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Temporal and Spatial Patterns of Gene Expression around Sites of Attempted Fungal Infection in Parsley Leaves

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We analyzed the expression patterns of several pathogen defense-related genes in primary leaf buds of parsley by in situ RNA hybridization. Labeled antisense RNA probes were generated from seven selected cDNAs detecting transcripts from genes that are rapidly and strongly activated in cultured parsley cells upon treatment with fungal elicitor. These genes encode two enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase and 4-coumarate:CoA ligase, a furanocoumarin-specific bergaptol *O*-methyltransferase, one pathogenesis-related protein, and three less well characterized proteins, designated as ELI 3, ELI 5, and ELI 7. In uninfected tissue, phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNA levels were high in epidermal cells, oil-duct epithelial cells, and cells of the developing xylem; bergaptol *O*-methyltransferase mRNA was confined to oil-duct epithelial cells; and the pathogenesis-related protein and ELI 3, ELI 5, and ELI 7 mRNAs were undetectable. All seven mRNAs accumulated transiently and locally around infection sites caused by the soybean-pathogenic fungus *Phytophthora megasperma* f. sp. *glycinea*, to which parsley is nonhost-resistant. The observed late appearance of bergaptol *O*-methyltransferase mRNA, as compared with all other mRNAs, is in accord with a similar relative timing of transient gene activation in elicitor-treated cell cultures. Sharp borders were observed between the infection center, where hypersensitive cell death had occurred in response to fungal penetration, the surrounding area of local gene activation, and the remainder of the tissue not showing any apparent response. Some of the genes were also activated, although less sharply localized, upon wounding of parsley leaves.

INTRODUCTION

Plants are continuously exposed to attempted invasion by potentially pathogenic microorganisms and have evolved efficient passive (preformed) as well as active (induced) defense mechanisms. In the majority of cases, the plant is nonhost-resistant. That is, all genotypic variants of the plant are resistant to all genotypic variants of the microorganism. This is in contrast to host resistance, where susceptibility is determined in certain cases by genotypic compatibility with a virulent strain of a pathogen. Broadly acting nonhost resistance, as opposed to narrowly specified host resistance, is, therefore, a highly desirable, genetically stable trait and an interesting research object for studies of the underlying defense mechanisms. An extensively analyzed example is the nonhost resistance response of parsley leaves to the fungus *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*), a pathogen of soybean (Scheel et al., 1987; Jahnen and Hahlbrock, 1988a).

Inoculation of parsley leaves with *Pmg* zoospores triggers a number of rapid, localized defense reactions in the vicinity of sites of attempted fungal penetration. Among the most obvious microscopically detectable events are hypersensitive cell death and the formation of small local lesions, along with the accumulation of wall-bound phenolics, callose deposition, and the subsequent formation of furanocoumarin phytoalexins (Jahnen and Hahlbrock, 1988a). Some of these reactions have been shown in situ to be associated with transient increases in biosynthetic capacity, including a key enzyme of general phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL), and an enzyme specific for the furanocoumarin pathway, *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase (BMT) (Jahnen and Hahlbrock, 1988a). PAL accumulates rapidly, and BMT somewhat later, at *Pmg* infection sites. In uninfected tissue, both occur at high levels in oil-duct epithelial cells, where furanocoumarins are produced constitutively with unknown function (Knogge et al., 1987; Jahnen and Hahlbrock, 1988b). PAL is also preferentially located in epidermal cells, the site of synthesis and accumulation of flavonoids. These compounds serve as UV-

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protective agents, and represent a different phenylpropanoid branch pathway that is unrelated to pathogen defense and regulated by light (Schmelzer, Jahnen, and Hahlbrock, 1988).

Studies of these phenomena at the gene expression level were initially carried out in a system of greatly reduced complexity using cultured parsley cells and an elicitor preparation derived from *Pmg* mycelial walls (Chappell and Hahlbrock, 1984; Somssich et al., 1986). One major result was the generation of a large number of probes for measuring gene expression at fungal infection sites with respect to both relative timing and spatial distribution. For this purpose, we have recently established the technique of in situ RNA hybridization in this system (Schmelzer, Jahnen, and Hahlbrock, 1988; Somssich et al., 1988).

We selected from the available collection probes specifically detecting putative defense-related transcripts from seven different genes or gene families whose large and transient activation in elicitor-treated parsley cells has been demonstrated: PAL, a small gene family consisting of about four members (Lois et al., 1989); 4-coumarate:CoA ligase (4CL), consisting of two genes encoding another, closely related key enzyme of general phenylpropanoid metabolism (Douglas et al., 1987; Lozoya et al., 1988); BMT, which may occur as a single-copy gene (K.D. Hauffe, K. Hahlbrock, and D. Scheel, *manuscript in preparation*); PR1, a gene family comprising approximately three members (Somssich et al., 1988); and three less extensively investigated, strongly and rapidly elicitor-activated genes, designated as ELI 3, ELI 5, and ELI 7 (Somssich et al., 1989). In situ hybridization of PR1 mRNA in *Pmg*-infected parsley leaves has been reported previously (Hahlbrock et al., 1987; Somssich et al., 1988) and is included here both for comparison with earlier results and as an internal control, exploiting its particularly high rate of induced, localized expression.

