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Local mechanical stimulation induces components of the pathogen defense response in parsley

(fungal infection/cytoplasmic rearrangement/local application of elicitor/reactive oxygen intermediate/oxidative burst)

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ABSTRACT **Cell suspension cultures of parsley (*Petroselinum crispum*) have previously been used as a suitable system for studies of the nonhost resistance response to *Phytophthora sojae*. In this study, we replaced the penetrating fungus by local mechanical stimulation by using a needle of the same diameter as a fungal hypha, by local application of a structurally defined fungus-derived elicitor, or by a combination of the two stimuli. Similar to the fungal infection hypha, the local mechanical stimulus alone induced the translocation of cytoplasm and nucleus to the site of stimulation, the generation of intracellular reactive oxygen intermediates (ROI), and the expression of some, but not all, elicitor-responsive genes. When the elicitor was applied locally to the cell surface without mechanical stimulation, intracellular ROI also accumulated rapidly, but morphological changes were not detected. A combination of the mechanical stimulus with simultaneous application of low doses of elicitor closely simulated early reactions to fungal infection, including cytoplasmic aggregation, nuclear migration, and ROI accumulation. By contrast, cytoplasmic rearrangements were impaired at high elicitor concentrations. Neither papilla formation nor hypersensitive cell death occurred under the conditions tested. These results suggest that mechanical stimulation by the invading fungus is responsible for the observed intracellular rearrangements and may trigger some of the previously demonstrated changes in the activity of elicitor-responsive genes, whereas chemical stimulation is required for additional biochemical processes. As yet unidentified signals may be involved in papilla formation and hypersensitive cell death.**

In contrast to various genetically defined host–pathogen interactions, in which pathogen recognition is determined by pairs of complementary genes, pathogen recognition in nonhost resistance is a multigenic trait that is difficult for potential pathogens to overcome (1). The plant must be capable of perceiving signals from a large variety of potential pathogens to trigger a broadly acting, non-race-specific resistance. Such a signal molecule has been isolated from the oomyceteous fungus *Phytophthora sojae* and identified as an extracellular glycoprotein (2). An oligopeptide fragment (Pep-25) from this glycoprotein, containing all of the signaling activity in a short amino acid sequence (Pep-13), elicits a battery of defense reactions in cultured cells of parsley, a nonhost plant to this fungus (3).

We previously used cultured parsley (*Petroselinum crispum*) cells infected by *Phytophthora infestans* (4) as a model system that closely mimics the nonhost interaction of parsley plants with *P. sojae* (5). Under appropriate conditions, the response of infected parsley cells included, in addition to all elicitor-induced reactions, major features of the hypersensitive reaction (6), as observed *in planta* in both the nonhost parsley–*P. sojae* (5) and the host potato–*P. infestans* interactions (7). We therefore used the cell culture system as a tool to further investigate the plant defense response at the single-cell level.

The multicomponent defense response of parsley cells to pathogen attack can be operationally grouped into two categories: morphological and biochemical changes (Fig. 1). Addition of elicitor to the medium of cultured parsley cells triggers a similar combination of biochemical changes (3). The question arises as to whether additional signals are required for induction of the complete defense response, including the morphological changes and hypersensitive cell death. It is conceivable that attempted penetration by the pathogen generates not only chemical but also mechanical signals and that this combination of signals has to be perceived by the plant cell to display the full complement of defense reactions. Here, we describe an approach to test this hypothesis. We applied micromanipulation to distinguish local mechanical triggers from chemical triggers that may simultaneously originate from a penetrating fungal hypha. We report the effects of the two types of signal on three characteristic defense-related reactions: the translocation of cytoplasm and nucleus as representative morphological changes, and the generation of intracellular reactive oxygen intermediates (ROI; "oxidative burst") and the effects on gene activity as typical early and late components of the infection-induced signal-reaction chain.

MATERIALS AND METHODS

Cultivation of Parsley Cells and Fungus. Cell suspension cultures of parsley were grown for 5 d in HA medium as described (8). Growth of *Phytophthora infestans* and production of zoospores have also been described (4).

Embedding of Parsley Cells. For micromanipulation, suspension-cultured parsley cells were immobilized by embedding in 2% (wt/vol) agarose (Sigma; type VII, low gelling temperature) that had been added to the medium prior to melting and mixing of the solution with the cell suspension to a final agarose concentration of 1% (wt/vol). Cells were plated as thin layers on the covers of 3.5-cm (diameter) Petri dishes (approximately 0.8 ml per plate) and stored at 4°C in a humid chamber.

