Plant Physiol. (1995) 109: 1259–1266

Evidence for a Mechanically Induced Oxidative Burst[1]

Tami Yahraus[2], Sreeganga Chandra, Laurent Legendre[3], and Philip S. Low*

Purdue University, Department of Chemistry, 1393 Brown Building, West Lafayette, Indiana 47907

Rapid release of H_2O_2 may constitute an initial defense response mounted by a plant. Inauguration of this oxidative burst is known to occur upon stimulation with chemical elicitors, but the possibility of mechanical elicitation arising from pathogen penetration/weakening of the cell wall has never been examined. To introduce an adjustable mechanical stress on the plasma membrane, cultured soybean (*Glycine max* Merr. cv Kent) cells were subjected to defined changes in medium osmolarity. Dilution of the medium with water or resuspension of cells in sucrose solutions of reduced osmolarity yielded an oxidative burst similar to those stimulated by chemical elicitors. Furthermore, the magnitude of oxidant biosynthesis and osmotic stress correlated directly. Upon return of the cells to normal tonicity, the oxidative burst abruptly halted, indicating that its expression depended on maintenance of the osmotic stress and not on any external chemical signal. To confirm the ability of soybean cells to respond to a mechanical stimulus with induction of an oxidative burst, cells were subjected to direct physical pressure. Application of pressure yielded a characteristic oxidative burst. Because neither these cells nor those subjected to osmotic pressure were damaged by their treatments, we conclude that plant cells can detect mechanical disturbances and initiate a classical defense reaction in response.

Production of reactive oxygen species has long been known to occur during a plant's defense response (for reviews, see Sutherland, 1991; Mehdy, 1994). Recently, the role of these plant-generated oxidants in disease resistance has risen into prominence as new contributions of H_2O_2 and $O_2^{-}\cdot$ to both local and systemic disease-resistance mechanisms have been discovered. Thus, host-generated H_2O_2 may not only serve as the mediator of salicylate's systemic effects (Chen et al., 1993; Dempsey and Klessig, 1994), but in the immediate vicinity of pathogen attack it may also act as a microbicidal agent (Keppler and Baker, 1989; Peng and Kuc, 1992; Legendre et al., 1993a), promote cell wall stabilization (Bradley et al., 1992; Olson and Varner, 1993), assist in induction of phytoalexin production (Apostol et al., 1989; Degousee et al., 1994), and help initiate the hypersensitive response (Doke, 1983; Keppler and Novacky, 1987; Greenberg et al., 1994; Levine et al., 1994), although the absolute requirement of active oxygen for the latter defense mechanism has recently been questioned (J.A. Glazner, E.W. Orlandi, and C.J. Baker, personal communication). Further, since rapid generation of oxidative species can occur within minutes of pathogen recognition (Low and Heinstein, 1986; Apostol et al., 1989; Levine et al., 1994; Mehdy, 1994), it may also constitute the earliest defense mechanism mounted by the besieged plant.

Elicitors of the oxidative burst are generally thought to be pathogen-derived macromolecules. Examples of such bioactive ligands include carbohydrates and/or polypeptides from *Verticillium dahliae* (Low and Heinstein, 1986; Davis et al., 1993), *Phytophthora megasperma* (Linder et al., 1988), *Erwinia amylovora* (Baker et al., 1993), *Heterobasidion annosum* (Schwacke and Hager, 1992), and *Cladosporium fulvum* (Vera-Estrella et al., 1992). Host cell-wall fragments released during microbial invasion may also stimulate oxidant biosynthesis. For example, an OGA fragment (degree of polymerization = 14 ± 3) can trigger production of approximately 10^{-14} mol H_2O_2 min^{-1} $cell^{-1}$ in soybean (*Glycine max*) cell suspension cultures, a rate almost identical to the biosynthesis of $O_2^{-}\cdot/H_2O_2$ by stimulated human neutrophils (Legendre et al., 1993a).

Although infectious bacteria and fungi may release cell-wall fragments during colonization of a plant, not all cell-wall fragments constitute elicitor-active species (Nothnagel et al., 1983; Bishop et al., 1984). Therefore, it is conceivable that certain microbes might escape early detection if the host plant is forced to rely solely on chemical signals to trigger its response. A potentially more universal sensor of pathogen invasion might arise if the plant were to transduce mechanical signals deriving from degradation, puncture, or deformation of its cell wall/plasma membrane. Since some physical distortion of the cell wall/plasma membrane may occur during even the most benign pathogen invasion, a mechanical trigger could explain why even compatible microbes trigger some resistance.

We have noted previously that plant cells in suspension culture can be stimulated to produce H_2O_2 simply by stirring the suspension very vigorously (Legendre et al., 1993a). Because mechanical stress was hypothesized to be the trigger of H_2O_2 biosynthesis, we have sought a more calibrated method of initiating this putative mechanical signal. In this paper, we impose the desired mechanical

[1] This work was supported by National Science Foundation grant MCB 9303929. T.Y. was supported by the Quest Fellowship Program.

[2] Present address: Department of Biochemistry, Cellular and Molecular Biology, Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205–2185.

[3] Present address: Université de Reims, Champagne-Ardenne, Laboratoire de Biologie et Physiologie Vegetales, UFR Sciences B.P. 347, Moulin de la Housse, 51062 Reims Cedex, France.

* Corresponding author; e-mail lowps@mace.cc.purdue.edu; fax 1–317–494–0239.