RESULTS

Cell Type-Specific Gene Expression

In view of previous results obtained at the protein level (Jahnen and Hahlbrock, 1988a, 1988b), we first analyzed the constitutive expression patterns of those genes encoding enzymes with known functions in disease resistance-related phenylpropanoid metabolism: PAL, 4CL, and BMT. Figure 1 shows that the expected, differential patterns of mRNA abundance were observed in cross-sections of uninfected primary parsley leaves hybridized in situ with ^{3}H-labeled PAL, 4CL, and BMT antisense RNA. Essentially the same patterns were obtained in several independent experiments. Comparison of Figures 1B and 1D demonstrates the very similar distribution of PAL and 4CL mRNAs. Both occurred preferentially in three different cell types: epidermal cells, cells of the developing xylem, and oil-duct epithelial cells. By contrast, BMT mRNA was exclusively detected in high amounts in oil-duct epithelial cells (Figure 1F).

The Infection Process

The growth of *Pmg* hyphae on the leaf surface and attempts to invade the tissue proceeded asynchronously. The average number and size of infection sites (autofluorescing cells) per leaf clearly increased with time post-inoculation. However, at any given time point measured (0 hr to 24 hr post-inoculation), the fungal mycelium continued growing under the experimental conditions used and generated new infection sites, causing considerable heterogeneity with respect to age and size of individual infection sites. Nevertheless, in the majority of cases, size and appearance in combination with time post-inoculation allowed an unequivocal age estimation of infection sites, and only those cases are considered in the following.

Gene Expression in Response to Fungal Infection

Most of the following results were obtained from serial sections through infection sites hybridized in situ alternately with the respective antisense RNAs at the indicated times post-inoculation. At the earliest stages tested (2 hr and 3 hr post-inoculation), small infection sites were clearly visible under a UV-epifluorescent microscope as bright yellow spots (Jahnen and Hahlbrock, 1988a) comprising a few dead cells around sites of attempted fungal penetration. No new hybridization signals above a low background were detectable at this stage using either PAL, 4CL, BMT, PR1, ELI 3, ELI 5, or ELI 7 antisense RNA as a probe.

By contrast, at 4 hr post-inoculation, strong signals were observed with all of these probes except BMT (Figure 2). Most of the signals, particularly those indicating PR1, PAL, and 4CL mRNA accumulation (Figures 2F to 2H), were sharply localized around the necrotic spot, which itself was free of signal in all cases. In these and several independent serial sections, PR1 was always included as an internal control and invariably gave the strongest signal (not shown for Figures 2A to 2D), except for very late time points (see below).

Subsequent analyses of later time points (6 hr, 8 hr, 18 hr, and 24 hr post-inoculation) are shown in Figure 3 for PAL, 4CL, PR1, and BMT. The induced mRNA levels of PAL, 4CL, and PR1 were highest and extended farthest at 6 hr (Figures 3A to 3D) to 8 hr and then declined. Similar results were obtained for ELI 3, ELI 5, and ELI 7 (data not shown). BMT mRNA, although very abundant in oil ducts, was not detectable at infection sites (Figures 3E and 3F) earlier than approximately 12 hr to 18 hr post-inoculation (Figure 3L). At this stage the necrotic spot had grown to