Mechanical Stimulation. Tungsten rods (Science Products, Hofheim, Germany) with a diameter of 20 μm were sharpened electrocatalytically in 1 M $NaNO_2$ and 0.6 M KOH at 5 V and 20 mA to give a final tip diameter of 3–5 μm. These rods were connected to a micromanipulator (DC3 KS; Märzhäuser, Wetzlar, Germany) that was mounted on the microscope and were moved by an STM 3 controller (Lang, Hüttenberg, Germany). A localized mechanical stimulus was applied to the

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Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; ROI, reactive oxygen intermediate(s).

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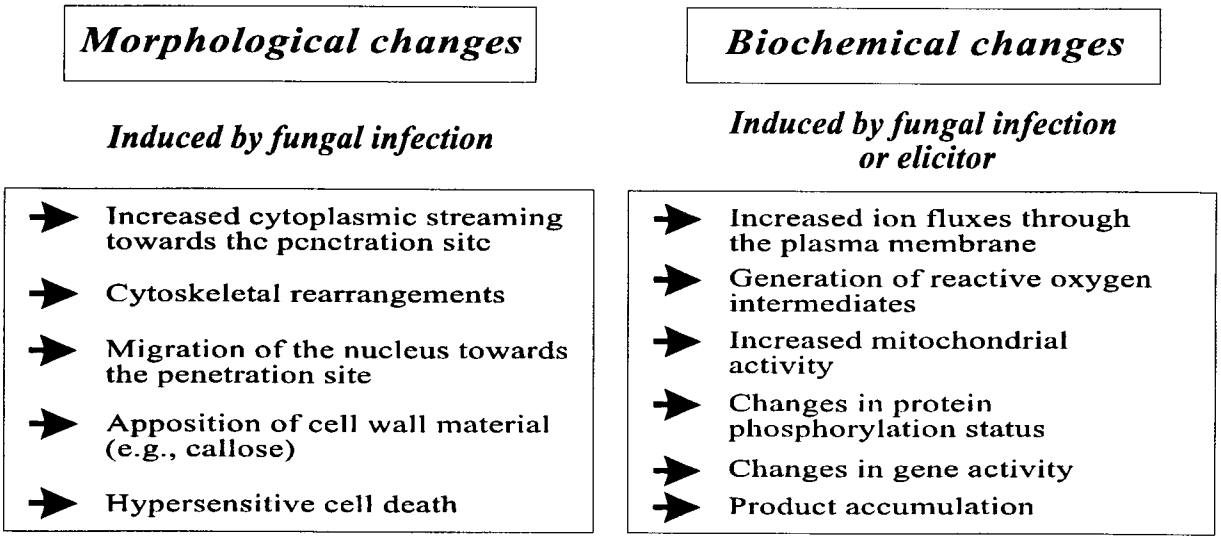

FIG. 1. Outline of previously established morphological and biochemical changes induced by fungal infection or fungal elicitor treatment of plant cells. References are given in the text.

embedded cells by touching them continuously with the tip of the tungsten rod.

The cells were treated with cytochalasin D by overlaying the agarose with a 40 μM solution for 4 h. For mechanical stimulation and elicitor treatment, the agarose containing the embedded cells was overlaid with varying concentrations of Pep-25 elicitor in HA medium and incubated for 10 min. For detection of local cell wall thickening when mechanically stimulated, the agarose layer containing the parsley cells was overlaid with 0.8 ml alkaline solution of aniline blue (0.05%, wt/vol, in HA medium, pH 8). Callose was detected after 5–10 min by UV light epifluorescence (excitation filter, 365 nm; dichroic mirror, 395 nm; barrier filter, 420 nm).

Gene Activation. Gene expression was assayed by reverse transcription-PCR with appropriate, gene-specific primers. The total RNA from parsley cells used as a template was prepared by using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA). Mechanical stress was applied (9) by placing cultured parsley cells between a microscope slide and a coverslip weighted with 0.025 g/mm^2 for 60–120 min. In control assays, a similar amount of cells was either left untreated or treated with the standard elicitor concentration (120 nM).