Abbreviations: OGA, oligogalacturonic acid; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid.

stress by two independent methods: direct physical pressure on the cultured cells and a change in osmotic pressure of the medium. We report that raising the turgor pressure within the plant cell not only induces the oxidative burst, but also probably initiates the response by a mechanical transducer. We further show that direct application of physical pressure to the cultured cells induces the same response. These data suggest that plant cells can detect a physical disturbance at their cell surfaces and initiate the oxidative burst reaction in response.

MATERIALS AND METHODS

Plant Cell Culture

Cell suspension cultures of soybean (*Glycine max* Merr. cv Kent) were maintained in W-38 medium as described previously (Low and Heinstein, 1986). Briefly, 9 cm^3 of filtered cells were transferred to 100 mL of fresh W-38 medium every 8 d. When an experiment was to be conducted, cells were allowed to grow for approximately 36 h before use unless stated otherwise in the figure legend.

Oxidative Burst Assay for Cells in Suspension

H_2O_2 production was determined by monitoring the oxidative quenching of the fluorescent peroxidase substrate pyranine (trisodium salt: excitation wavelength 405 nm, emission wavelength 512 nm; Molecular Probes, Eugene, OR), as described previously (Legendre et al., 1992). Briefly, 1.5 mL of cells were treated with 7 μL of pyranine (0.2 mg/mL) and maintained in suspension by mild stirring in a spectrofluorimeter cuvette. Under these conditions, no oxidant production was detectable until addition of elicitor or dilution with water. Then, production of H_2O_2 was monitored by following the decrease in pyranine fluorescence due to H_2O_2-mediated oxidation of the dye by plant peroxidases. The fluorescence signal of the pyranine-labeled cell suspension was set arbitrarily at 80% of full scale, and the rate of pyranine quenching was assessed from the steepest portion of the quenching curve. All studies reported in a single figure were obtained from the same flask of cells to assure consistency in cell behavior. Additionally, all studies were repeated at least once on a separate flask of cells. Peroxidase activity was also evaluated to assure that it was never rate limiting in the oxidative burst assays. Several experiments used varying amount of cells, and these changes are mentioned in the figure legends. Suc, a common additive in these studies, was shown not to influence the oxidative burst assay.

Elicitor Preparation

An OGA fraction (degree of polymerization = 14 $\pm$ 3) that contained 0.5 mg/mL uronic acid equivalents as determined by the method of Blumenkrantz and Asboe-Hansen (1973) was prepared as described earlier (Nothnagel et al., 1983; Legendre et al., 1993a). This oligogalacturonide has been shown to be a potent elicitor of the oxidative burst (Legendre et al., 1993a).

Osmometry

Osmotic strength measurements were conducted using a Wescor model 5100C vapor pressure osmometer (Wescor Inc., Logan, UT) as described previously (Premachandra et al., 1994).

Fluorescence Microscopy

Experiments were conducted essentially as described previously (Legendre et al., 1993a). Briefly, 0.75 mL of a soybean suspension was mixed with 5 μL of a 100 mg/mL 2′,7′-dichlorofluorescin diacetate solution (Molecular Probes) or 50 μL of a 1 mg/mL pyranine solution and observed under a fluorescence microscope (10×). For application of physical pressure, circular metal washers were placed on the coverslip such that the cells under pressure could be directly viewed through the central hole. Release of pressure was achieved by removal of the metal washers.

RESULTS

General Characterization of the Osmotic Pressure-Induced Oxidative Burst

To generate a calibrated mechanical stress on the plant cell wall, suspension-cultured soybean cells were diluted with known volumes of distilled water and examined for production of H_2O_2. As shown in Figure 1, cells suspended in their growth medium (control) gave no oxidative burst, indicating that neither iso-osmotic media nor the stirring conditions of the assay trigger the burst. However, addition of as little as 0.33 volumes of water (one-fourth dilution) promoted the biosynthesis of H_2O_2, leading to the oxidative quenching of the pyranine fluorescence. Supplementation of the medium with even greater water volumes, while keeping the volume of cells constant, resulted in increasingly stronger oxidative bursts. Importantly, the H_2O_2 burst was not instantaneous, but followed imposition of the osmotic stress by 1 to 3 min, a delay analogous to that seen with elicitor-induced bursts (Legendre et al., 1993a). Since the diluted cells remained intact, as judged by Evans blue exclusion, we conclude that oxidant release is the response of a living cell to osmotic pressure, not the property of a ruptured cell running oxidative reactions out of control. It is interesting that hyperosmotic conditions did not induce an oxidative burst in the soybean cell cultures.

The response of cell cultures to imposition of osmotic stress varied with the stage of the cells in their growth cycle. Immediately after transfer to fresh growth medium, cells responded to dilution and generated small quantities of H_2O_2 upon addition of water (Fig. 2). For the next approximately 50 h, a completely refractory period ensued (i.e. between 50 and 100 h post-transfer) during which the cells were totally unresponsive to osmotic stress. Finally, at >100 h post-transfer, the cells became highly responsive to changes in osmotic pressure. In contrast, stimulation of the oxidative burst by OGA was maximal approximately 20 h after subculturing in fresh medium, after which the cells lost sensitivity to this plant cell-wall component (Fig. 2).