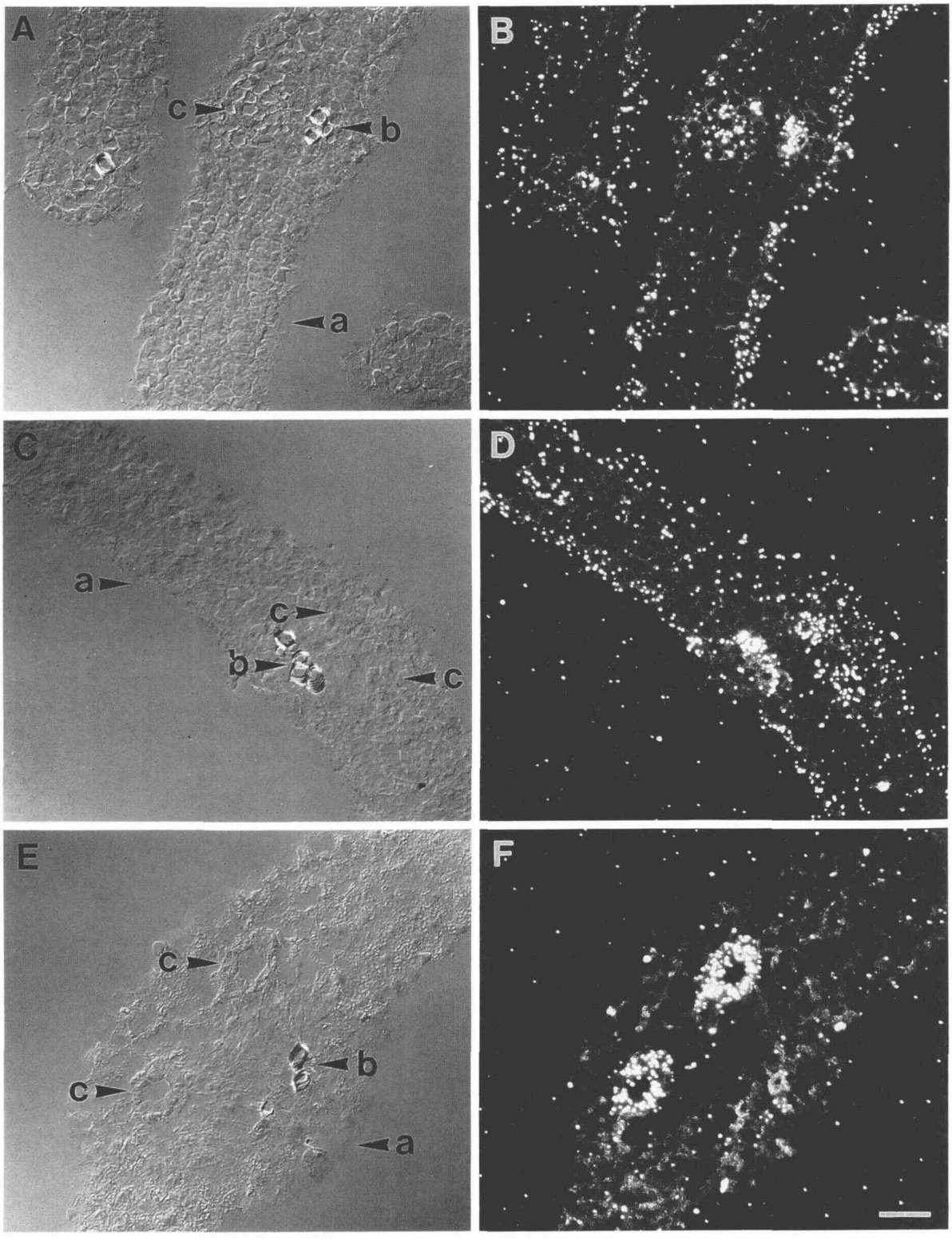

Figure 1. Localization of PAL [**(A)** and **(B)**], 4CL [**(C)** and **(D)**], and BMT [**(E)** and **(F)**] mRNAs in Cross-Sections of Uninfected Primary Leaf Buds by in Situ Hybridization with Antisense RNA.

(A), **(C)**, and **(E)** Structural features are shown in photomicrographs taken under conditions of differential interference contrast.
(B), **(D)**, and **(F)** Hybridization is visualized by autoradiography and dark-field microscopy of the same sections.
Arrows indicate upper epidermis (a), xylem (b), and oil-duct epithelial cells (c).
Bar = 25 μm.

