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Rapid Changes in Plasma Membrane Protein Phosphorylation during Initiation of Cell Wall Digestion[1]

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ABSTRACT

Plasma membrane vesicles from wild carrot cells grown in suspension culture were isolated by aqueous two-phase partitioning, and ATP-dependent phosphorylation was measured with [γ-^{32}P]ATP in the presence and absence of calcium. Treatment of the carrot cells with the cell wall digestion enzymes, driselase, in a sorbitol osmoticum for 1.5 min altered the protein phosphorylation pattern compared to that of cells treated with sorbitol alone. Driselase treatment resulted in decreased phosphorylation of a band of M_r 80,000 which showed almost complete calcium dependence in the osmoticum treated cells; decreased phosphorylation of a band of M_r 15,000 which showed little calcium activation, and appearance of a new band of calcium-dependent phosphorylation at M_r 22,000. These effects appeared not to be due to nonspecific protease activity and neither *in vivo* nor *in vitro* exposure to driselase caused a significant loss of Coomassie blue-staining bands on the gels of the isolated plasma membranes. However, protein phosphorylation was decreased. Adding driselase to the *in vitro* reaction mixture caused a general decrease in the membrane protein phosphorylation either in the presence or absence of calcium which did not mimic the *in vivo* response. Cells labeled *in vivo* with inorganic ^{32}P also showed a response to the Driselase treatment. An enzymically active driselase preparation was required for the observed responses.

The plasma membrane of plant cells is a potential site for the transduction of signals from the external environment to the cell interior. The role of the plant plasma membrane in signal transduction has been under scrutiny for some time.

The regulatory role of protein phosphorylation in plants is becoming increasingly apparent (for review see Ref. 13), and calcium-dependent protein kinases could provide a means of signal transduction across the plasma membrane. A calcium and calmodulin-activated, autophosphorylating protein kinase of M_r 18,000 has been characterized in the plasma membranes isolated from pea shoots (2, 3). Thus, there is the potential for the plant plasma membrane to respond to a stimulus and alter cellular metabolism.

To determine whether changes in protein phosphorylation occur in the plasma membranes of suspension culture cells in response to the cell wall digestion enzymes used to isolate protoplasts, studies of protein phosphorylation of the carrot suspension culture cells were undertaken. These cells are normally plasmolysed in a solution of 0.4 molal sorbitol during cell wall digestion; therefore, the sorbitol osmoticum was used as a control. Dramatic and rapid changes were seen in the calcium-dependent *in vitro* phosphorylation patterns of plasma membranes isolated from the cells exposed to Driselase compared to the sorbitol controls. Rapid changes in protein labeled *in vivo* with inorganic ^{32}P also were observed.

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]ATP (specific activity 185 TBq/mmol) and ^{32}P (acid free, 370 MBq/ml) were obtained from Amersham International plc (Amersham, Bucks., UK). Other chemicals were obtained from Sigma London Chemical Co (Poole, Dorset, UK). Driselase was obtained from Plenum Scientific Co.

Treatment of Cells and Isolation of Plasma Membrane. Wild carrot cells were maintained by weekly serial transfer into fusion inducing medium as previously described (4). For *in vivo* labeling with ^{32}P, the cells were grown on phosphate-free medium and 6 to 10 MBq ^{32}P were added for 15 h prior to harvesting. For all experiments, the cells were harvested 4 d after transfer by filtration on filter paper, washed once with approximately 10 ml of water, and placed in the designated solutions at a concentration of approximately 0.4 g fresh weight per 20 ml of solution. Incubations were performed at room temperature in 0.4 molal sorbitol in 1 mM MES (pH 4.8) with or without 2% (w/v) driselase. Stock driselase was prepared at 4% and the supernatant from a 1,000*g* centrifugation collected. At indicated times the cells or protoplasts were collected by centrifugation and washed twice by resuspension and centrifugation in approximately 5 ml of 0.45 molal sorbitol 1 mM MES (pH 6.0).

The washed cells and protoplasts were homogenized in a ground glass homogenizer in a medium consisting of 8% sucrose, 95 mM LiCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM $MgCl_2$, and 50 mM Tris (pH 7.5). The homogenate was centrifuged at 1,000*g* for 4 min and the 1,000*g* supernatant centrifuged at 40,000*g* for 1 h. The 40,000*g* pellet was resuspended in 0.5 ml of water and the plasma membrane-rich fraction isolated by aqueous two phase partitioning (1) using 6.3% PEG (approximate mol wt 3,350) and dextran (average mol wt 500,000) as the polymers as previously described for the wild carrot cells (16) except that 10% water of hydration was assumed for the dextran. The upper phase was collected, diluted with approximately 30 ml of homogenizing buffer without the lithium or sucrose, and centrifuged at 40,000*g* for 1 h.