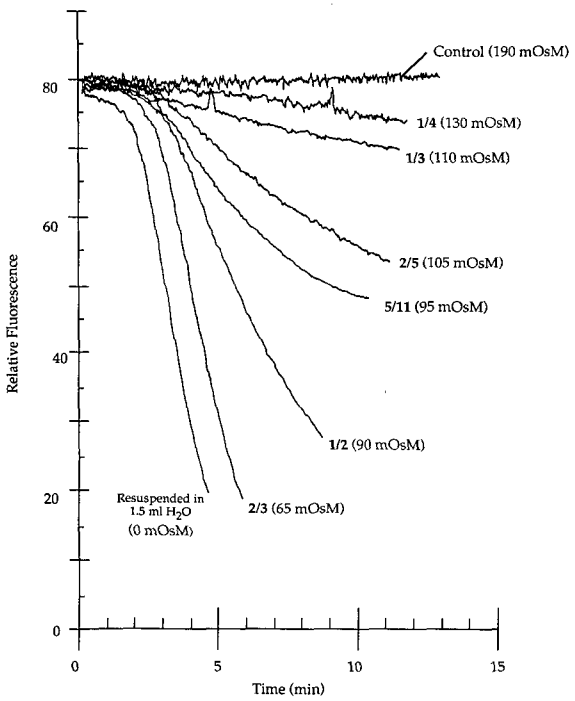

Figure 1. Kinetics of the dilution-induced oxidative burst. A soybean suspension culture (0.75 mL; 10–15% packed cell volume) was mixed with varying amounts of distilled water in a fluorimeter cuvette to obtain the dilutions indicated. Following dilution, 7 μL of pyranine was immediately added and oxidative quenching of the dye was monitored spectrofluorimetrically. Cell suspensions that were not diluted with water served as the control. After monitoring the H_2O_2 burst, the cells were pelleted and the osmotic strength of the supernatant was measured. Similar results were obtained in three independent experiments at each dilution. mOsM, Milliosmolar.

Whether developmental separation of the two bursts occurs in planta still remains to be investigated. Only between 10 and 50 h post-transfer did cultured soybean cells respond to both stimuli.

Although there was no significant pH change in the medium during the course of either the osmotically induced burst (data not shown) or the OGA-induced burst (Horn et al., 1992), external pH was nevertheless found to modulate both bursts. Lowering medium pH from its normal value of 4.8 to 3.8 promoted a more rapid release of H_2O_2 in response to dilution with water (data not shown). This pH dependence contrasts with the OGA-induced burst, where lowering the pH over the same range inhibited H_2O_2 production (Legendre et al., 1993a).

The Osmotically Induced Burst Is Promoted by a Mechanical Signal

Previous work from our laboratory has demonstrated that citrate, a common metabolite of cultured plant cells, is a potent inhibitor of the oxidative burst and other defense responses (Apostol et al., 1987). To ensure that the osmotically induced burst was not a trivial consequence of dilution of some endogenous inhibitor such as citrate, soybean cells were filtered and resuspended in Suc solutions of decreasing osmolarity. As shown in Figure 3A, iso-osmotic Suc solution (approximately 200 milliosmolar) promoted no measurable synthesis of oxidant, whereas hypo-osmotic solutions induced a burst similar to that seen upon direct dilution of the cell suspension. Figure 3B compares the magnitudes of the H_2O_2 burst in Suc and growth medium of different osmotic strengths. Within reasonable limits, the two dependencies are similar, suggesting that dilution of an inhibitor or any other medium component is not a causative factor of the dilution-triggered burst.

It was also conceivable that osmotic stress might promote release of a previously sequestered chemical inducer or an enzyme (e.g. polygalacturonase) that could liberate an oxidative burst agonist. To test this possibility, cells were induced to produce H_2O_2 by one-half aqueous dilution, and following termination of the burst, the cells were removed and replaced with unstimulated cells (Fig. 4). As expected, exposure of the unstimulated cells to the same diluted growth medium induced their participation in the oxidative burst. However, supplementation of this previously "active" medium with Suc to restore its native osmolarity eliminated any induction of the burst. Thus, stable inducing factors do not appear to be released during an osmotically stimulated oxidative burst.

To unequivocally establish the absence of a chemical induction mechanism in the dilution-triggered burst, soybean cells were induced to generate H_2O_2 by 50% dilution with water and then treated at various times with Suc to restore osmolarity. As shown in Figure 5, regardless of the duration of engagement in burst activity, restoration of osmolarity rapidly halted H_2O_2 biosynthesis. These data thus demonstrate that no stable chemical agonist is present

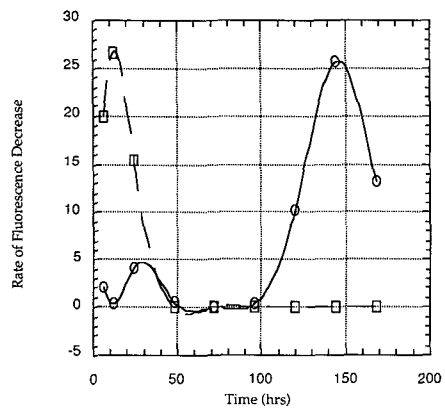

Figure 2. Comparison of the cell age dependence of the OGA- and dilution-induced bursts. The rate of H_2O_2 production (determined as the rate of fluorescence decrease) was measured over the course of the 8-d growth cycle of the cells. Briefly, 1.5 mL of cells were taken at different times post-transfer and treated with either 4.8 μg/mL OGA (□) or diluted with an equal volume of water (○), and the rate of decrease in pyranine fluorescence was monitored. Rates observed at early time points (<10 h) represent autoelicitation, i.e. production of H_2O_2 in the absence of a stimulus. A similar profile was seen in two separate experiments. Zero time is taken as the time of cell transfer to fresh medium.