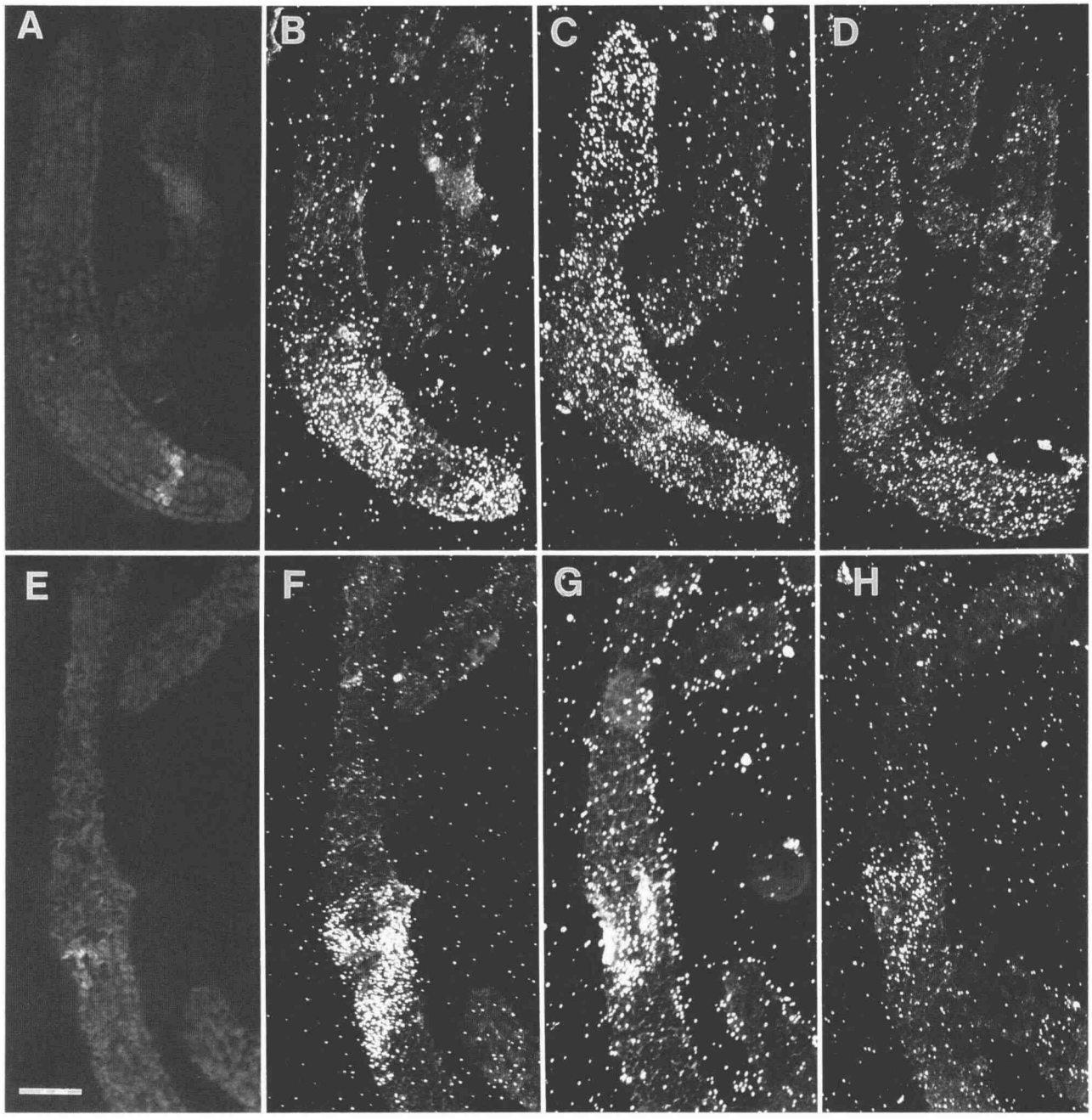

Figure 2. Differential Expression of Various Defense-Related Genes around *Pmg* Infection Sites 4 hr Post-Inoculation.

(A) and **(E)** Infection sites were identified by visualizing autofluorescing cells under UV-epifluorescent light in the same sections as shown in **(B)** and **(F)**, respectively.

(B), **(C)**, **(D)** and **(F)**, **(G)**, **(H)** Serial sections through two independent infection sites [**(A)** through **(D)** and **(E)** through **(H)**] were hybridized in situ with ELI 7 **(B)**, ELI 3 **(C)**, ELI 5 **(D)**, PR1 **(F)**, PAL **(G)**, and 4CL **(H)** antisense RNAs.

Bar = 100 μm.