Local Application of Elicitor. Micropipettes were prepared from 100-mm-long borosilicate glass (outer diameter, 1.5 mm; inner diameter, 0.87 mm; omega dot, 0.2 mm; Hilgenberg, Malsfeld, Germany) by using a vertical pipette puller with a solenoid-type heating filament made of platinum wire according to the manufacturer's instructions (Sutter P30; Bachofer, Reutlingen, Germany) and were examined under the microscope for appropriate shape and tip diameter (10). Micropipettes with a tip diameter of approximately 0.5 μm were filled with solutions containing Pep-25 and fluorescein isothiocyanate-dextran 10,000 or Lucifer yellow (final concentrations 2.5 mg/ml and 0.2 mg/ml, respectively) for visualization of pipette contents under the epifluorescence microscope and connected to a microinjector (Transjector 5246 basic; Eppendorf).

Microscopy and Video Equipment. Light microscopy was performed with an inverted microscope (Axiovert 135 TV; Zeiss) equipped for epifluorescence microscopy. Micrographs were taken on Kodak Ektachrome 1600 ASA film with an Olympus (New Hyde Park, NY) OM-2 camera. The response of cells was recorded on video tape by using a charge-coupled device video camera (model C 3077; Hamamatsu Photonics, Hamamatsu City, Japan) mounted to the microscope and connected to an S-VHS video recorder (Panasonic AG-6720-E; Matsushita Electric Industrial, Osaka). Individual images from the video scenes were photographed on Kodak Ektachrome 400 film.

ROI Detection. Parsley cells were embedded in agarose as described above. Immediately after a cell was touched with the tungsten needle, the agarose layer was covered with HA medium containing 4 μg/ml 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes). Fluorescence was monitored after 10 min of incubation under blue light (excitation filter, 450–490 nm; dichroic mirror, 510 nm; barrier filter, 520 nm). Green fluorescence indicated oxidation of the nonfluorescent DCFH-DA by ROI to the fluorescing dye 2′,7′-dichlorofluorescein (DCF). Before stimulation with Pep-25, the embedded cells were loaded with DCFH-DA as described above. The dye solution was replaced after 10 min with HA medium containing Pep-25, and the generation of ROI was monitored after an additional 5–10 min as described above. The inhibitor diphenylene iodonium (10 μM) was applied 20 min before the addition of DCFH-DA. Photooxidation of the dye in nonstimulated cells under constant irradiation with blue light for approximately 5 min was used as a control for equal loading of the cells with DCFH-DA.

RESULTS

Local Mechanical Stimulation. Parsley cells were subjected to local mechanical stimulation by being touched gently and continuously with a tungsten needle. The diameter of the tip of the needle was 3–5 μm, similar to the diameter of a fungal hypha. If such a needle was moved carefully, the cell wall was never penetrated. The response of the mechanically stimulated cells was recorded by video microscopy (Fig. 2). A few minutes after the first contact of the needle with the cell wall, enhanced cytoplasmic streaming, the appearance of cytoplasmic strands aiming toward the site of stimulation, and accumulation of a small portion of the cytoplasm at this site could be detected (Fig. 2 *B* and *C*). Nuclear migration toward this site commenced 15–20 min later and was completed within 1–1.5 h. Essentially the same response was observed when cells were stimulated locally with a micropipette instead of the tungsten needle. However, because of difficulties in handling, e.g., frequent breakage of the micropipette or wounding of cells with the sharp glass tip, use of the metal needle was preferred. When the mechanical stimulus was removed, the cytoplasmic rearrangements disappeared within 15–30 min. Timing and morphological appearance of the cytoplasmic rearrangements

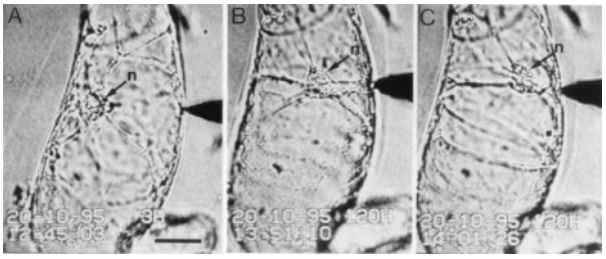

FIG. 2. Video sequence demonstrating cytoplasmic rearrangements in an agarose-embedded, mechanically stimulated parsley cell. The cell was continuously touched with a tungsten needle (right of each panel) for 76 min. n, nucleus. (Bar = 10 μm.)

were similar to those induced by fungal penetration (4, 7). Likewise, the response to 40 μM cytochalasin D was similar (4), completely inhibiting cytoplasmic streaming and nuclear migration toward the site of mechanical stimulation.