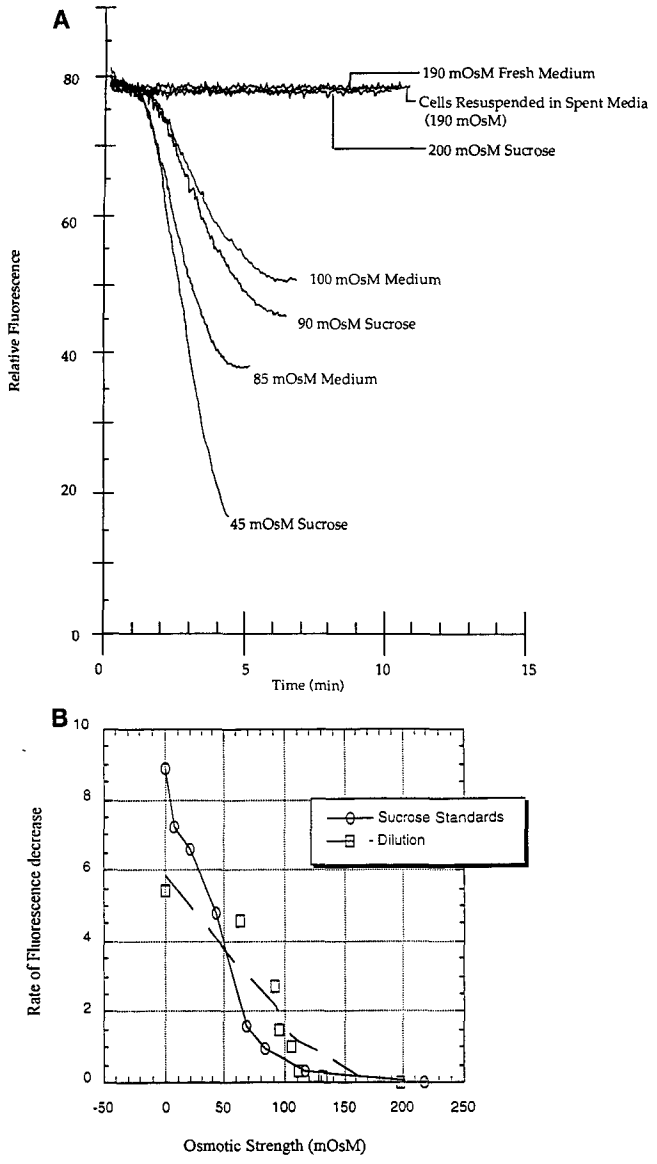

Figure 3. Response of filtered soybean cells upon resuspension in iso-osmotic and hypo-osmotic Suc solutions. A, Cells (1.5 mL; 10–15% packed cell volume) were filtered and resuspended into 1.5 mL of diluted media or Suc solutions of different osmotic strengths. The oxidative burst was followed by the protocol described in "Materials and Methods." Cells resuspended in fresh growth medium and spent growth medium served as iso-osmotic controls. B, Graphic representation of the osmotic strength dependence of the oxidative bursts triggered by dilution (□; data from Fig. 1) and by resuspension into hypo-osmotic-strength Suc solutions (○; data from Fig. 3A and from other, unpublished scans). The curves fitted to the data show similar trends over the same range of osmotic strengths. mOsM, Milliosmolar.

at any time during the dilution-induced burst and that initiation of the pathway is likely promoted by a mechanical signal. The results further establish that the induction mechanism is rapidly reversible and that soybean cells are exquisitely aware of changes in the osmotic status of their environment.

Induction of the Oxidative Burst by Direct Application of Physical Pressure

To confirm by an independent technique that a plant cell has the ability to respond to a mechanical disturbance, suspension cultured soybean cells were treated with the oxidant-sensitive dye used in Figures 1 to 5 (pyranine) and placed under a glass coverslip on a microscope slide. In the absence of any exogenous physical pressure, no change in fluorescence of the pyranine was detected by fluorescence microscopy (Fig. 6, top left). However, upon application of weight (0.039 g/mm^2) to the coverslip, a rapid diminution of fluorescence arising from oxidative quenching of the dye was observed (Fig. 6, middle left). Furthermore, when the weight was removed, the quenching reaction ceased, suggesting that continuous pressure on the cell surface was essential for continued expression of the burst. For comparison, soybean cells treated with OGA chemical elicitor were similarly examined by fluorescence microscopy, and an analogous dye-quenching reaction localized to the vicinities of the activated cells was also seen (Fig. 6, bottom left).