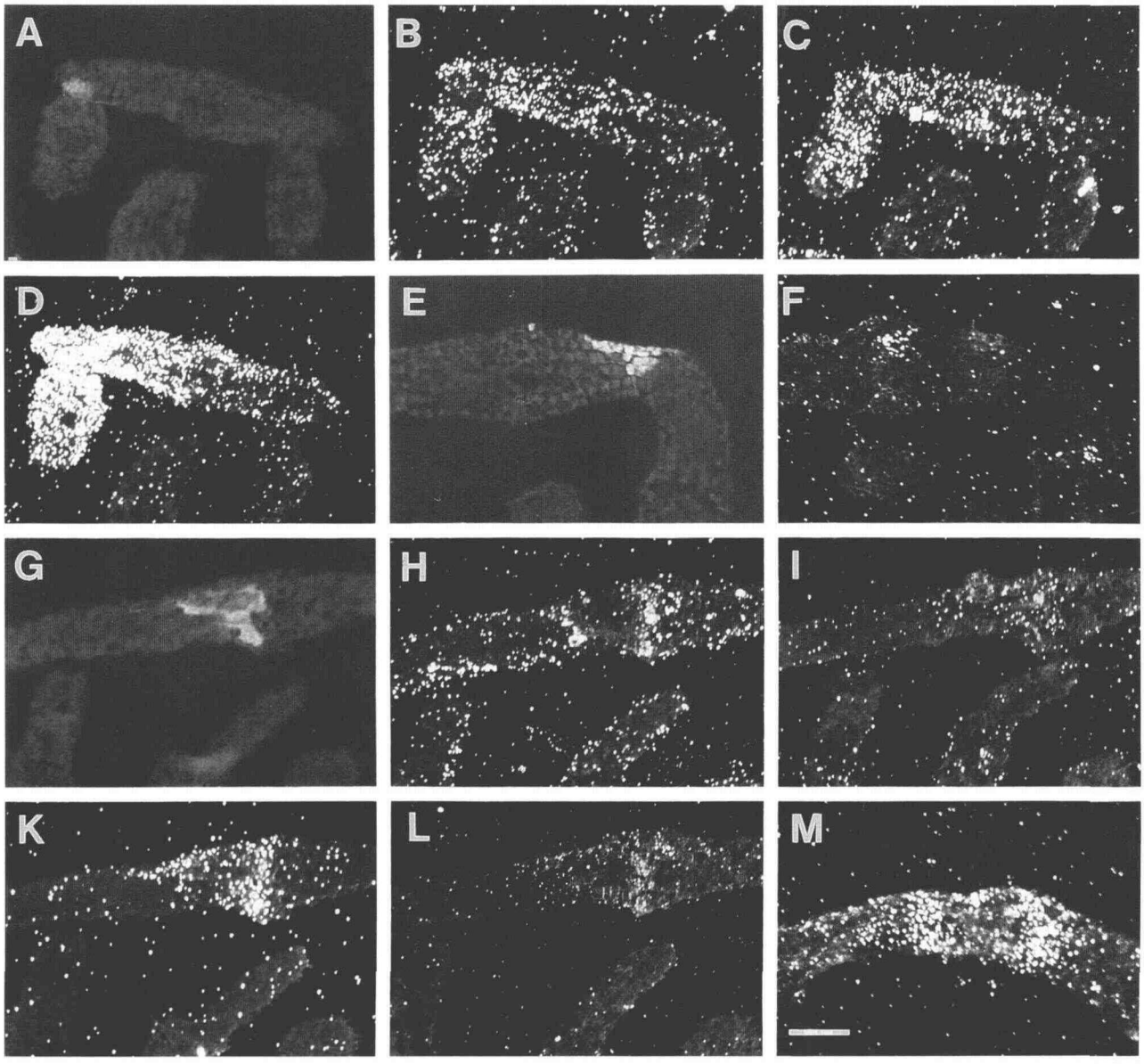

Figure 3. Selected Timepoints for Comparative Analysis of PAL, 4CL, PR1, and BMT mRNA Accumulation at *Pmg* Infection Sites.

(A), **(B)**, **(C)**, and **(D)** Serial sections through an infection site 6 hr post-inoculation hybridized with PAL **(B)**, 4CL **(C)**, and PR1 **(D)** antisense RNA.

(E) and **(F)** Section hybridized with BMT antisense RNA **(F)** 8 hr post-inoculation.

(G), **(H)**, **(I)**, **(K)**, and **(L)** Serial sections through an infection site 18 hr post-inoculation hybridized with PAL **(H)**, 4CL **(I)**, PR1 **(K)**, and BMT **(L)** antisense RNAs.

(M) Section hybridized with BMT antisense RNA 24 hr post-inoculation.

(A), **(E)**, and **(G)** Infection sites were identified by UV epifluorescence.

Bar = 100 μm.

an appreciable size (Figure 3G), and the mRNA levels of PAL, 4CL, and PR1 had decreased considerably (Figures 3H to 3K). High levels of BMT mRNA were not reached until about 24 hr post-inoculation, and were centered around a relatively large, signal-free necrotic area (Figure 3M).

Cell Type Specificity of the Response

Results from numerous experiments indicate that gene activation around infection sites affects all microscopically identifiable types of leaf cell, with the notable exception of oil-duct epithelial cells. These cells contain high levels of PAL, 4CL, and particularly BMT mRNAs (Figure 1), but do not at all participate in the induced accumulation of PR1 mRNA in the tissue surrounding fungal penetration sites (Figure 4). With this exception, our results indicate that induced levels of all mRNAs tested are equally distributed over the whole area of affected cells. The boundaries of this area were usually very sharp, with fully activated and completely unaffected cells lying directly adjacent to one another. A typical example is given in Figure 5A.

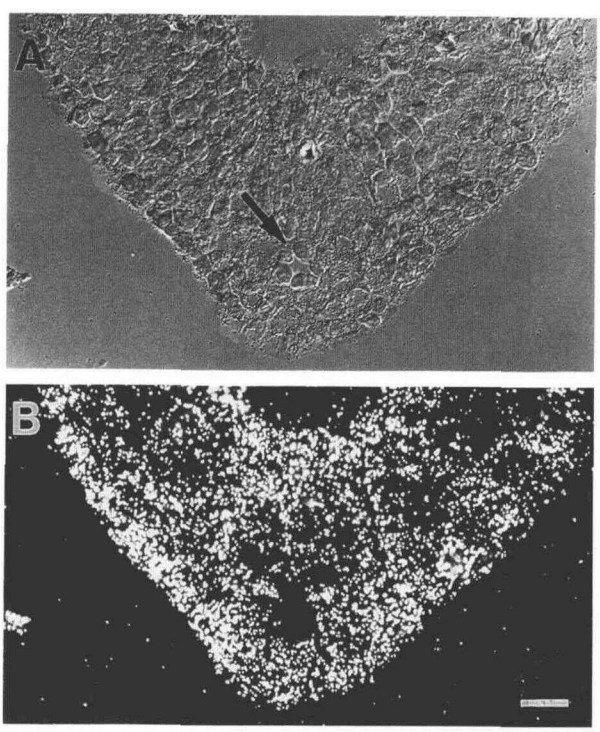

Figure 4. Absence of PR1 mRNA in Oil-Duct Epithelial Cells.

