Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4145–4149, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Oligogalacturonide defense signals in plants: Large fragments interact with the plasma membrane *in vitro*

(polygalacturonic acid/pectin/tomato/protein phosphorylation/pp34)

PHILIPPE REYMOND, SUZANNE GRÜNBERGER, KALANETHEE PAUL, MATHIAS MÜLLER*, AND EDWARD E. FARMER†

Université de Lausanne, Institut de Biologie et de Physiologie Végétales, Bâtiment de Biologie, 1015 Lausanne, Switzerland

ABSTRACT **Oligogalacturonides are plant cell wall-derived regulatory molecules which stimulate defense gene expression during pathogenesis. *In vitro*, these compounds enhance the phosphorylation of an ≈34-kDa protein (pp34) in purified plasma membranes from potato and tomato leaves. We now show that polygalacturonate-enhanced phosphorylation of pp34 occurs in plasma membranes purified from tomato roots, hypocotyls, and stems and from undifferentiated potato cells. Furthermore, a similar phosphorylation is detected in leaf plasma membranes from soybean, a plant distantly related to tomato. Purified oligogalacturonides 13 to at least 26 residues long stimulate pp34 thiophosphorylation *in vitro*. This stimulation pattern differs from the induction of many known defense responses *in vivo*, where a narrower range of smaller fragments, between approximately 10 and 15 residues long, are active. On the basis of these differences we suggest that observed effects of applied exogenous oligogalacturonides on defense responses may not necessarily reflect the situation during pathogenesis. The cell wall could act as a barrier to many exogenous oligo- and polygalacturonides as well as other large regulatory ligands.**

The plant cell wall is a source of regulatory molecules which are capable of controlling defense and developmental processes (1, 2). Perhaps the best-characterized class of plant cell wall-derived signals is the oligogalacturonides (OGAs), homopolymers of α-1,4-linked D-galacturonic acid. These molecules derive from a parent polysaccharide, homogalacturonan, which resides in the pectic matrix. OGAs are thought to be released by degradative enzymes during pathogenesis. These molecules were first shown to stimulate accumulation of phytoalexin (1, 3) and proteinase inhibitor (4), but subsequent studies have revealed a wider variety of effects of exogenous oligogalacturonides. For example, these molecules can stimulate the formation of flowers and inhibit the formation of roots in tobacco thin cell layers (5) and inhibit root initiation/development on tobacco leaf explants (6). These effects are, in most cases, dependent on the degree of polymerization (DP)—i.e., OGAs must have an approximate length of 10 to 14 to be biologically active. This largely unexplained feature of OGA signaling deserves further study, as does signal transduction for OGA-stimulated responses.

Relatively little is known about signal transduction for OGA responses. OGAs stimulate ion flux (7, 8) and an oxidative burst in which G proteins may participate (9). There is evidence that linolenic acid hydroperoxide is a necessary intermediate in the OGA-stimulated expression of proteinase inhibitor genes in tomato leaves (10). These genes, however, do not obey the "DP rule," and there is as yet no indication of how general the requirement for fatty acid hydroperoxidation is for other OGA-responsive genes.

We have reported the OGA-stimulated *in vitro* phosphorylation of a small family of leaf plasma membrane-associated proteins from tomato and potato (11, 12). These results would be consistent with a role for protein phosphorylation in the plant's response to pectin-derived cell wall fragments. Other groups have also reported results strongly implicating protein phosphorylation/dephosphorylation in the induction of plant defense responses (13–16).

Following the initial report (11) of the OGA-stimulated *in vitro* phosphorylation of plasma membrane-associated proteins, the phosphorylation of one of these proteins of molecular mass ≈34 kDa ("pp34") has been investigated in some detail (12). The phosphorylation of pp34 shows some features that suggest that it might be somehow involved in OGA signal transduction. For example, enhanced pp34 thiophosphorylation so far appears to be specific for α-D-1,4-linked galacturonic acid polymers and is only weakly stimulated by α-L-1,4-guluronic acid, a stereochemically close relative of these polymers (12). Additionally, the concentrations of a tomato leaf-derived oligogalacturonic acid fraction required to stimulate pp34 phosphorylation are in the range of those necessary to stimulate a number of biological responses to this ligand (17). A chain length of OGA of approximately 14–15 was shown to be required to stimulate pp34 thiophosphorylation *in vitro* (12). This length does not appear to correlate with the length required to stimulate any known defensive or developmental response.

