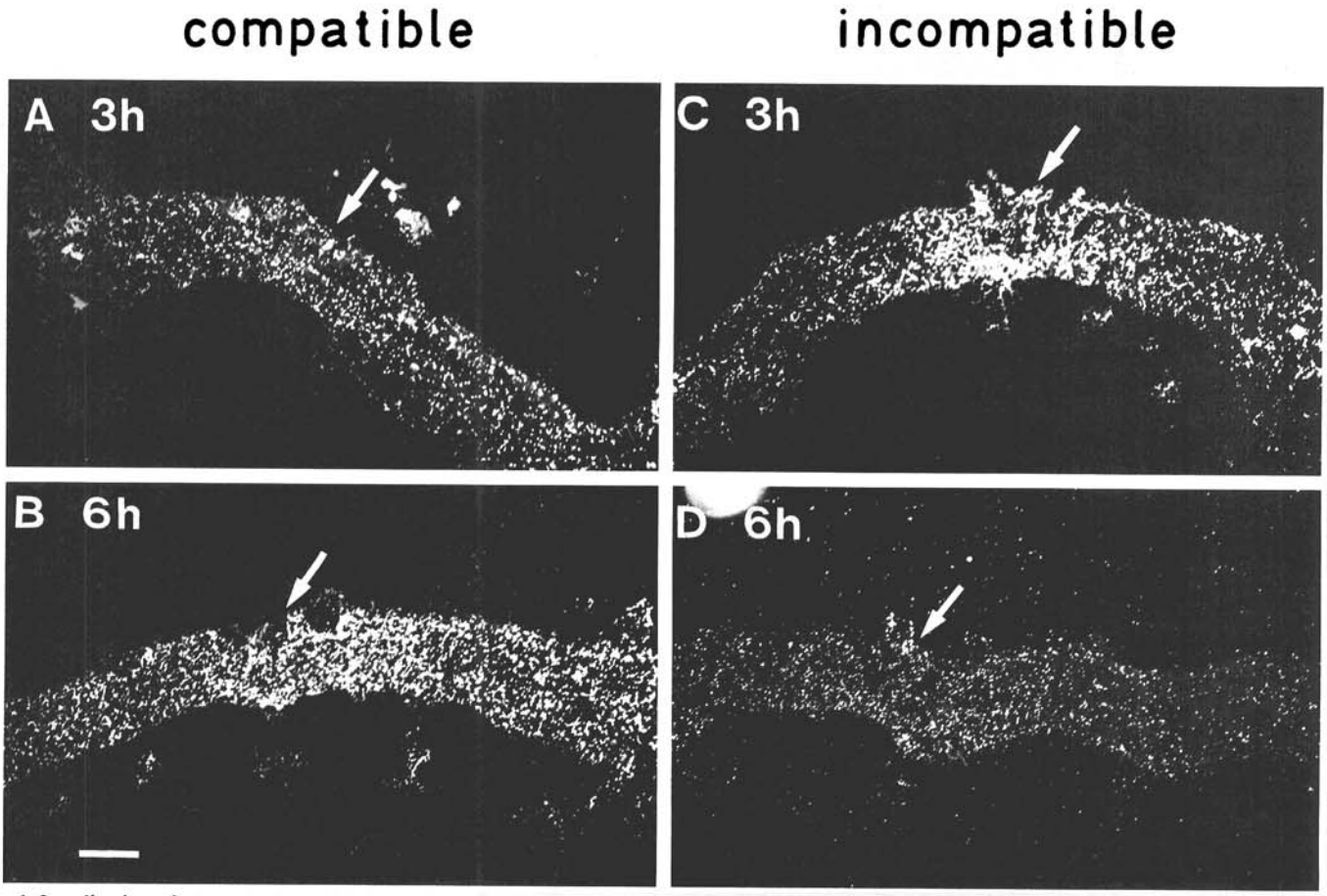

Fig. 1. Localization of phenylalanine ammonia-lyase mRNA in cryo-sections of young potato (*Solanum tuberosum* L. cv. Datura) leaves infected with zoospores of *Phytopthora infestans* race 1 (compatible interaction) and race 4 (incompatible interaction). Leaves were harvested at 3 hr **(A, C)** and 6 hr **(B, D)** following inoculation, and phenylalanine ammonia-lyase mRNA was hybridized *in situ* with ^{3}H-labeled "antisense" RNA. Following autoradiography photomicrographs were taken under dark-field conditions. The centers of infection sites are marked with arrows. The bar represents 50 μm.