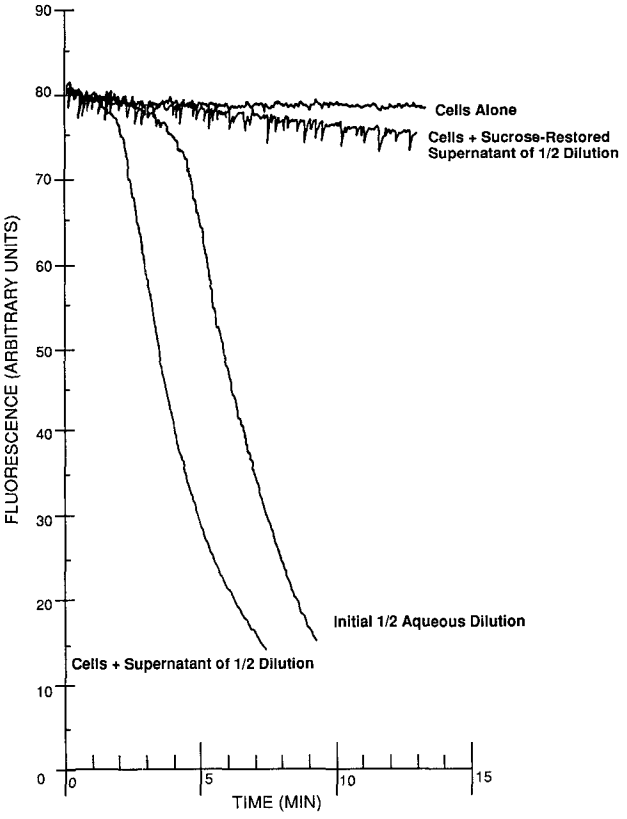

Figure 4. Response of filtered soybean cells to resuspension into hypo-osmotic and iso-osmotic cell culture supernatant. An oxidative burst was initiated by diluting 1 mL of cells with an equal volume of distilled water. After completion of the burst, the suspension was filtered and the diluted supernatant was collected. Fresh cells (1.5 mL; 10–15% packed cell volume) were then filtered and added to 1.5 mL of the previously collected supernatant in the presence or absence of sufficient Suc to restore the osmotic strength of the medium. The oxidative burst was followed by the protocol described in "Materials and Methods."

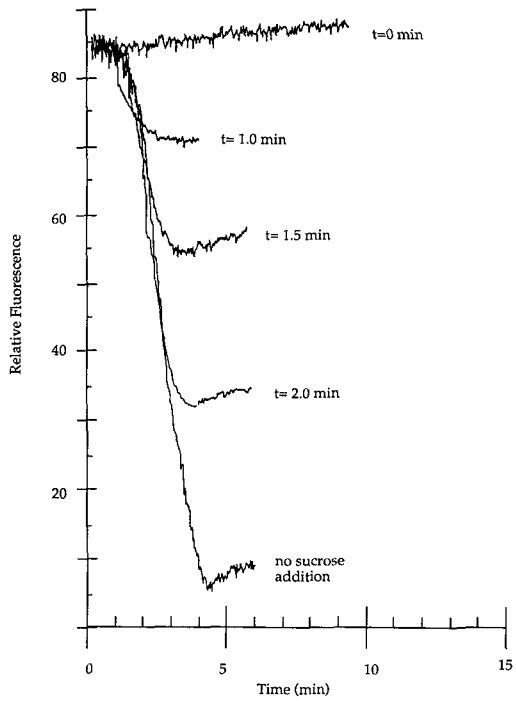

Figure 5. Arrest of the dilution-induced oxidative burst upon restoration of iso-osmolarity. An oxidative burst was initiated by diluting 1 mL of cells with an equal volume of distilled water. At the specified times after dilution, 160 μL of a 0.5 g/mL Suc solution (final concentration 200 mM) were added to restore iso-osmolarity.

To assure that the loss of fluorescence was not a trivial consequence of extrusion of the dye from the cell clusters, a second dye that becomes fluorescent only upon oxidation with H_2O_2 (2′,7′-dichlorofluorescin diacetate) was substituted for pyranine in the same study. As seen in the right panels of Figure 6, little fluorescence was generated upon application of the coverslip alone (Fig. 6, top right). However, after addition of 0.039 g/mm^2 to the coverslip, a strong fluorescence localized to the cell clusters was seen (Fig. 6, middle right). Again, the change in fluorescence closely resembled that observed upon application of elicitor (OGA) to the same cell suspension (Fig. 6, lower right). These data confirm that soybean cells can detect a mechanical perturbation of their cell walls and respond with a classical oxidative burst.

In the course of these studies we had hoped to establish participation of stretch-activated channels in the mechanically induced oxidative burst by demonstrating sensitivity to gadolinium ion, a well-characterized, stretch-activated channel blocker in both plants and animals (Yang and Sachs, 1989; Ding and Pickard, 1993). Although gadolinium indeed inhibited the osmotically triggered burst with 50%-inhibitory concentration of approximately 0.5 mM (Fig. 7), it also inhibited the OGA-triggered burst with the same potency. Thus, an unequivocal evaluation of the involvement of stretch-activated channels in the mechanically triggered burst was not possible. Nevertheless, the data are not inconsistent with such a possibility.

DISCUSSION

We have shown by application of direct pressure and by dilution of the osmoticum bathing a plant cell that mechanical forces acting at the cell wall/membrane can induce the rapid synthesis of H_2O_2. The trigger for the osmotically stimulated oxidative burst was suggested to be mechanical in nature, since (a) it is independent of the osmotic species present, (b) it cannot be transferred from stimulated to unstimulated cells, and (c) it is rapidly neutralized upon restoration of native osmotic strength. The transducer for the physical pressure-induced oxidative burst was similarly proposed to be mechanical, since (a) no change in chemical content was required to promote or augment the burst and (b) oxidant production was halted upon removal of the applied weight from the cells. Therefore, we conclude that plant cells can sense a mechanical perturbation at their cell surfaces and respond with an oxidative burst.