We chose to investigate the degree of polymerization dependence of pp34 phosphorylation in more detail, using quantitative analysis by phosphoimaging. Additionally, we studied the distribution of OGA-stimulated phosphorylation of pp34 throughout the vegetative tissues of tomato and in other, nonsolanaceous, species.

METHODS

Plant Materials. Two-week-old tomato (*Lycopersicon esculentum* cv. Bonny Best) plants were grown as described (12). Maize plants (*Zea mays* cv. LG11) were grown for 7 days in a greenhouse with a 17-h daylength and a minimum daytime temperature of 25°C. Three-week-old soybean (*Glycine max* var. Maple Arrow) plants were grown under similar conditions

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: OGA, oligogalacturonide; PGA, polygalacturonate; DP, degree of polymerization.

*Present address: Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064.
†To whom reprint requests should be addressed.

in a growth chamber. Potato cell suspension cultures (derived from *Solanum tuberosum* cv. Bintje meristem tissue) were maintained in MS medium (Sigma; containing 2,4-dichlorophenoxyacetic acid at 3 mg·liter^{-1} and kinetin at 0.1 mg·liter^{-1}) in low light [≈60 μE·cm^{-2}·sec^{-1} (1 einstein, E, = 1 mol of photons)] at 22°C with a weekly cycle.

***In Vitro* Thiophosphorylation.** *In vitro* thiophosphorylation of plasma membrane fractions was according to the protocol of Farmer *et al.* (12). The specific activity of the adenosine 5′-[γ-[^{35}S]thio]triphosphate (DuPont/NEN) was adjusted to 23.5 TBq·mmol^{-1} by dilution with unlabeled adenosine 5′-[γ-thio]triphosphate. The concentration of ATP used in the *in vitro* thiophosphorylation assays was 0.25 μM. After electrophoresis in 10% polyacrylamide gels containing SDS, gels were dried and analyzed by autoradiography or phosphoimaging, taking care to work in the region of linearity. OGAs are not degraded in the thiophosphorylation assay (K.P. and E.E.F., unpublished results). Western blotting using a polyclonal antibody to pp34 was carried out as described by Jacinto *et al.* (18).

Preparation of Membrane Fractions. Plasma membranes from tomato, soybean, and maize leaves were prepared as previously described (12), using a dextran/polyethylene glycol two-phase system with both polymers at a concentration of 5.9% (wt/vol). Unless otherwise indicated we used plasma membranes purified by one two-phase partition ("U_1"). Plasma membranes purified by two consecutive two-phase partitions were referred to as "U_2" and were used for data in Table 1 and Fig. 3.

OGAs. OGAs were prepared from sodium polygalacturonate (sodium PGA; Sigma) by the procedure of Spiro *et al.* (19) with modification. We simplified the anion exchange chromatography step on Q-Sepharose. Sodium PGA (4 g) was digested for 8 h at 24°C in 200 ml of 20 mM NaOAc, pH 5.0, containing 1 mg of bovine serum albumin, with 30 units of endopolygalacturonase from *Fusarium moniliforme* (a gift from C. Bergmann, University of Georgia). After digestion the PGA solution was autoclaved and selectively precipitated (19). The pellet was resuspended in 50 mM ammonium formate and exhaustively dialyzed against 300 mM ammonium formate, pH 6.5, in molecular weight 2000 cut-off dialysis tubing (Spectra-Por). The dialysate was loaded onto a 2.2 × 36 cm Q-Sepharose column preequilibrated in 300 mM ammonium formate, pH 6.5 at room temperature (22–25°C). The column was initially washed with 200 ml of 300 mM ammonium formate, pH 6.5, at a flow rate of 5 ml/min. The column was then developed with a 600-ml linear gradient of 300 mM to 750 mM ammonium formate, pH 6.5. Fractions of 10 ml were collected and quantitated by uronic acid analysis (20). Depending on the DP range of OGAs required, fractions early in the gradient (smaller oligouronides) or late in the gradient were pooled. For example, fractions 20–40, 41–48, and 49–52 were enriched in OGAs of DPs ranging from 4 to 12, 6 to 19, and 12 to 30, respectively.