One of the morphological changes induced by fungal penetration is the formation of a collar-like wall thickening, consisting at least in part of callose, around the progressing fungal infection tube (4, 6). In those cells in which enhanced cytoplasmic streaming or nuclear migration was observed, staining for callose with aniline blue was performed at different times after the onset of constant mechanical stimulation. By using the same local mechanical stimulus as described above (Fig. 2), we could not detect any local cell wall thickening or callose apposition in 40 independent experiments.

Preincubation of parsley cells with Pep-25 elicitor suppressed the response to local mechanical stimulation in a dose-dependent manner. At the standard concentration of 120 nM Pep-25, only 20% of the cells showed enhanced cytoplasmic streaming and less than 5% nuclear migration toward the site of mechanical stimulation, whereas the respective values for control cells were 70 and 45%. This inhibitory effect was weaker at one-third (40 nM) and disappeared at one-tenth (12 nM) of the standard Pep-25 concentration.

Accumulation of ROI. Recently, we demonstrated the intracellular generation of ROI in cultured parsley cells when infected with fungi (6) and the release of ROI from elicitor-treated cells (3, 11). Here, we monitored the intracellular accumulation of ROI at the single-cell level when the cell was mechanically stimulated or treated with elicitor. ROI were visualized by using DCFH-DA, which is converted to the green fluorescent compound DCF by intracellular peroxides (12, 13).