(A) Oil-duct identification (arrow) under differential interference contrast conditions.

(B) The same section is shown for PR1 mRNA localization under dark-field conditions. Leaf-bud tissue containing a *Pmg* infection site (not visible) was hybridized with PR1 antisense RNA 8 hr post-inoculation.

Bar = 25 μm.

Wounding

Several of the genes related to pathogen defense are also rapidly activated upon wounding of parsley leaves. A typical example is 4CL, whose mRNA accumulation patterns in *Pmg*-infected and wounded leaves are compared in Figure 5. In contrast to the highly localized accumulation of 4CL mRNA at infection sites, e.g., 8 hr post-inoculation (Figure 5A), a strong and rapid wound response, e.g., 6 hr after wounding (Figure 5B), involves a much larger area and levels out with a gentle slope toward the leaf edge. Essentially the same results were obtained with PR1.

DISCUSSION

We have previously reported initially high and then rapidly declining levels of PAL activity in growing parsley leaf buds (Hahlbrock et al., 1971). In developing parsley cotyledons, we observed coordinated decreases of PAL and 4CL activities concomitant with transient increases in BMT activity (Knogge et al., 1987). Here we show that constitutively high levels of all three mRNAs in primary leaf buds are preferentially localized in those cell types where the respective enzymes have been detected immunohistochemically (Jahnen and Hahlbrock, 1988b). Differential, cell type-specific expression of the corresponding genes is in agreement with the multiple, closely related functions of PAL and 4CL in general phenylpropanoid metabolism. This is in contrast to a very specific role of BMT in the furanocoumarin branch pathway (Hauffe, Hahlbrock, and Scheel, 1986). High concentrations of PAL and 4CL mRNAs in epidermal cells are attributed to their involvement in flavonoid biosynthesis. Expression of the flavonoid pathway is strictly confined to this part of the tissue (Schmelzer, Jahnen, and Hahlbrock, 1988), and is most active in young, opening leaf buds (Hahlbrock et al., 1971; Jahnen and Hahlbrock, 1988b).

General phenylpropanoid metabolism, in connection with at least two different branch pathways, is also activated in response to attempted fungal penetration. One branch is biochemically less well defined and is indirectly detected by the rapid appearance of PAL and 4CL mRNAs at infection sites (Figures 2G and 2H) and the subsequent accumulation of phenolics in the cell wall (Jahnen and Hahlbrock, 1988a). The furanocoumarin branch pathway

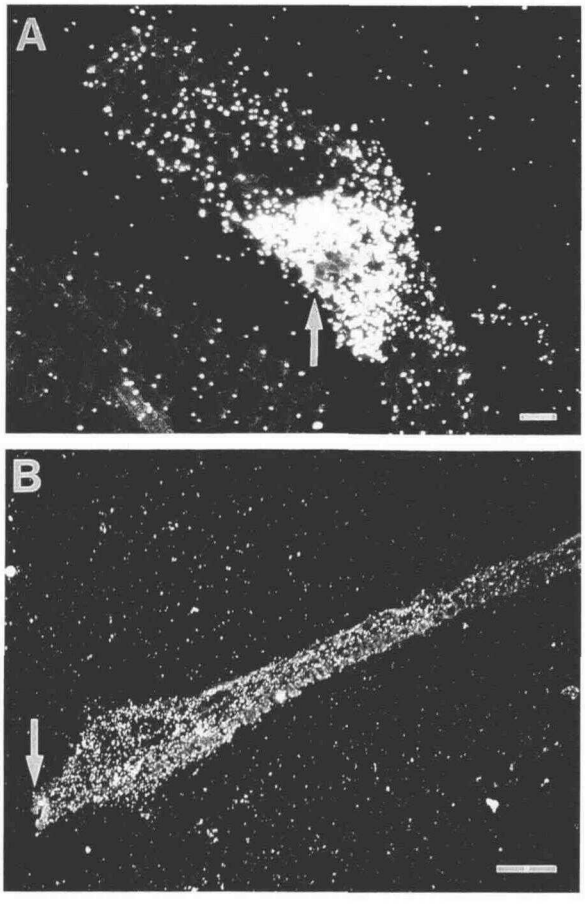

Figure 5. Spatial Distribution of 4CL mRNA Accumulated in Response to *Pmg* Infection or Wounding.