OGA pools from Q-Sepharose chromatography were further resolved on a semipreparative CarboPac PA-100 (Dionex) column of 9 × 250 mm according to Spiro *et al.* (19). The pulsed amperometric detection monitor was set at E_1 = +150 mV; E_2 = +700 mV; E_3 = −300 mV; T_1 = 480 msec; T_2 = 120 msec; T_3 = 360 msec; sensitivity was set at 300 nA. Each peak from this column was collected and the degree of polymerization of the OGAs it contained was estimated by comparison of retention times with standards of known length as well as by uronide (20) and reducing-end assays (21). After exhaustive dialysis against water (19) oligouronides were analyzed for purity on an analytical Carbo-Pac PA-1 column (Dionex) and by gel electrophoresis in gels containing 15% acrylamide and 0.75% bisacrylamide in 89 mM Tris base/89 mM boric acid/2 mM Na_2EDTA and run and stained as described (12). Oligouronides were estimated to be approximately 85% pure as judged by gel electrophoresis as well as by pulsed amperometric detection on HPLC. It should be noted that other contaminants invisible to these two techniques may also be present in the samples. Purified oligouronides were stored at −20°C frozen in water. When OGA concentrations are given as molarities these values are calculated for potassium salts expected to be the principal salt species in the fractions used. Tomato leaf PGA and fractionated pectin were gifts from C. A. Ryan (Washington State University, Pullman) and are, respectively, the "TFA-PIIF" and "G50-PIIF" referred to by Bishop *et al.* (4). Citrus pectin was from Sigma.

RESULTS AND INTERPRETATION

Polygalacturonide-Stimulated pp34 Phosphorylation Is Distributed Throughout the Vegetative Tissues of Tomato. Polygalacturonides and OGAs were shown previously to stimulate the *in vitro* phosphorylation of an ≈34-kDa protein (pp34) in plasma membrane from tomato and potato leaves (11, 12). The tomato protein migrates as if its mass were about 32.5 kDa but is termed "pp34" for simplicity since it is immunologically and biochemically related to potato pp34 (18). To extend our information on the phosphorylation of pp34 we first decided to look at the distribution of polygalacturonide-stimulated thiophosphorylation in plasma membrane fractions from throughout the vegetative tissues of tomato. Plasma membrane fractions from leaf, hypocotyl, stem, and root were prepared from 14- to 15-day-old tomato plants. Fig. 1*A* shows that the *in vitro* thiophosphorylation of pp34 in tomato leaf, hypocotyl, stem, and root plasma membrane is stimulated by tomato leaf PGA. In each case the most pronounced effect of the polygalacturonide was on pp34 thiophosphorylation, with little effect on "background" phosphorylations. These results provide an indication that most of the vegetative tissues of tomato might be OGA sensitive. The presence of 0.2 μg of α-D-galacturonic acid (Fig. 1*A*) did not enhance root plasma membrane pp34 thiophosphorylation, indicating that the action of tomato leaf PGA was not simply due to an ionic strength effect. The OGA-enhanced phosphorylation of pp34 from root plasma membrane may be a first indication that one or more biological activities of OGAs exist

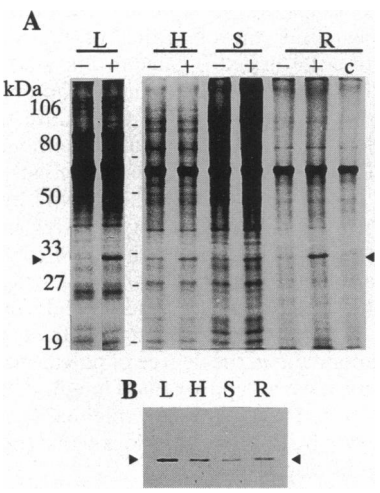

FIG. 1. Distribution of a PGA-responsive phosphoprotein, pp34 (arrowhead), in plasma membranes from the vegetative tissues of tomato. (*A*) *In vitro* thiophosphorylation of plasma membrane (2 μg of protein) from leaf (L), hypocotyl (H), stem (S), and root (R) in the absence (−) or presence (+) of 0.2 μg of tomato leaf PGA. In the root panel c is a sample thiophosphorylated in the presence of 0.2 μg of galacturonic acid. (*B*) Western blot of tomato plasma membranes (10 μg of protein) probed with anti-pp34 polyclonal antibody showing cross-reactivity in the ≈32-kDa (arrowhead) region.

in the tomato root. Western blot analysis of leaf, hypocotyl, stem, and root plasma membrane fractions (Fig. 1*B*) revealed a cross-reacting polypeptide at ≈32 kDa, a position corresponding to the position of tomato pp34.