The pelleted plasma membrane-rich fraction (50–80 μg protein) was resuspended in approximately 50 μl of water and used for *in vitro* phosphorylation as previously described (9) or for other treatments as described in the text. Protein was determined

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according to Lowry (10) with BSA as standard.

Gel Electrophoresis. SDS-PAGE was performed using 12% polyacrylamide gels 20 × 16 × 0.1 cm as previously described (8). Approximately equal amounts of protein were added to each lane.

Protein Kinase Assays. Protein kinase assays for the isolated plasma membrane fraction were carried out using TCA precipitation onto cellulose discs as previously described (9).

RESULTS

Driselase Treatment of Cells Alters *in Vitro*/*in Vivo* Protein Phosphorylation of Plasma Membrane. An analysis of the effects of driselase treatment upon subsequent *in vitro* calcium-activated protein phosphorylation is presented in Figure 1A. Plasma membrane preparations were isolated from cells treated for various times in driselase. After 1.5 min incubation of the cells in a solution of 2% driselase in 0.4 molal sorbitol, the protein phosphorylation pattern of the isolated plasma membranes had changed compared to control cells (compare Fig. 1A, lanes a and b with e and f). The isolated membranes were labeled with [γ-^{32}P]ATP in the presence and absence of calcium ions. Results are shown for 1.5 and 5 min of driselase treatment. The 1.5 min point was the shortest time point possible to collect and wash the cells.

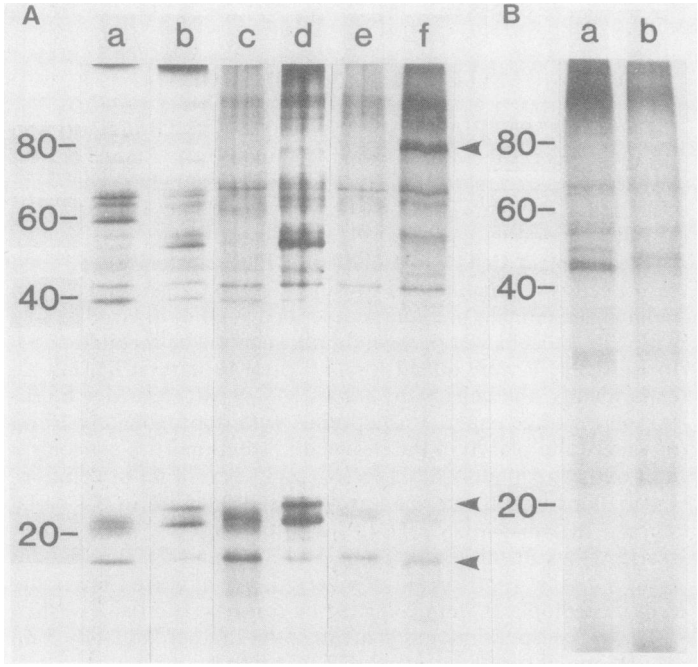

FIG. 1. (A) Gel electrophoretic analysis of the proteins phosphorylated in plasma membranes isolated from cells incubated with and without driselase. Cells were treated for 1.5 (lanes a and b) and 5 min (lanes c and d) at room temperature in osmoticum with 2% (w/v) driselase in osmoticum or in osmoticum alone (lanes e and f). A plasma membrane fraction was isolated from the washed cells and phosphorylated for 3 min at 4°C using [γ-^{32}P]ATP in the presence (lanes b, d, and f) and absence (lanes a, c, and e) of approximately 100 μM free calcium ions. Reactions were terminated by the addition of sample buffer and heating at 100°C for 10 min. (B) Gel electrophoretic analysis of *in vivo* labeled plasma membrane proteins. Cells were labeled with 6 MBq ^{32}P for 15 h, collected by filtration, and treated with osmoticum (lane a) or 2% (w/v) driselase in osmoticum (lane b) for 5 min. The treated cells were washed in sorbitol and the ^{32}P-labeled plasma membranes were isolated and heated in sample buffer at 100°C for 10 min. Preparations were separated by SDS-PAGE and the dried down gels autoradiographed. Arrows refer to bands discussed in the text. Numbers on the left of figures are mol wt × 10^{-3} based on standard proteins.