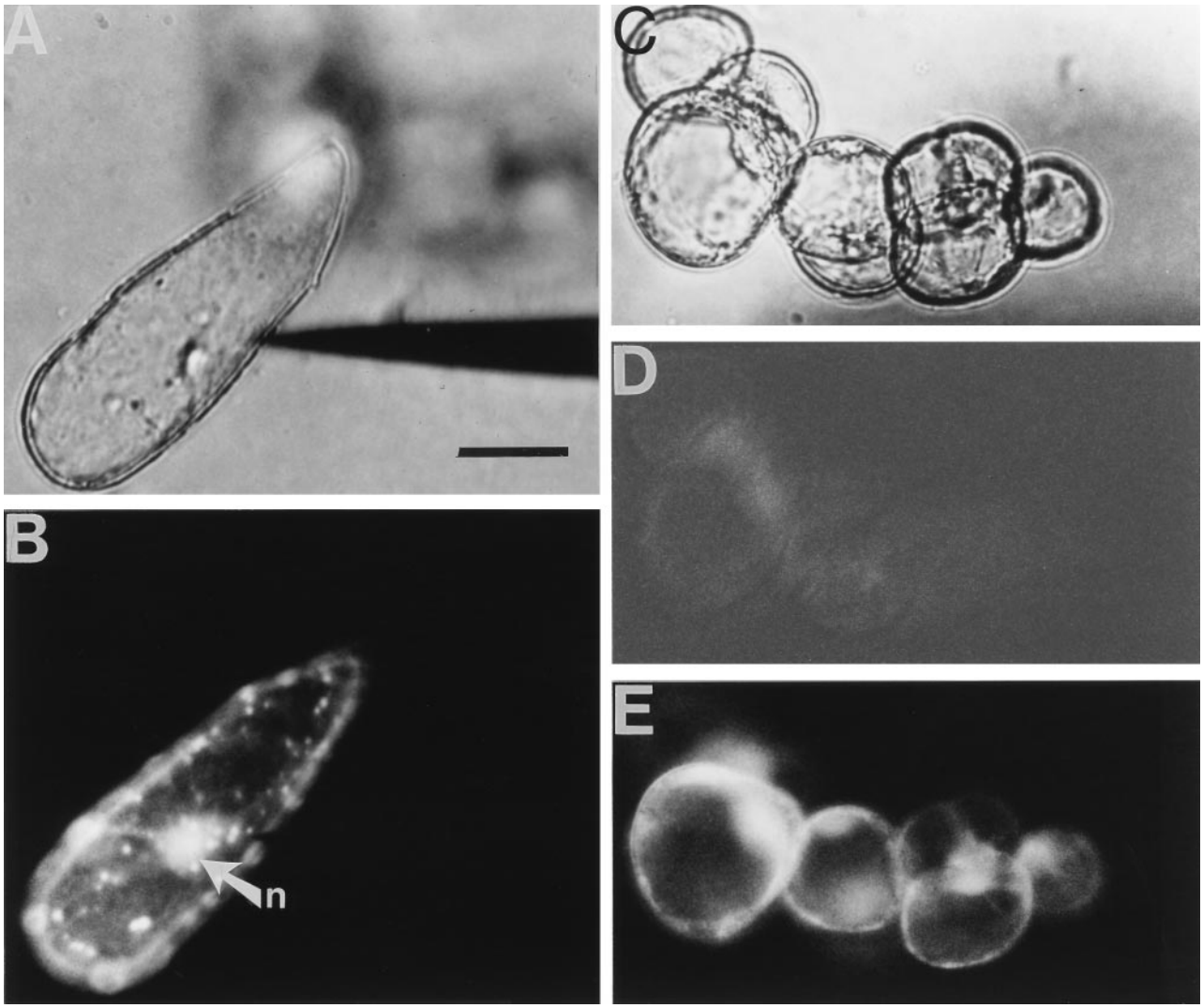

FIG. 3. Intracellular ROI accumulation of embedded parsley cells after local mechanical stimulation. Immediately after touching with a tungsten needle, cells were loaded with DCFH-DA for 7 min and subsequently irradiated with blue light for epifluorescence analysis. (*A*) Bright-field image; cell and needle in focal plane. (*B*) Blue light epifluorescence after 7 min of mechanical stimulation. (*C*) Bright-field image; unstimulated background cells of *A* in focal plane. (*D*) Blue light epifluorescence at the same time as shown in *B*. (*E*) Same as *D* but after 5 min of irradiation with blue light. n, nucleus. (Bar = 20 μm.)

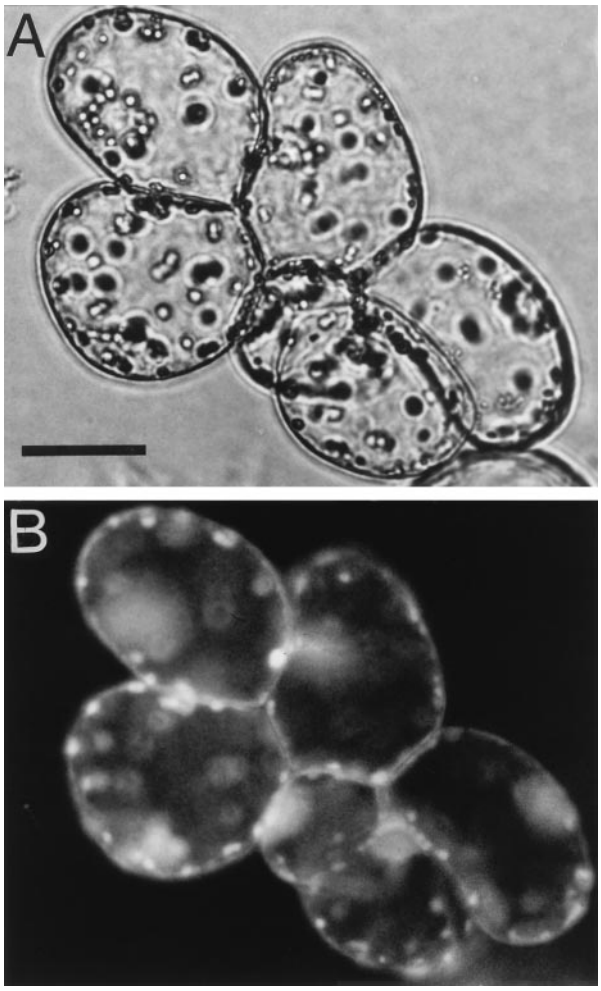

FIG. 4. Intracellular ROI accumulation of embedded parsley cells when treated with elicitor. The cells had been loaded with DCFH-DA and then treated with 120 nM Pep-25. After 5–10 min the generation of ROI was monitored by blue light epifluorescence. (*A*) Bright-field image. (*B*) Fluorescence image. (Bar = 20 μm.)

Parsley cells were embedded in agarose and stimulated mechanically with a tungsten needle. Only 5–10 min of constant mechanical stimulation was required to evoke the intracellular generation of ROI, as detected by the bright green fluorescence of the stimulated cell (Fig. 3*A* and *B*). The fluorescence was observed throughout the cytoplasm, particularly around organelles such as amyloplasts and the nucleus. Nonstimulated cells (Fig. 3*C*; present also in *A* and *B*, although out of focus) showed only weak background fluorescence (Fig. 3*D*). After 5 min of continuous irradiation with blue light, nonstimulated cells also fluoresced intensely because of photooxidation of the dye (Fig. 3*E*). This internal control demonstrated that the dye was present in all cells but was converted to the fluorescent form by ROI only in stimulated cells. The local mechanical stimulus had to be continuous for at least 5–10 min. A brief touch with the tungsten needle for a few seconds failed to induce any of the observed reactions.