The age of the tomato plants from which leaves were used to prepare plasma membranes had no significant effect on *in vitro* pp34 thiophosphorylation (data not shown). Thus pp34 phosphorylation is apparently not related to a specific developmental stage of the expanding leaf. Previous studies on a wide variety of plants have shown that different vegetative tissues are OGA responsive. These organs include leaves (4), stem tissue (22), thin cell layers (5), seedlings (cotyledon and hypocotyl) (3), and cell suspension cultures (see ref. 17 for review). It now appears that tissues sensitive to PGA-derived signals exist throughout the vegetative parts of plants. This is true of the responses of intact tissues to OGA and to the *in vitro* phosphorylation of pp34.

Polygalacturonide-Stimulated pp34 Phosphorylation Occurs in Soybean Leaf Plasma Membranes. We extended our search for PGA-stimulated pp34 thiophosphorylation to two other species, namely soybean and maize. These plants were chosen for the following reasons. OGAs induce phytoalexin accumulation in soybean cotyledons and hypocotyls (1), and this species is one of the most highly studied plants in terms of defense responses. Maize is a graminaceous monocot widely diverged from tomato or soybean. So far as we are aware no reports of OGA-induced defense gene expression have been published for these plants. A maize defense gene is induced in response to wounding and fungal infection (23), but the authors give no indication of the nature of the elicitor(s) involved in this defense mechanism.

In vitro thiophosphorylation of soybean plasma membranes in the presence and absence of tomato leaf PGA revealed a PGA-enhanced thiophosphorylation of a protein of about 34 kDa (Fig. 2*A*). Western blots of soybean plasma membranes probed with polyclonal anti-pp34 (Fig. 2*B*) revealed a single cross-reacting band at this position. The finding of PGA-stimulated pp34 phosphorylation in soybean extends the observation of this response to a second dicot family distantly related to the Solanaceae: the Fabaceae. These data suggest that PGA-stimulated pp34 phosphorylation might be more widespread among dicotyledonous plants. In contrast to the case of soybean, no PGA-stimulated pp34 thiophosphorylation was observed in plasma membrane preparations from the monocot maize (Fig. 2*A*). Western blotting with anti-pp34, however, revealed a cross-reacting band of approximately 32 kDa (Fig. 2*B*). We conclude that maize contains a polypeptide immunologically related to pp34 from solanaceous plants. Failure to observe a PGA-stimulated phosphorylation of proteins in maize plasma membranes may reflect a genuine difference between this plant and certain dicots. Alternatively, our membrane preparation or thiophosphorylation conditions may not have been optimized for this species.

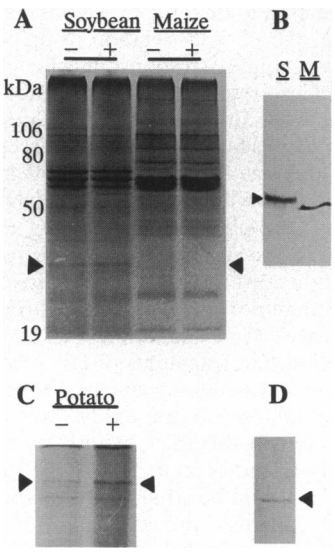

FIG. 2. Occurrence and PGA-stimulated phosphorylation of pp34 (arrowhead) in plasma membranes from non-solanaceous species and in undifferentiated cells. (*A*) *In vitro* thiophosphorylation of leaf plasma membranes (2 μg of protein) from soybean and maize in the presence (+) and absence (−) of 0.2 μg of tomato leaf PGA. (*B*) Western blot of soybean (S) and maize (M) leaf plasma membranes (10 μg of protein) probed with anti-pp34 polyclonal antibody. (*C*) Potato cell plasma membranes (4 μg of protein) thiophosphorylated in the absence (−) and presence (+) of tomato leaf PGA (0.2 μg). (*D*) Western blot of potato cell plasma membranes (30 μg of protein) probed with anti-pp34 polyclonal antibodies. Arrowhead indicates a molecular mass of ≈34 kDa.