There is abundant evidence that mechanical forces can stimulate biologically relevant responses in both the plant and animal kingdoms. In animals, mechano-sensitive detectors are involved in such processes as hearing, the sense of touch, control of balance, and osmoregulation. Importantly, stretch-activated channels are thought to play a predominant role in initiation of the signal transduction pathways for each of these responses (Sachs, 1989). In plants, mechanically triggered pathways are also believed to contribute to osmoregulation (Schroeder and Hedrich, 1989), but they may be further involved in thigmotropism, geotropism, ethylene generation, and callose biosynthesis (Jaffe, 1973; Jaffe et al., 1985; Pickard and Ding, 1992). As was the case with animal systems, stretch-activated ion channels are again hypothesized to help inaugurate these responses via sites of contact between the cytoskeleton and the cell wall (Trewavas and Knight, 1994). In fact, stretch-activated channels have already been identified in the plasma membranes of onion epidermal cells (Ding and Pickard, 1993), *Vicia faba* guard cells (Schroeder and Hedrich, 1989), and cultured tobacco cells (Falke et al., 1988). Similar channels have been characterized in plant vacuolar membranes, where they have been demonstrated to respond to changes in osmotic pressure (Alexandre and Lassalles, 1991). Based on these observations, it would not be difficult to imagine that a stretch-activated channel might participate in initiating an oxidative burst.

In an attempt to simulate a mechanical force that might arise during penetration of plant tissue by a pathogen, we have imposed two types of physical pressure whose relevance to the mechanical forces during fungal/bacterial invasion in planta is difficult to assess. Thus, little information is currently available concerning the magnitudes and directions of the physical pressures that arise during microbe penetration/infestation of a plant. Further, extrapolation of the behavior of cells in culture to their behavior in intact tissues requires assumptions that remain to be tested. Nevertheless, an estimate of the pressure imposed by a growing fungal appressorium has been obtained and reported to be in excess of 80 bars (Howard et al., 1991). Comparison of this value with the direct pressure employed in our studies (0.039 g/mm^2 = 0.0039 bars) indi-

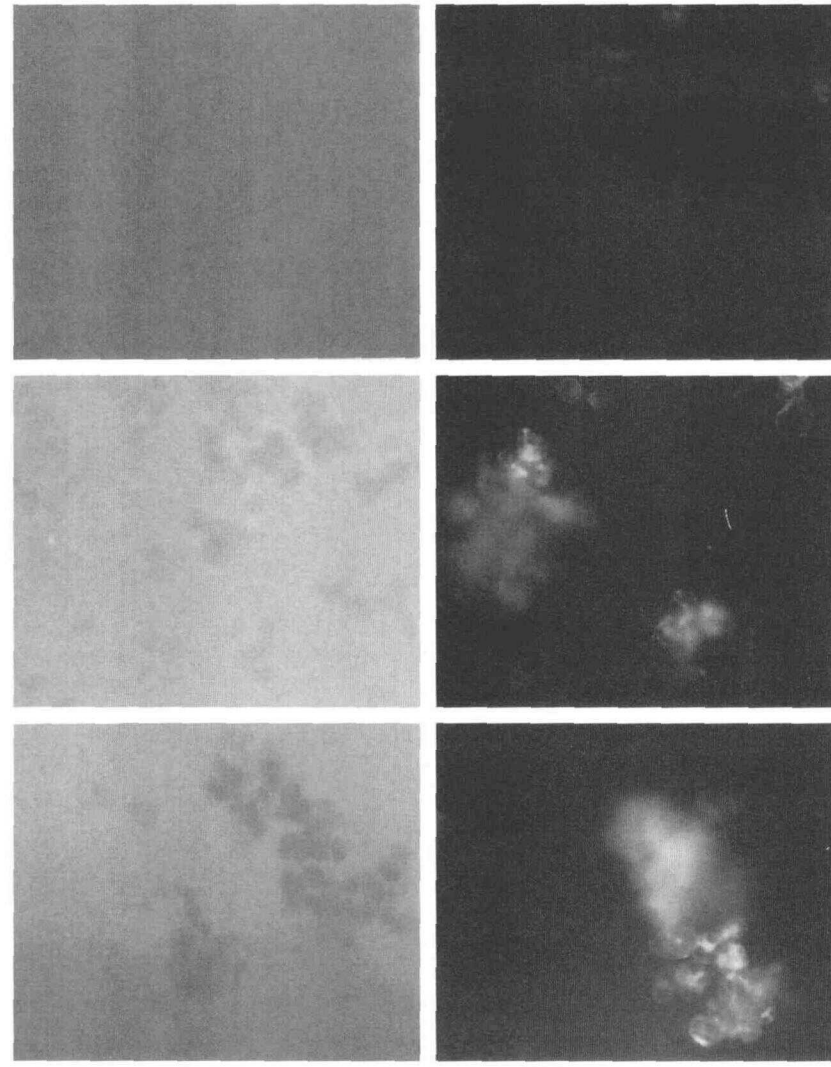

Figure 6. Fluorescence microscopy of mechanically stimulated cells. Cells (36 h old, i.e. responsive to both elicitor and osmotic stimuli) were treated with either pyranine (left panels) or 2′,7′-dichlorofluorescin diacetate (right panels), placed on a glass slide under a coverslip, and observed using a fluorescence microscope as elaborated in "Materials and Methods." Pictures were taken either 4 min (2′,7′-dichlorofluorescin) or 10 min (pyranine) after placement of the cells on the glass slides. The top photographs are of untreated cells, the middle photographs are of cells exposed for 4 min to 0.039 g/mm^2 pressure, and the bottom photographs are of cells treated at the time of mounting with 13.3 μg/mL OGA elicitor. The exposure time for all micrographs was 10 s. Kinetics of elicitation were also examined on the same cell suspension using the macroscopic oxidative burst assay to ensure that the cells were normally responsive. The pyranine dye (left panels) loses fluorescence upon oxidation, whereas the 2′,7′-dichlorofluorescin dye (right panels) gains fluorescence upon oxidation.

cates that our applied pressure is only a fraction of the value reported above. It is, therefore, conceivable that plant cells have developed the ability to detect mechanical insults substantially more subtle than those involved in penetration of a fungal appressorium, perhaps even the changes that occur during bacterial invasion.