The effect of elicitor treatment on the intracellular ROI level was also examined in the embedded parsley cells (Fig. 4). Five to 10 min after addition of 120 nM Pep-25 elicitor almost all cells (about 95%) fluoresced brightly under blue light

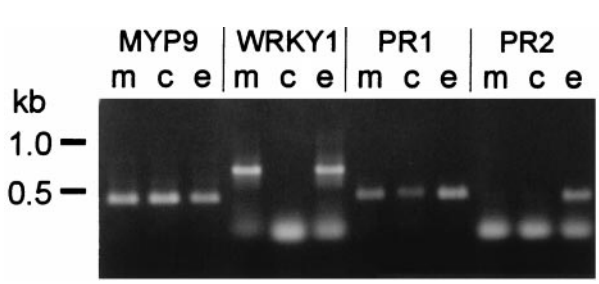

FIG. 5. Effects on mRNA accumulation rates. Total RNA was extracted from mechanically stimulated cells (m), untreated control cells (c), or elicitor-treated cells (e) and used as a template for reverse transcription-PCR with specific primers as indicated.

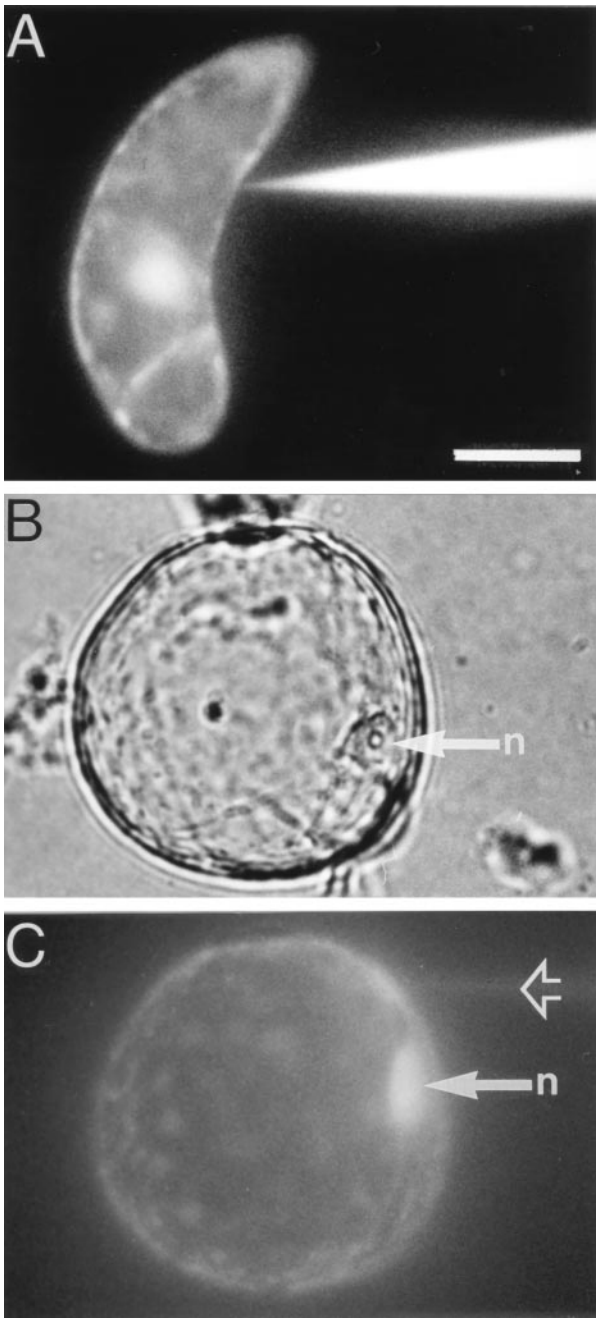

FIG. 6. Intracellular ROI accumulation and nuclear migration on simultaneous local mechanical stimulation and elicitor application. The elicitor solution (*A,* 120 nM; *B* and *C*, 12 nM) was applied by the microcapillary pipette visualized by the fluorescing dye in *A* and indicated by the open arrow in *C*. ROI generation was monitored by DCFH-DA staining 20 min (*A*) or 35 min (*C*) after the onset of stimulation. (*A* and *C*) Fluorescence image. (*B*) Bright-field image of the cell shown in *C*, however, prior to the onset of stimulation. n, nucleus. (Bar = 15 μm.)

throughout the cytoplasm, indicating the intracellular accumulation of ROI (Fig. 4*B*). Untreated cells fluoresced only weakly (Fig. 3*D*). The generation of ROI when the elicitor was applied was not instantaneous but was delayed by approximately 3–5 min.