To establish whether or not PGA-stimulated pp34 thiophosphorylation exists only in plasma membranes isolated from differentiated cells, we tested this membrane from potato cell suspension cultures. A protein of ≈34 kDa shows weakly enhanced *in vitro* thiophosphorylation in response to tomato cell wall PGA (Fig. 2*C*) and a cross-reacting polypeptide of the same molecular mass is revealed by developing Western blots of potato cell plasma membranes with anti-pp34 (Fig. 2*D*). These results indicate that PGA-stimulated pp34 phosphorylation exists in plasma membranes isolated from nondifferentiated tissues and suggest that, if pp34 phosphorylation is biologically important, its role will not be restricted to one or two highly differentiated cell types.

Degree of Polymerization Dependence of OGA-Stimulated *in Vitro* Phosphorylation of pp34. The OGA-enhanced phosphorylation of tomato and potato leaf pp34 was previously investigated in some detail (12). An initial study showed that the phosphorylation of tomato leaf pp34 is dependent on the DP of the OGAs, being stimulated by fragments approximately equal to or larger than 14 galacturonic acid units long (12). We decided to apply quantitative techniques to investigate this DP dependency over a large range of purified OGAs. We also wished to test whether purified oligogalacturonides were as active as tomato leaf PGA in enhancing pp34 thiophosphorylation. A series of initial tests were performed to compare different batches of plasma membranes. After a large number of experiments (not shown) we now know that batch-to-batch variation in the sensitivity of tomato leaf plasma membranes to tomato leaf PGA and to oligogalacturonides exists. This appears to be due to at least two causes. First, the purity of the membrane: the more highly purified the greater the response to tomato leaf PGA and to purified OGAs. We have tested this as far as plasma membranes purified by two consecutive two-phase partitions (Fig. 3 and Table 1) but not with even more highly purified membranes. Second, freezing the membranes reduces their response to tomato leaf PGA and to purified OGAs (each freeze/thaw cycle reducing by about 20% the tomato leaf PGA-stimulated thiophosphorylation of pp34 compared with fresh plasma membrane samples). We also established that concentrations of purified oligogalacturonides of around 10 μM gave near maximum OGA-stimulated pp34 thiophosphorylation, and this was not critically dependent on the DP of OGA fragments.

Having established basic requirements for a quantitative measure of pp34 thiophosphorylation, we tested a range of purified OGAs. Fig. 3 shows that pp34 thiophosphorylation is slightly inhibited by fragments of DPs between 6 and 12. Thiophosphorylation of pp34 begins to be stimulated at a DP of about 13, in agreement with previously published results (12). The level of ^{35}S incorporation into pp34 is similar when the protein is thiophosphorylated in the presence of OGAs between DPs of about 15 and 26. Fragments around DP 26 were slightly more active than those around a DP of 16 but the general pattern was one of a plateau with no indication of

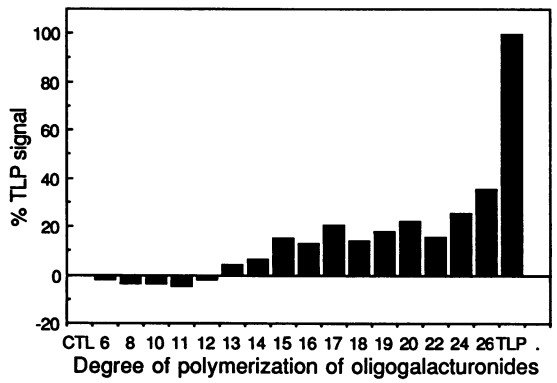

FIG. 3. Effect of the DP of purified OGAs on thiophosphorylation of pp34 in freshly prepared U_2 plasma membranes from tomato leaves. Results are represented as the percentage increase in the thiophosphorylation of pp34 over the water control when incubated with tomato leaf PGA. In each case the reaction mixtures contained 2 μg of plasma membrane protein. The concentration of the purified OGAs (which were approximately 85% pure and added as potassium salts) was 7.5 μM in the assay. Results are the average from three experiments. Variation between measurements with oligogalacturonides of the same length was between 2% and 12%. TLP, tomato leaf PGA; CTL, water control.

lower activity of larger fragments. A second important observation from Fig. 3 is that it is clear that pp34 phosphorylation in the presence of tomato leaf PGA is greater than in the presence of purified OGAs, which represent only 20–40% of the tomato leaf PGA activity. Similar results were found with potato plasma membranes. The smallest fragment that induced pp34 phosphorylation was DP 13, and OGAs between DP 14 and DP 26 were slightly more active in potato than in tomato (data not shown).