Many alterations in calcium-activated protein phosphorylation, as a result of the short driselase treatments, were clearly visible. Three of these changes will be taken and discussed in more detail. One band, of M_r 80,000, in the plasma membrane from osmoticum-treated cells showed almost complete dependence upon calcium ions for its phosphorylation (arrowed in Fig. 1A). The driselase treatment of the cells dramatically reduced the phosphorylation of this band (Fig. 1A, lanes b and d). Phosphorylation in a band of M_r 15,000 (arrowed in Fig. 1A) showed little calcium activation in the osmoticum-treated cell plasma membrane. After driselase treatment of the cells, the phosphorylation of this band increased in the absence of calcium. Finally, a novel calcium-dependent band of phosphorylation at M_r 22,000 was seen to appear in the plasma membrane from cells treated with driselase (arrowed in Fig. 1A, lanes b and d).

Driselase treatment of cells labeled *in vivo* with ^{32}P also was performed (Fig. 1B). Inorganic ^{32}P was added for 15 h to cells grown in phosphate-free medium. The cells were treated for 5 min with either sorbitol (Fig. 1B, lane a) or sorbitol plus driselase (Fig. 1B, lane b); the ^{32}P-labeled plasma membranes were isolated, and the proteins were separated by SDS-PAGE. While many of the phosphorylated bands are common to both the *in vitro* and *in vivo* labelings, the response to driselase is less dramatic in the latter. The relative intensity of the bands is seen to differ between the *in vitro* and *in vivo* labelings. These differences in the pattern of phosphorylation and driselase response of the *in vitro* and *in vivo* labeled membranes are not surprising since the plasma membrane is exposed to an entirely different enzymatic environment in each instance during its phosphorylation.

To determine whether or not the driselase treatment was affecting the amount of *in vitro* protein phosphorylation, membranes were isolated from cells which had been treated with either osmoticum alone or osmoticum plus driselase for 30 min and the specific activity of protein phosphorylation was measured (Fig. 2). Driselase treatment of carrot cells for a period of 30 min at room temperature is not sufficient to produce a true protoplast and much of the cell wall still remains. The calcium-activated protein phosphorylation in plasma membrane isolated from cells treated for 30 min at room temperature in osmoticum alone was only marginally different from those treated with osmoticum with driselase. It was evident that the specific activity of phosphorylation, both in the presence and absence of calcium, had been reduced by the driselase treatment.

The pattern of *in vitro* phosphorylation of the plasma membrane proteins after 30 min treatment of the cells is shown in Figure 3. A comparison of Figures 1A and 3 indicates that the band at M_r 15,000 (arrowed in Fig. 3) was reduced in both the presence and absence of calcium.

When cells were treated with driselase for 90 min, single protoplasts were recovered. The plasma membrane isolated from these protoplasts and from cells treated with osmoticum only, gave the same pattern of calcium-dependent protein phosphorylation as that depicted in Figure 3 for the 30 min driselase and osmoticum treatments, respectively (data not shown).

It should be noted that the sorbitol treatment alone had a slight effect upon protein phosphorylation when compared to that of membranes isolated from cells freshly isolated from medium. These differences were minor when compared to those when Driselase was added and were necessarily a part of the sorbitol/driselase treatment (compare lanes a and b of Fig. 5 with lanes a and b of Fig. 3).

Is Driselase Treatment Nonspecifically Degrading All Membrane Proteins? To test whether driselase treatment was leading to major degradation of membrane protein rather than more specific changes, isolated plasma membranes were treated with or without driselase for 30 min at room temperature. The plasma

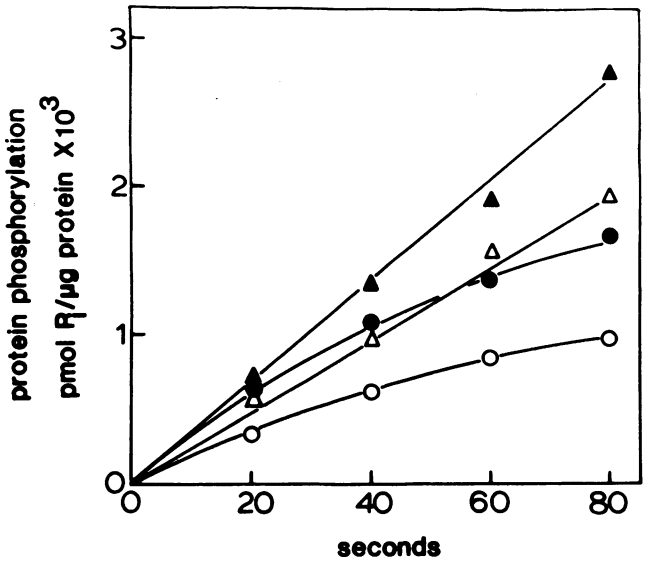

FIG. 2. Decrease in specific activity of *in vitro* plasma membrane phosphorylation by driselase treatment of cells. Cells were treated for 30 min at room temperature in osmoticum with (circles) or without (triangles) 2% (w/v) driselase. A plasma membrane fraction was then isolated from the washed cells and used in an *in vitro* phosphorylation assay. Plasma membranes was incubated with [γ-^{32}P]ATP in the presence (closed symbols) or absence (open symbols) of aproximately 100 μM free calcium ions. Aliquots were removed at the times indicated and incorporated phosphate estimated.