Gene Activation. Because the previously used method of *in situ* RNA hybridization (4) was not applicable to the agarose-embedded cells, the effects of mechanical stimulation on gene expression could not be analyzed under the conditions used above. We therefore took the alternative approach of placing cells between a microscope slide and a weighted coverslip (9) and measuring the resulting changes in mRNA amounts by reverse transcription–PCR. This method of gently squeezing cells likewise exerts mild physical pressure without wounding, although less highly localized, and enables mRNA determinations with high sensitivity. We used probes for three selected, strongly elicitor-responsive genes encoding the putative transcription factor WRKY1 (14) and two "pathogenesis-related" proteins, PR1 (14) and PR2 (15), as well as the myosin isoform MYP9 (A. Tietze and E.S., unpublished results) as a control. When compared with the expected, clear-cut response to elicitor, the WRKY1 mRNA was strongly, the PR1 mRNA moderately, and the PR2 mRNA not induced at a detectable rate. All controls behaved as predicted (Fig. 5).

Local Application of Elicitor. So far, the elicitor had always been applied uniformly to cultured cells through the medium. For local application to agarose-embedded parsley cells, a microcapillary pipette was filled with a solution containing 120 nM Pep-25 and a fluorescent marker dye (Lucifer yellow) and was directed through the agarose toward a cell until the tip was positioned close to the cell wall without touching it. As visualized by the marker fluorescence, a small volume of the solution was released from the capillary pipette by adjusting the compensation pressure accordingly. Under these conditions of local elicitor application, we never observed any cytoplasmic rearrangements within the cells, whereas approximately 40% of them accumulated intracellular ROI, as demonstrated by DCFH-DA staining.

In another set of experiments, the elicitor-containing capillary pipette was moved as close as possible to the cell wall, until the tip touched the cell but did not penetrate the cell wall, to apply a mechanical stimulus together with the local release of elicitor. Again, 120 nM Pep-25 impaired the cytoplasmic rearrangements caused by mechanical stimulation alone. However, in contrast to the experiments described above, in which the cells were treated with elicitor prior to mechanical stimulation, inhibition of the response to the mechanical stimulus was not complete. In about 20% of the cells the nucleus still moved toward the site of local mechanical and chemical stimulation, and ROI generation was observed in the majority (75%) of stimulated cells (Fig. 6*A*). When the elicitor was applied locally at a low concentration of 12 nM, the rate of nuclear migration was similar to that obtained by mechanical stimulation alone (Fig. 6 *B* and *C*) and a major proportion of treated cells again showed ROI accumulation (Fig. 6), in contrast to only 5–10% of the cells treated with 12 nM Pep-25 without mechanical stimulation (see above).

DISCUSSION

The major question of this study concerns the role of the physical pressure exerted by an invading fungus as a signal involved in triggering the plant's defense response. We provide evidence for such a role by demonstrating that even a very gently applied local mechanical stimulus alone induces some of the reactions occurring during fungal invasion of cultured parsley cells by *P. infestans* (4, 6) or *P. sojae* (S.G.-M. and E.S., unpublished results). Two of the processes analyzed, the structural changes monitored as directed migration of nucleus and cytoplasm and the biochemical changes represented by the oxidative burst, were essentially the same in response to touch by a tungsten needle or to penetration by a fungal hypha, whereas the changes in gene expression overlapped only partially.

Nuclear migration has previously been shown to be a characteristic response of plant cells not only to fungal infection (7, 16) but also to other types of tissue damage (17). Our data indicate that the plant cell wall must not be penetrated to evoke the translocation of cytoplasm and nucleus, particularly because we never observed any obvious wound effect. Even when the mechanical pressure toward the cell wall was increased to simulate more closely the actual penetration process rather than the mere physical touch, the cell was never perforated, probably because the tip of the needle was not sharply pointed. Another argument against possible wounding by the needle tip was the lack of callose apposition at the site of mechanical stimulation. It has been shown that puncturing of barley cells with a glass microneedle caused the formation of a callose-containing wound plug during repair of the perforation site (18). In our experiments, we never observed any visible wall thickening at the site of mechanical stimulation, and as soon as the needle was removed, the cytoplasmic aggregation disappeared and the nuclear migration stopped.