The observation that purified OGAs are less active than tomato leaf PGA might suggest that OGAs contain much but not all of the structural information necessary to efficiently stimulate pp34 phosphorylation. However, since it has been suggested that biologically active oligouronides may be intermolecular "egg box" complexes (12), the counterion composition of the OGAs used may be critical, and this will need to be tested in the future before solid conclusions concerning the relative activity of tomato leaf PGA and purified OGAs are drawn. The facile explanation, that the purified OGAs we used contained substances which inhibit pp34 thiophosphorylation, may be true to some extent. Coaddition of OGA of DP = 20 had a slight inhibitory effect on tomato leaf PGA-enhanced pp34 thiophosphorylation (data not shown). This effect was, however, not sufficient to fully explain the quantitative difference between OGAs and tomato PGA in their abilities to stimulate pp34 thiophosphorylation. There is another possibility, that the combination of fragments of different DP leads to the total activation of the pp34 phosphorylation in a synergistic manner. Such a finding has been reported for the induction of phytoalexin accumulation by purified OGAs in soybean cotyledons (24). Preliminary experiments (not shown) suggest that this is not the case for pp34 thiophosphorylation. A simpler explanation for the apparent weaker efficacy of purified OGA fragments to stimulate pp34 phosphorylation would be that very large fragments, bigger than DP = 26, are necessary to fully activate the system, and this will be tested. However, the possibility that another more effective structure resides in the tomato leaf PGA can also be considered. As a preliminary test we compared the relative abilities of pectin and related substances to stimulate pp34 thiophosphorylation. Table 1 shows that pectin, the sodium salt of PGA, and tomato leaf PGA all stimulate pp34 thiophosphorylation in tomato leaf plasma membranes to a similar extent (87–100%). It is likely that unmethylated galacturonide chains are essential for the *in vitro* stimulation of pp34 thiophosphorylation since fractionated pectin, which is 80% methylated (4), shows only half the activity of these effectors (Table 1). Furthermore, chemically methylated tomato leaf PGA is inactive in stimulating pp34 thiophosphorylation (data not shown). Pectin is also heavily methylated but is heterogeneous and may contain unmethylated stretches of PGA. It has to be mentioned that in certain cases, for reasons we cannot explain, sodium PGA was inactive in enhancing the phosphorylation of pp34. Further experiments are necessary to establish if other structural features of tomato leaf PGA, or copurifying counterions, are important in stimulating pp34 thiophosphorylation. If an active molecule is characterized, its biological activity, if any, would need to be established.

Table 1. Effect of galacturonic acid-containing polymers on the *in vitro* thiophosphorylation of pp34 in tomato plasma membranes

Effector	pp34 thiophosphorylation, %
Tomato leaf PGA	100
Pectin	88
Fractionated pectin	52
Sodium PGA	87
Control	0

Effectors (0.2 μg) were incubated with plasma membranes (2 μg of protein) in the presence of adenosine 5′-[γ-thio]triphosphate (0.25 μM) for 10 min at 30°C. Reactions were analyzed by SDS/PAGE and the intensity of the pp34 band was quantitated by phosphoimaging. The increase of phosphorylation relative to the water control was set to 100% for tomato leaf PGA. Each value represents the mean of five experiments.