damage caused in the incompatible interaction.

Additional changes in tissue morphology occurred in the immediate surrounding of infection craters. Cells in this region were often less closely arranged than in healthy tissue, a likely reason for the encounter of tissue rupture upon cryo-sectioning preferentially at infection sites. Difficulties in sectioning were also encountered with browned cells, especially at later stages of the infection process. Three hr after inoculation, browning of cells in the incompatible interaction was best visible following pretreatment for *in situ* hybridization. Under these conditions, a ring of brownish cells, usually consisting of one layer, was observed even at this early stage of lesion formation. The infection craters had enlarged at 6 hr following inoculation and often reached the other side of the leaf after a total period of 12 hr.

***In situ* hybridization.** In all of the following experiments, serial sections were alternatingly used for hybridization of PAL mRNA with antisense RNA and localization of the fungus with fluorescence-labeled antibodies. Similar low levels of PAL mRNA were reproducibly detectable in uninfected leaves or leaf tissue clearly separated from infection sites. Typical pictures taken from central areas of four independent infection sites are shown in Figures 1 and 2. The fungus and necrotic plant tissue surrounding fungal penetration sites were localized in cross-sections used for RNA hybridization (arrows in Fig. 1) by immunohistochemistry in adjacent sections (Fig. 2) and microscopic examination under bright-field conditions (not shown). Results were obtained at two early stages (3 and 6 hr) for both compatible and incompatible interactions of young cv. Datura leaves with *P. infestans* races 1 and 4, respectively.

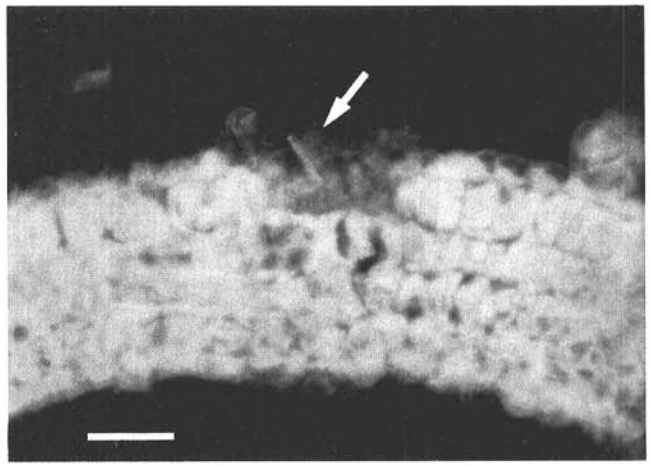

Fig. 2. Identification of an infection site (3 hr incompatible) by immunohistochemical localization of fungal structures and visualization of damaged plant tissue. Cross-sections of a leaf were incubated with antibodies raised against *Phytophthora megasperma* (Jahnen and Hahlbrock 1988). Fungal material was visualized by fluorescence microscopy after staining with anti-rabbit FITC-coupled antibodies (greenish-yellow fluorescence using a 450–490 nm excitation filter with a dicroic mirror of 510 nm and a barrier filter of 520 nm). The arrow points at the center of the infection site and demonstrates how positions of arrows in Figures 1 and 3 were determined. The bar represents 25 μm.