(A) *Pmg* infection.
(B) Wounding.
Sections either from a primary leaf bud 8 hr post-inoculation or from a secondary leaf half 6 hr after wound-cutting were hybridized with 4CL antisense RNA. Arrows indicate the centers of infection and wound sites.
Bars = 25 μm **(A)** and 100 μm **(B)**.

is apparently stimulated several hours later (Figures 3F, 3L, and 3M), in agreement with earlier observations at both the enzyme and product levels (Scheel et al., 1986; Jahnen and Hahlbrock, 1988a). In accordance with the method of identification (Somssich et al., 1989), the three selected ELI clones detected rapid and transient mRNA accumulation with about the same kinetics as observed for PAL, 4CL, and PR1.

This differential behavior of PAL, 4CL, PR1, ELI 3, ELI 5, and ELI 7 on the one hand, and BMT on the other hand, is remarkable for several reasons. First, all seven of these clones were originally identified by the strong induction of the corresponding mRNAs in cultured parsley cells treated with *Pmg*-derived elicitor (Kuhn et al., 1984; Lois et al., 1989; Somssich et al., 1989). We now find that all seven mRNAs also accumulate transiently in *Pmg*-infected leaves.

Moreover, the relative timing of induction is essentially the same in elicitor-treated cells and infected leaf tissue. In both cases, PAL, 4CL, PR1, and the three ELI mRNAs appear rapidly and more or less simultaneously (Lois et al., 1989; Somssich et al., 1989), whereas BMT mRNA accumulates considerably later (K.D. Hauffe, K. Hahlbrock, and D. Scheel, manuscript in preparation). This demonstrates how closely elicitor treatment of cultured cells mimics the response of infected, whole leaves. Similar observations were made earlier with light-induced flavonoid biosynthesis in cultured (Chappell and Hahlbrock, 1984) and leaf epidermal cells (Schmelzer, Jahnen, and Hahlbrock, 1988).

Finally, the late expression of BMT, relative to all other gene products analyzed, suggests that furanocoumarin phytoalexins are not involved in the early defense response triggered by gene activation. Rather, it seems that individual defense reactions are induced, at least in part, sequentially, with phytoalexin accumulation occurring at a remarkably late time point in this system. An interesting question in this connection concerns the overall temporal and spatial development of the necrotic spot and the mechanism of lesion limitation. In particular, one could speculate that the phytoalexins exert their toxic effects on both fungus and plant tissue and thus participate in determining the final size of the lesion. However, the apparent variability in timing and extent of formation of individual lesions around fungal penetration sites has so far prevented the establishment of causal connections with the results obtained by in situ RNA (Figures 2 and 3), protein, or product localization (Jahnen and Hahlbrock, 1988a).

Another interesting but unexplained finding is the absence of an infection-triggered response of oil-duct epithelial cells (Figure 4). Two alternative explanations are equally conceivable at the present state of knowledge. Either these highly specialized cells are not responsive to the putative signals triggering defense-related gene activation, or such signals do not reach the cells.

One important result with respect to the possible nature and action of signal molecules is most clearly visible in Figure 5A. At all examined infection sites containing sufficiently large mRNA amounts, the borders between stimulated and surrounding cells were very sharp, both toward the central necrotic spot and toward the unaffected tissue outside the area of local gene activation. This allows two general conclusions to be drawn. First, it seems that hypersensitive cell death at the actual site of fungal penetration is too rapid to involve activation to a measurable extent of any of the genes investigated. The hypersensitive response is more likely to be the result of a direct interac-

tion of the invading fungus with plant cells and, hence, is highly localized (Jahnen and Hahlbrock, 1988a; Cuypers and Hahlbrock, 1988). Second, the sharp border between stimulated and unstimulated cells may indicate a threshold phenomenon in the signalling mechanism.

Such a sharp border was not observed in the wound response, despite a partial overlap of the induced biosynthetic pathways. Because specific probes have now become available to measure individual members of gene families, e.g., for PAL (Lois et al., 1989), 4CL (Lozoya et al., 1988), or PR1 (Somssich et al., 1988), an interesting question in future studies will be whether or not differential expression of gene family members occurs in response to infection, wounding, and other types of stress. So far, all members of individual gene families were detected together under the conditions used. The results presented here demonstrate that the method of in situ RNA hybridization has a large potential for such studies.

METHODS

Materials

[5,6-^{3}H]Uridine 5′-triphosphate was purchased from Amersham, Braunschweig, Federal Republic of Germany. Paraplast was obtained from Monoject Scientific Inc., Kildare, Ireland; glutaraldehyde solution, 25%, for electron microscopy was from Merck, Darmstadt, FRG, and pronase (RNAse-free) was from Calbiochem, Frankfurt, FRG.