The finding that many of the OGAs which stimulate pp34 phosphorylation are of a different size than those which elicit biological responses might suggest that other, as-yet-undiscovered, biological effects of oligo- and polygalacturonides exist in plants. This would require careful investigation and experiments might be difficult to set up and analyze for reasons discussed in the next section. We have three principal hypotheses that might be used to investigate the possible role of pp34 in signaling. First, the phosphorylation of pp34 may act as a way to negatively regulate a response to oligogalacturonides, for example, a defense response. The stimulated *in vitro* phosphorylation of pp34 occurs with galacturonides of DP > 13, while living plant tissues begin to show decreased responses to oligouronides of this size and larger. Perhaps there is a form of negative regulation which prevents long OGAs, such as those existing in the wall, from stimulating defense in the absence of pathogenesis. Second, the shift in sensitivity of pp34 thiophosphorylation toward fragments of greater DP might reflect the loss of an important component during the isolation of plasma membranes. An explanation must be sought for the puzzling observation that fragments of DP > 15 are active in the *in vitro* system but generally not *in vivo*. Perhaps large, potentially active OGAs cannot easily gain access to the surface of living cells. If this third hypothesis turns out to be correct, the results of studies on the *in vitro* phosphorylation of pp34 highlight what could be a barrier to research on "large" regulatory ligands in plants: the cell wall.

The Plant Cell Wall as a Potential Barrier to Exogenous OGA Defense Signals. Many biological responses to OGAs are characterized by a dependence on the degree of polymerization of this ligand. There are several interesting features of this activation. First is the fact that relatively large fragments, generally 10 or more residues long, are necessary for activity (17, 25). It has already been suggested (12) that the ligands which activate many defense responses may be intermolecular "egg box" complexes of OGAs and calcium ions, known to form in solution with OGAs of DP > ≈9 (26). This is one potential explanation for the minimum size requirement of OGA-stimulated responses. This hypothesis, however, does

not explain why OGAs larger than a DP of about 14 tend to be inactive when applied to living tissues.

We suggest that the permeability of the cell wall to larger fragments may limit their access to the cell surface. In other words, if the cell wall were removed, plant tissues would be sensitive to a greater range of OGAs. The undamaged cell wall might then represent the case in which there is a window only large enough to allow the passage of OGAs of DPs up to ≈14 or 15. There are some indications in the literature that this might be the case. Experiments on the sensitivity of protoplasts to OGAs and PGA have been conducted, and the results have been compared with those of similar experiments on walled cells (27, 28). It appears that PGA, in the presence of calcium, can exert strong effects on membrane potential, weak effects on cytosolic pH, and strong effects on cytosolic calcium levels in protoplasts (27). Similar experiments on walled cells indicate that PGA is less active than on protoplasts (27). These results suggest (to us) that the cell wall may exclude most of the exogenous PGA from contact with the plasma membrane.

If it is the cell wall that limits the size range of active, endogenous OGAs, this may have consequences in pathogenesis. Pectinolytic enzymes operating within the plant cell wall would release oligo- and polygalacturonides. As pathogenesis progresses and cell wall degradation proceeds, larger and larger fragments would have access to the cell surface, increasing the number of signal-active molecules available to activate defense gene expression.

More generally, it is possible that, due to the presence of the cell wall, we may not easily detect the presence of high molecular mass signal molecules in plants. In animals a number of very large signals exist in the extracellular matrix. These include proteins such as fibronectin and complex molecules such as proteoglycans (29, 30). Similar molecules may determine adhesion events in plants (31), but evidence for roles as regulators is not yet available. It is noticeable that most signal molecules so far discovered in plants tend to be relatively small; there are few exceptions (for example, see ref. 32). It is possible that *in vitro* studies in the absence of the cell wall may help lead to the discovery of larger regulatory ligands in plants.

We thank P. Albersheim, A. Darvill, C. Bergmann, and M. Spiro of the Complex Carbohydrate Research Center, University of Georgia, for their generosity in providing endopolygalacturonase, OGA standards, and purification protocols prior to publication. C. A. Ryan (Washington State University) provided tomato leaf PGA, and A. Buchala and J.-P. Métraux (University of Fribourg, Switzerland) provided potato cell suspension cultures. We thank B. Künstner for maintenance of plant material. This work was supported by Swiss National Science Foundation Grant 31-36472.92.