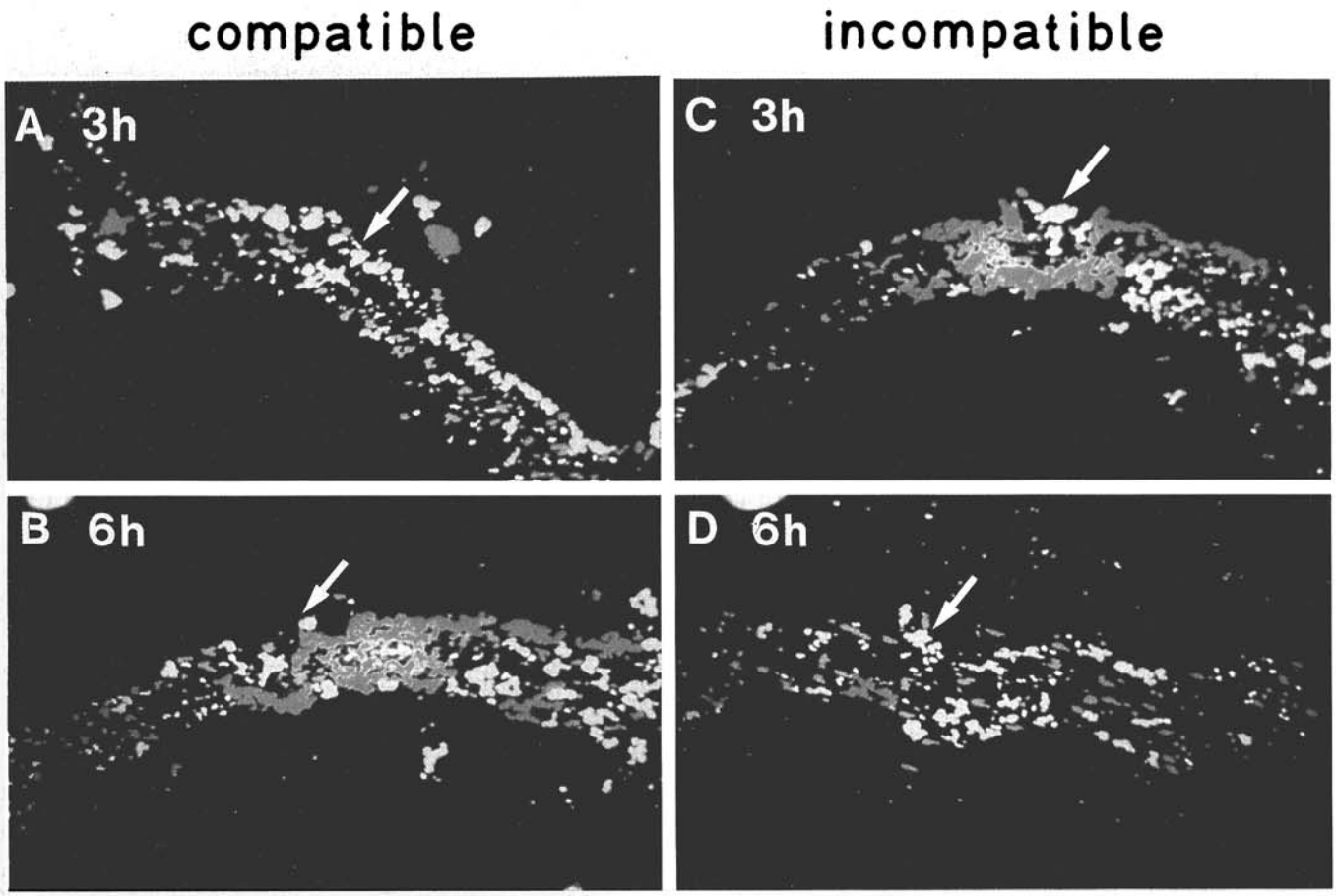

Fig. 3. False-color automatic microscopic image analysis of autoradiographs. The distribution and agglomeration of silver grains on the autoradiographs of Figure 1 were analyzed by automatic gray image evaluation and editing (IBAS 2000 computer image analysis system, Kontron Electronics). The silver grain agglomerates were divided into five size classes and related to five different colors. The color order with increasing size of silver grain agglomerates is as follows: blue, violet, green, yellow, red. Infection sites are marked by arrows as in Figure 1.