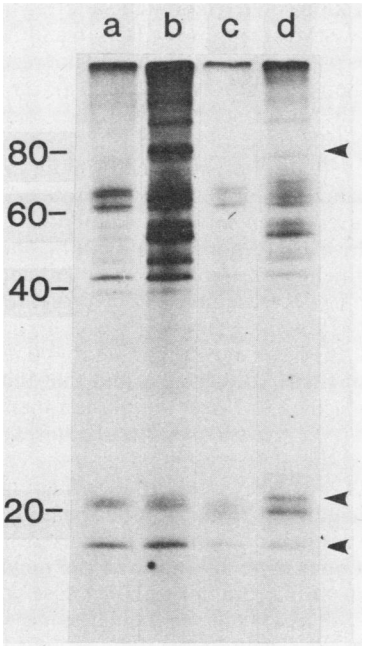

FIG. 3. Gel electrophoretic analysis of the proteins phosphorylated in plasma membranes isolated from cells incubated 30 min with osmoticum in the presence and absence of driselase. Cells were treated for 30 min at room temperature in osmoticum with (lanes c and d) or without (lanes a and b) 2% (w/v) driselase. *In vitro* phosphorylation was as per Figure 1A. Lanes b and d were plus 100 μM free calcium and a and c were in the absence of added calcium. Arrows refer to bands discussed in the text. Numbers on left are mol wt $\times$ 10^{-3} based on standard proteins.

membrane was washed through a sorbitol cushion prior to analysis of the proteins by SDS-PAGE. The effects of *in vitro* driselase treatment on the Coomassie blue-stained proteins were compared to that of the *in vivo* treatment (Fig. 4). Only two differences were readily visible for the *in vivo* treatment—notably the loss of staining of two proteins at M_r 20,000 (arrowed in Fig. 4, lanes a and b). Driselase treatment *in vitro* (lanes c and d) leads to similar changes. A single protein of M_r 15,000 appears after *in vitro* driselase treatment of membranes (arrowed in Fig. 4, lane c). Presumably this band represents a degradation product or a driselase component which has co-isolated with the washed membranes.

The above *in vitro* driselase treatment completely abolished all protein kinase activity in the resultant washed membrane fractions. In addition, it was found that simply including driselase in the phosphorylation assay mixture led to decreased phosphorylation as indicated in Figure 5. Driselase alone contained no measurable protein kinase activity (data not shown). Taken *in toto*, these results indicate that neither major degradation of plasma membrane proteins nor phosphorylation of driselase components contributed to the changes in protein phosphorylation seen after *in vivo* driselase treatment.

The reduced phosphorylation when driselase was added to the *in vitro* reaction mixture was similar but not identical to the response to *in vivo* driselase treatment (*e.g.*, compare Fig. 5, lanes c and d, to Fig. 1A, lanes a and b). Phosphorylation of the band of M_r 80,000 in the presence of calcium was reduced but not eliminated (arrowed in Fig. 5). This band was characteristic of the membranes isolated from the control cells and was reduced