The ability of a plant to respond to mechanical as well as chemical signals may be highly adaptive. Expression of receptors for ligands secreted by all possible pathogenic fungi, bacteria, and viruses would clearly represent a formidable task for any plant. Mutation of these receptors to retain high affinity for such ligands as the microbes mutated would further complicate this assignment. Since plants are not able to rapidly develop new high-affinity receptors for pathogens, as mammals do in the case of antibodies, etc., an alternative strategy must be developed to sense a perturbation common to all pathogens. Because many pathogens must penetrate the cell wall to parasitize a plant cell, a mechanical transducer that could detect cell wall/plasma membrane perturbations might constitute such a sensor. However, since such a sensor would not readily discriminate a compatible from an incompatible interaction, other defense responses would obviously also be required to confer resistance.

Although the mechanical and OGA-induced oxidative bursts are clearly initiated by different stimuli, they nevertheless appear to exhibit many common features. Thus, both pathways are characterized by the same 1- to 2-min interval between stimulation and oxidant production (Figs. 1 and 3). Additionally, both stimuli promote a similar rate of H_2O_2 biosynthesis under optimum conditions (Fig. 2). As shown in Figure 6, both pathways exhibit the same sensitivity to Gd^{3+}, and other, unpublished observations suggest that the two pathways are also similarly sensitive to citrate (Apostol et al., 1987; T. Yahraus, unpublished data), various kinase inhibitors (Chandra and Low, 1995; T. Yahraus, unpublished data), and Ca^{2+} channel blockers (T. Yahraus, unpublished data). Taken together, it is conceivable that the mechanical and chemical signaling pathways may in fact converge and promote assembly of the same activated oxidase complex on the membrane.

Although the focus of this research has been on plant defense, it is not inconceivable that mechanically triggered generation of H_2O_2 might serve other, unrelated functions.

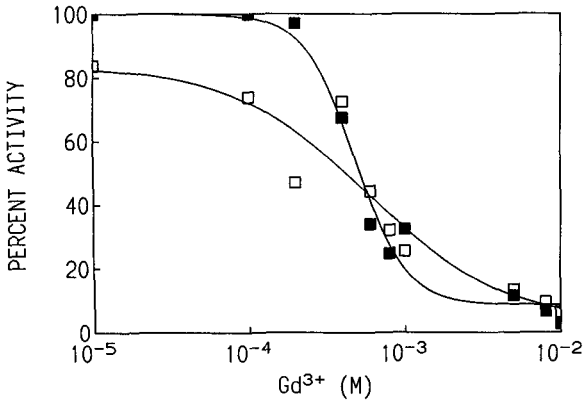

Figure 7. Inhibition of the dilution and OGA-induced bursts by gadolinium. Assays were conducted essentially as described in "Materials and Methods." Cells were mixed with 7 μL of pyranine and the desired concentration of gadolinium (III) chloride (Aldrich) in a fluorimeter cuvette. The cells were then either diluted 1:1 with distilled water (□) or treated with 6.6 μg/mL OGA (■), and the oxidative burst that ensued was monitored spectrofluorimetrically. Percent activity refers to the rate of H_2O_2 generation in the presence of the indicated concentration of gadolinium relative to the rate in its absence. Data presented here are the mean values of three independent experiments. The curves were drawn using a nonlinear regression program.

Thus, distortions of the cell wall/plasma membrane during freeze/thaw injury, or accompanying water stress, or in response to hormone-induced cell elongation could conceivably initiate some level of oxidant production. However, further studies under better-defined conditions will be required to verify this hypothesis.

Finally, it should not go unnoticed that the mechanical trigger for the H_2O_2 burst constitutes yet a fourth pathway for inducing the oxidative response. Previously characterized pathways include two distinct signal transduction cascades initiated by oligogalacturonides (Legendre et al., 1993b) and a third signaling pathway inaugurated by an extract from *Verticillium dahliae* (S. Chandra, P. Heinstein, and P.S. Low, unpublished observations). Importantly, activation of phospholipase C but not phospholipase A was found to be essential for the OGA-induced burst, whereas stimulation of phospholipase A was important for the *V. dahliae*-triggered response. The role of these phospholipases in the osmotically promoted H_2O_2 burst is currently being investigated. As with the neutrophil oxidative burst, which can also be triggered by each of the above pathways, the plant oxidative burst may be regulated by a complex network of second messengers and signaling intermediates.

ACKNOWLEDGMENT

We thank Dr. R.J. Joly for use of his osmometer.

Received June 26, 1995; accepted August 28, 1995.