Computer analysis (Fig. 3) facilitated interpretation of the autoradiographs depicted in Figure 1.

The timing of PAL mRNA accumulation differed markedly between the two types of interaction. Whereas a relatively slow accumulation from 3 to 6 hr following inoculation was observed in the compatible interaction (Figs. 1 and 3, A and B), a high level at 3 hr was followed by a rapid decline to a level slightly above background at 6 hr in the incompatible interaction (Figs. 1 and 3, C and D). The same result was consistently obtained in several independent experiments. Typically, the total area comprising cells with high PAL mRNA levels was larger and less clearly defined at 6 hr in the compatible interaction than at 3 hr in the incompatible interaction.

Subsequent sections of these four and other 3- and 6-hr series demonstrated that the longitudinal appearance of the PAL mRNA-rich area is essentially the same as shown here in lateral view. Regardless of whether infected or uninfected sections were examined, control experiments using sense RNA gave much less signal than obtained with the antisense probe in uninfected tissue.

DISCUSSION

These results demonstrate that the procedure developed recently for *in situ* RNA hybridization in parsley leaves (Hahlbrock *et al.* 1987; Schmelzer *et al.* 1988; Somssich *et al.* 1988) is applicable to young potato leaves and probably to various other plant tissues. The method differs from others (Martineau and Taylor 1986; Cornish *et al.* 1987; Smith *et al.* 1987; Meyerowitz 1987; McFadden *et al.* 1988) by combining tissue prefixation in a mixture of formaldehyde and glutaraldehyde with a subsequent pronase treatment. From our experience with this method, leaves are not a particularly easy tissue to work with. Young, densely structured leaves are more suitable than old leaves, presumably to a large part because of a relatively high RNA content as well as effective tissue preservation upon freezing, cutting, and subsequent chemical treatments. An unbiased interpretation of the data was greatly facilitated by false-color computer analysis.

In this study, we were primarily concerned with the early detection of changes in PAL gene activity in *P. infestans*-infected potato leaves. We have demonstrated a marked difference in the timing of PAL mRNA accumulation between compatible and incompatible interactions. The rapid appearance and disappearance of a clearly confined halo around the fungal penetration site in the incompatible interaction contrasts with the much slower development of a comparatively diffuse halo in the compatible interaction. Whether the halo subsequently spreads further in the latter case has not been investigated extensively. Preliminary results suggest that this is indeed the case.

An alternative mechanism for PAL mRNA accumulation not involving increased rates of transcription, but, for example, changes in the rate of processing or PAL mRNA stability, was most likely ruled out by Fritzemeier *et al.* (1987) by using isolated nuclei for measurements of run-off transcription rates *in vitro*. However, a combination of both mechanisms was not excluded.

Thus far, wherever PAL or PAL mRNA have been tested, their rapid accumulation in infected tissue has been demonstrated, supporting arguments in favor of a role in pathogen defense (Cramer *et al.* 1985; Fritzemeier *et al.* 1987; Jahnen and Hahlbrock 1988). However, the temporal and functional relation between lesion development and the area with increased PAL mRNA levels is not known in detail. It remains open at present whether differences in cultivar-race specificity in the plant's defense response are causally connected with phenylpropanoid metabolism as measured through PAL mRNA levels around infection sites. Alternatively, cultivar-race specificity might be more directly related to the efficiency of hypersensitive cell death or other defense reactions at the very site of fungal penetration. More data obtained by *in situ* localization and measurements of concentration changes in various defense-related and other metabolites will be required to draw final conclusions. In any case, the rapid spread of the observed response undoubtedly requires transmission of a signal triggering PAL gene activation in advance of the fungus. It will be interesting to see whether such a signal is of fungal or plant origin, or a combination of both.

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