1. Darvill, A. G. & Albersheim, P. (1984) *Annu. Rev. Plant Physiol.* **35,** 243–275.
2. Ryan, C. A. & Farmer, E. E. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 651–674.
3. West, C. A., Bruce, R. J. & Jin, D. F. (1984) in *Structure, Function and Biosynthesis of Plant Cell Walls*, eds. Dugger, W. M. & Bartinicki-Garcia, S. (Williams and Wilkins, Baltimore), pp. 359–375.
4. Bishop, P. D., Makus, D., Pearce, G. & Ryan, C. A. (1984) *J. Biol. Chem.* **259,** 13172–13177.
5. Mohnen, D., Eberhard, S., Marfà, V., Doubrava, N., Toubart, P., Gollin, D. J., Gruber, T., Nuri, W., Albersheim, P. & Darvill, A. G. (1990) *Development (Cambridge, U.K.)* **108,** 191–201.
6. Bellincampi, D., Salvi, G., De Lorenzo, G. & Cervone, F. (1993) *Plant J.* **4,** 207–213.
7. Thain, J. F., Doherty, H. M., Bowles, D. J. & Wildon, D. C. (1990) *Plant Cell Environ.* **13,** 569–574.
8. Mathieu, Y., Kurkdjian, A., Xia, H., Guern, J., Koller, A., Spiro, M. D., O'Neill, M., Albersheim, P. & Darvill, A. (1991) *Plant J.* **1,** 333–343.
9. Legendre, L., Rueter, S., Heinstein, P. F. & Low, P. S. (1993) *Plant Physiol.* **102,** 233–240.
10. Farmer, E. E., Caldelari, D., Pearce, G., Walker-Simmons, MK. & Ryan, C. A. (1994) *Plant Physiol.* **106,** 337–342.
11. Farmer, E. E., Pearce, G. & Ryan, C. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1539–1542.
12. Farmer, E. E., Moloshok, T. D., Saxton, M. J. & Ryan, C. A. (1991) *J. Biol. Chem.* **266,** 3140–3145.
13. Dietrich, A., Mayer, J. E. & Hahlbrock, K. (1990) *J. Biol. Chem.* **265,** 6360–6365.
14. Felix, G., Grosskopf, D. G., Regenass, M. & Boller, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8831–8834.
15. Felix, G., Regenass, M., Spanu, P. & Boller, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 952–956.
16. MacKintosh, C., Lyon, G. D. & MacKintosh, R. W. (1994) *Plant J.* **5,** 137–147.
17. Darvill, A., Augur, C., Bergmann, C., Carlson, R. W., Cheong, J.-J., Eberhard, S., Hahn, M. G., Lo, V.-M., Marfa, V., Meyer, B., Mohnen, D., O'Neill, M. A., Spiro, M. D., van Halbeek, H., Work, W. S. & Albersheim, P. (1992) *Glycobiology* **2,** 181–198.
18. Jacinto, T., Farmer, E. E. & Ryan, C. A. (1993) *Plant Physiol.* **103,** 1393–1397.
19. Spiro, M. D., Kates, K. A., Koller, A. G., O'Neill, M. A., Albersheim, P. & Darvill, A. G. (1993) *Carbohydr. Res.* **247,** 9–20.
20. Blumenkrantz, N. & Asboe-Hansen, G. (1973) *Anal. Biochem.* **54,** 484–489.
21. Lever, M. (1972) *Anal. Biochem.* **47,** 273–279.
22. Branca, C., De Lorenzo, G. & Cervone, F. (1988) *Physiol. Plant.* **72,** 499–504.
23. Cordero, M. J., Raventos, D. & San Segundo, B. (1994) *Plant J.* **6,** 141–150.
24. Davis, K. R., Darvill, A. G., Albersheim, P. & Dell, A. (1986) *Plant Physiol.* **80,** 568–577.
25. Ryan, C. A. (1988) *Biochemistry* **27,** 8879–8883.
26. Kohn, R. (1985) *Pure Appl. Chem.* **42,** 371–397.
27. Messiaen, J., Read, N. D., Van Cutsem, P. & Trewavas, A. J. (1993) *J. Cell Sci.* **104,** 365–371.
28. Messiaen, J. & Van Cutsem, P. (1994) *Plant Cell Physiol.* **35,** 677–689.
29. Juliano, R. L. & Haskill, S. (1993) *J. Cell Biol.* **120,** 577–585.
30. Damsky, C. H. & Werb, Z. (1992) *Curr. Opin. Cell Biol.* **4,** 772–781.
31. Zhu, J.-K., Shi, J., Singh, U., Wyatt, S. E., Bressan, R. A., Hasegawa, P. M. & Carpita, N. C. (1993) *Plant J.* **3,** 637–646.
32. Hayashi, T. & Yoshida, K. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2618–2622.