LITERATURE CITED

Alexandre J, Lassalles J (1991) Hydrostatic and osmotic pressure activated channel in plant vacuole. Biophys J **60:** 1326–1336

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **90:** 109–116

Apostol I, Low PS, Heinstein PF, Stipanovic RD, Altman DW (1987) Inhibition of elicitor-induced phytoalexin formation in cotton and soybean cells by citrate. Plant Physiol **84:** 1276–1280

Baker CJ, Orlandi EW, Mock NM (1993) Harpin, an elicitor of the hypersensitive response in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells. Plant Physiol **102:** 1341–1344

Bishop PD, Pearce G, Bryant JE, Ryan CA (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. J Biol Chem **259:** 13172–13177

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem **54:** 484–489

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Chandra S, Low PS (1995) Role of protein phosphorylation in elicitation of the oxidative burst in cultured soybean cells. Proc Natl Acad Sci USA **92:** 4120–4123

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262:** 1883–1886

Davis D, Merida J, Legendre L, Low PS, Heinstein PF (1993) Independent elicitation of the oxidative burst and phytoalexin formation in cultured plant cells. Phytochemistry **32:** 607–611

Degousee N, Triantaphylides C, Montillet J (1994) Involvement of oxidative processes in the signaling mechanisms leading to the activation of glyceollin synthesis in soybean (*Glycine max*). Plant Physiol **104:** 945–952

Dempsey DA, Klessig DF (1994) Salicylic acid, active oxygen species and systemic acquired resistance in plants. Trends Cell Biol **4:** 334–338

Ding JP, Pickard BG (1993) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J **3:** 83–110

Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and the hyphal wall components. Physiol Plant Pathol **23:** 345–357

Falke LC, Edwards KL, Pickard BG, Stanley M (1988) A stretch-activated anion channel in tobacco protoplasts. FEBS Lett **237:** 141–144

Greenberg JT, Guo A, Klessig DF, Ausubel FM (1994) Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell **77:** 551–563

Horn MA, Meadows RP, Apostol I, Jones CR, Gorenstein DG, Heinstein PF, Low PS (1992) Effect of elicitation and changes in extracellular pH on the cytoplasmic and vacuolar pH of suspension-cultured soybean cells. Plant Physiol **98:** 680–686

Howard RJ, Ferrari MA, Roach DH, Money NP (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. Proc Natl Acad Sci USA **88:** 11281–11284

Jaffe MJ (1973) Thigmomorphogenesis: the response of plant growth and development to mechanical stimulation. Planta **114:** 143–157

Jaffe MJ, Huberman M, Johnson J, Telewski FW (1985) Thigmomorphogenesis: the induction of callose formation and ethylene by mechanical perturbation in bean stems. Physiol Plant **64:** 271–279

Keppler LD, Baker CJ (1989) $O_2^{\cdot-}$-initiated lipid peroxidation in bacteria-induced hypersensitive reaction in tobacco cell suspensions. Phytopathology **79:** 555–562

Keppler LD, Novacky A (1987) The initiation of membrane lipid peroxidation during bacteria-induced hypersensitive response. Physiol Mol Plant Pathol **30:** 233–245

Legendre L, Heinstein PF, Low PS (1992) Evidence for the participation of GTP-binding proteins in elicitation of the rapid oxi-

dative burst in cultured soybean cells. J Biol Chem **267:** 20140–20147

Legendre L, Rueter S, Heinstein PF, Low PS (1993a) Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. Plant Physiol **102:** 233–240

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS (1993b) Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J Biol Chem **268:** 24559–24563

Levine A, Tenhaken A, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593

Linder WA, Hoffman C, Grisebach H (1988) Rapid elicitor-induced chemiluminescence in soybean cell suspension cultures. Phytochemistry **27:** 2501–2503

Low PS, Heinstein PF (1986) Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. Arch Biochem Biophys **249:** 472–479

Mehdy MC (1994) Active oxygen species in plant defense against pathogens. Plant Physiol **105:** 467–472

Nothnagel EA, McNeil M, Albersheim P, Dell A (1983) Host pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol **71:** 916–926

Olson PD, Varner JE (1993) Hydrogen peroxide and lignification. Plant J **4:** 887–892

Peng M, Kuc J (1992) Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf discs. Phytopathology **82:** 696–699

Pickard BG, Ding JP (1992) Gravity sensing in higher plants. *In* F Ito, ed, Advances in Comparative and Environmental Physiology, Vol 10: Comparative Aspects of Mechanoreceptor Systems. Springer Verlag, Berlin, pp 81–110

Premachandra GS, Hahn DT, Joly RJ (1994) Leaf water relations and gas exchange in two grain sorghum genotypes differing in their pre- and post-flowering drought tolerance. J Plant Physiol **143:** 96–101

Sachs F (1989) Ion channels as mechanical transducers. *In* WD Stein, F Bronner, eds, Cell Shape: Determinants, Regulation and Regulatory Role. Academic Press, San Diego, CA, pp 63–92

Schroeder JI, Hedrich R (1989) Involvement of ion channels and active transport in osmoregulation and signaling of higher plant cells. Trends Biochem Sci **14:** 187–192

Schwacke R, Hager A (1992) Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein-kinase activity. Planta **187:** 136–141

Sutherland MW (1991) The generation of oxygen radicals during host plant responses to infection. Physiol Mol Plant Pathol **39:** 79–93

Trewavas A, Knight M (1994) Mechanical signalling, calcium and plant form. Plant Mol Biol **26:** 1329–1341

Vera-Estrella R, Blumwald E, Higgins VJ (1992) Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Plant Physiol **99:** 1208–1215

Yang X, Sachs F (1989) Block of stretch-activated ion channels in *Xenopus* oocytes by gadoliniun and calcium ions. Science **243:** 1068–1071