Plant Material and Inoculation

Cultivation of parsley seedlings (*Petroselinum crispum* cv Hamburger Schnitt) under aseptic conditions, growth of the fungus (*Phytophthora megasperma* f. sp. *glycinea*, race 1), isolation of zoospores, and inoculation were carried out as described by Knogge et al. (1987) and Jahnen and Hahlbrock (1988a). Primary leaf buds were inoculated, without wounding, with small droplets (5 μL to 10 μL) containing approximately 10^3 zoospores. Under the conditions used (100% relative humidity, 16 hr illumination per day), this spore density gave rise to several distinct lesions per inoculum.

Tissue Fixation, Paraffin Embedding, and Sectioning

To obtain morphological resolution at the single cell level, paraffin sections were prepared instead of the cryosections previously described (Schmelzer, Jahnen, and Hahlbrock, 1988). Primary leaf buds were fixed in freshly prepared 2% paraformaldehyde and 0.2% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.0, for 2 hr on ice. This aldehyde fixative proved to be very useful for young plant tissue. Older tissue containing large vacuoles and intercellular spaces was fixed in 100 mM sodium phosphate buffer containing 1% glutaraldehyde for 1 hr on ice. The fixed tissue was dehydrated in a series of aqueous ethanol solutions (v/v): 20 min at 0°C in 30%, 20 min at 0°C in 50%, overnight at 4°C in 70%, 20 min at room temperature in 85%, 20 min at room temperature in 90%, and finally twice for 20 min at room temperature in 100% ethanol. Subsequently, the tissue was infiltrated with tertiary butanol and paraplast, first for 30 min each at room temperature with mixtures of ethanol/tertiary butanol at the ratios 3:1, 1:1, 1:3 (v/v), followed by 30 min in 100% tertiary butanol at 30°C. The tertiary butanol was then replaced by tertiary butanol/paraplast (saturated at 30°C) for 4 hr to 6 hr at 30°C. In a second 30-min treatment with the same solution, the temperature was raised to 42°C. The infiltration with paraffin was continued overnight at 42°C with tertiary butanol/paraplast (saturated at 42°C), followed by a 30-min imbibition in this solution at 60°C. Finally, the tissue was transferred to small Petri dishes and incubated in paraplast for 2 days at 60°C with several changes of paraplast. After cooling and hardening of the paraplast, a small paraffin block enclosing a leaf bud was mounted to a holder, trimmed, and sectioned at 5 μm thickness with a standard rotary microtome (Leitz microtome 1512). The sections were transferred to water droplets on microscopic slides coated with poly-L-lysine (Angerer, Cox, and Angerer, 1988). After relaxation of the sections, the water was carefully removed and the slides were exposed for 2 hr to 40°C on a heating plate. For deparaffinization of the sections, the slides were immersed in xylol for 10 min, in xylol/ethanol (1:1, v/v) for 5 min, and succeedingly rehydratized stepwise in 100%, 95%, 80%, 60%, and 30% aqueous ethanol for 5 min each.

In Situ Hybridization

Prior to hybridization the sections were treated with pronase and postfixed (Somssich et al., 1988). Conditions for in situ hybridization with ^{3}H-labeled sense (control) and antisense RNA transcripts obtained from the various parsley cDNAs and detection of the resulting double-stranded RNA by microautoradiography were the same as described by Somssich et al. (1988) and Cuypers and Hahlbrock (1988). With the exception of a recently identified 800-bp BMT cDNA (K.D. Hauffe, unpublished results), all cDNAs were used as described: PR1, ELI 3, ELI 4 (= PAL; see Lois et al., 1989), ELI 5, ELI 7 (Somssich et al., 1989), and 4CL (Lozoya et al., 1988). Most of the cDNAs (not ELI 5 and BMT) contained short oligo(T) sequences (4 bp to 26 bp) complementary to poly(A) tails of the mRNAs. Prior to in vitro transcription, all cDNAs were subcloned in pBS (Vector Cloning Systems, San Diego, CA), except 4CL, which was subcloned in sp6 (Amersham, Braunschweig, FRG). The ^{3}H-labeled transcripts were partially hydrolyzed in alkali to give fragments with an average length of 50 to 150 nucleotides (Somssich et al., 1988). Control treatments using sense transcripts gave in all cases very low background signals randomly distributed on glass and tissue surfaces.

Photomicrographs were taken under a Zeiss IM 35 microscope equipped with darkfield and epifluorescence optics or a Zeiss Axiophot microscope equipped with epifluorescence and differential interference contrast (Numarsky) optics. Kodak Ektachrome X-160, X-200, and Agfa PAN 25 films were used.

Wounding

Secondary leaves of ½-year-old parsley plants were wounded by cutting off one half. At several timepoints after wounding, the

remaining halves of the leaves were harvested, fixed, and embedded in paraplast as described above. Paraffin sections were sliced perpendicularly to the wounded edge of the leaf and used for in situ hybridization.

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