A very sensitive indicator of mechanical stimulation of plant cells appears to be the oxidative burst. Cultured soybean cells have been shown to produce intracellular peroxides in response to physical pressure exerted by placing them between a microscope slide and a weighted coverslip (9). According to our results, which confirm and extend these earlier findings, local application of gentle physical pressure to plant cells is enough to stimulate within minutes the generation of intracellular ROI. In this context we noted that the DCF fluorescence of stimulated cells decreased strongly within about 20 min, whereas in control cells the fluorescence resulting from photooxidation lasted much longer. The same phenomenon occurred in elicitor-treated cells. This effect may be ascribed to rapid changes in membrane permeability, a frequently observed immediate stress response of plant cells, particularly in plant–pathogen interactions (19). As a result, the dye may leak out of the cells and/or diffuse into the vacuole, where under acidic conditions the nonfluorescing DCF cation would probably be formed.

So far the elicitor has always been applied uniformly to the medium of cell suspensions, making it extremely unlikely that the cells underwent any directed or localized processes, such as translocation of cytoplasm and nucleus or local wall apposition. We attempted to simulate as closely as possible the plant cell–fungus interaction by combining the local mechanical stimulus with the elicitor as a locally applied chemical signal. Local application of the elicitor alone did not induce cytoplasmic rearrangements. On the contrary, regardless of how the elicitor was applied, either locally or in suspension, this chemical signal interfered in a dose-dependent manner, up to total inhibition, with the physical signal triggering the translocation of cytoplasm and nucleus, whereas the oxidative burst was not affected to a detectable extent. When only low doses of the elicitor were used, both the morphological and biochemical reactions occurred in a manner similar to the effects of the local mechanical stimulus alone or of fungal penetration.

A possible explanation for this phenomenon may be deduced from the recent finding that a Ca^{2+}-permeable ion channel in the plasma membrane of parsley cells was specifically activated by elicitor (20). The authors concluded from their data that this channel is causally involved in the signal transduction mediating pathogen defense. Their electrophysiological approach also revealed a mechanosensitive ion channel with very similar properties. Thus, it is possible that this ion channel represents a common constituent of two at least partly distinct signaling cascades, one of which would operate via elicitor receptor binding to trigger various biochemical reac-

tions and the other would operate via the perception of a local mechanical stimulus, which may lead to partly overlapping responses as well as cytoplasmic rearrangements. Saturating elicitor concentrations would then abolish mechanosensitivity of this ion channel and, hence, the induction of cytoplasmic rearrangements.

Although the method of mechanically stimulating cells with the tip of a needle could not be combined with direct *in situ* hybridization of the induced gene products for technical reasons, the alternative approach used here provided clear-cut evidence for differential behavior of the three selected elicitor-responsive genes. More detailed analysis will have to clarify whether individual members of gene families can also respond differentially. The genes analyzed in this study were selected such that either they occurred in only one copy [WRKY1 (T. Eulgem and I. E. Somssich, unpublished results) and PR2 (15)] or no more than three copies [PR1 (14)] were detected under the conditions used. These results allow us to draw the following conclusions concerning the signaling involved in the nonhost resistance response of parsley to *P. sojae* or *P. infestans*. At an initial stage of attempted fungal colonization, probably during appressorium formation immediately before penetration of the plant cell wall, the perception of a local mechanical signal from the emerging infection tube is sufficient to induce the oxidative burst and cytoplasmic rearrangements. For the triggering of local cell wall thickening and papilla formation, penetration of the plant cell wall by the fungal infection tube may be required, similarly to the formation of a wound plug when puncturing cells (18). As soon as the fungal infection tube is in contact with the plasma membrane of the plant cell, the glycoprotein elicitor or a derived peptide may have an essential role in the activation of additional biochemical defense reactions.

Concentrations as low as a few nanomolar of the smallest fully active peptide elicitor, Pep-13, have been shown to be sufficient for strong activation of various biochemical defense reactions in suspension-cultured parsley cells (3). Because antibodies specific for the *P. sojae* glycoprotein immunohistologically stained exclusively the wall of fungal structures outside as well as inside the plant tissue (21), the glycoprotein probably represents a surface constituent of the fungal wall. Our present results suggest that the functionally active elicitor occurs *in vivo* in amounts small enough to exclude interference with the triggering of cytoplasmic rearrangements.

Thus, the combination of appropriately coordinated intensities and spatial distribution patterns of the physical and chemical stimuli may be responsible for triggering intracellular rearrangements as well as the various biochemical changes involved in gene activation and product accumulation, whereas additional signals are apparently required for the induction of papilla formation and hypersensitive cell death.

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