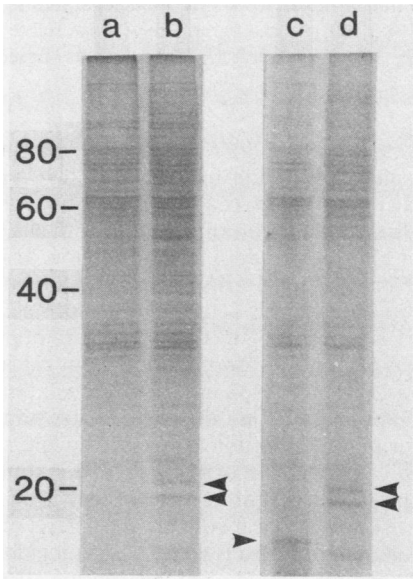

FIG. 4. A comparison of the protein content of plasma membrane after *in vivo* and *in vitro* treatment with driselase. For *in vivo* treatment (lanes a and b) cells were incubated for 30 min at room temperature in the presence (lane a) and absence (lane b) of 2% (w/v) Driselase. A plasma membrane preparation was then isolated from the washed cells, added to sample buffer and separated by SDS-PAGE. The *in vitro* treatment consisted of a 30 min incubation at room temperature of plasma membrane isolated from untreated cells in the presence (lane c) and absence (lane d) of 2% (w/v) driselase in the osmoticum buffer without sorbitol. Membranes were then spun through a neutral pH sorbitol cushion at 11,500*g*, added to sample buffer heated at 100°C for 10 min and separated by SDS-PAGE. The resultant polyacrylamide gels were stained with Coomassie blue, dried down and photographed. Arrows relate to bands discussed in the text. Numbers on left are mol wt $\times$ 10^{-3} based on standard proteins

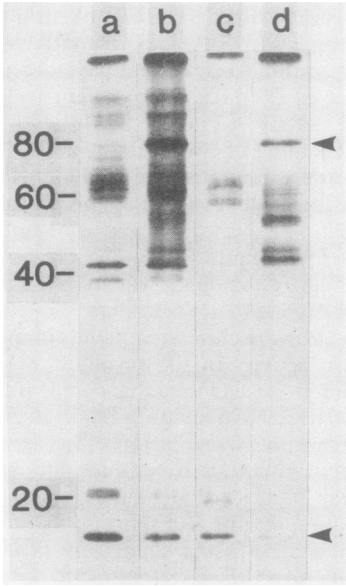

FIG. 5. Demonstration of the lack of phosphorylation of driselase proteins by the isolated plasma membrane fraction. A plasma membrane fraction was isolated from untreated cells and phosphorylated by incubaiton in [γ-^{32}P]ATP in the presence (lanes c and d) and absence (lanes a and b) of 2 % (w/v) driselase in the assay mix. Additionally, labelings were performed in the absence (lanes a and c) and presence (lanes b and d) of approximately 100 μM free calcium ions. Reactions were terminated after 3 min at 4°C by the addition of sample buffer and heating at 100°C for 10 min. Preparations were then separated by SDS-PAGE and the dried down gels autoradiographed. Arrows relate to bands discussed in the text. Numbers on left are mol wt $\times 10^{-3}$ based on standard proteins.

to a greater extent with an *in vivo* driselase treatment of only 1.5 min. The phosphorylation of the M_r 15,000 protein in the presence of calcium was reduced by the *in vitro* driselase treatment. Thus, direct interaction of driselase with the membranes was capable of altering protein phosphorylation. It is important to note, however, that long term *in vitro* treatment led to complete loss of protein kinase activity, while even after 90 min exposure to driselase *in vivo*, the distinct pattern of protein phosphorylation was evident both with and without calcium present.

What Alters Protein Phosphorylation? Incubation of cells in osmoticum containing a boiled driselase supernatant had no effect on protein phosphorylation compared to the sorbitol controls. This indicated that an active enzymatic component was required for the response. Furthermore, incubation of cells with a boiled 'once used' driselase mixture had no effect on the protein phosphorylation pattern of the isolated plasma membranes. Thus, any cell wall fragments remaining in the solution after the normal 90 min cell wall digestion period were not acting as elicitors for this response.

An initial attempt was made to identify the component(s) in the driselase which might cause such changes in protein phosphorylation. Since driselase is a crude mixture of fungal enzymes, several hydrolytic enzymes were tested to see if they would give a similar response. Cells were treated with either ribonuclease (1 μg/ml), trypsin (1 μg/ml), or pectinase (2 mg/ml) for 1.5 min. None of the treatments gave detectable changes in the protein phosphorylation pattern compared to the sorbitol controls. While longer (30 min) treatment with trypsin decreased protein phosphorylation, the response was general and the pattern of phosphorylation did not vary either with or without calcium added to the *in vitro* reaction mixture. In further attempts to eliminate the effects of nonspecific proteases present in the driselase, cells were treated for 5 min with driselase containing 1.0% BSA. The addition of BSA did not inhibit the driselase effect on protein phosphorylation (data not shown).

If extracellular calcium was acting as a second messenger during the response to driselase *in vivo*, then removing extracellular calcium with the calcium chelator, EGTA, should decrease the response and increasing intracellular calcium with the ionophore, A23187, should mimic the response. Neither EGTA (2.5 mM) with driselase, nor A23187 (10 μM) in the absence of driselase affected the pattern of protein phosphorylation of the isolated membranes. Thus, the *in vivo* status of calcium appeared not to be critical for the driselase-induced changes in the plasma membrane.

DISCUSSION

While differences in the structure (15, 17) and function (11, 14) of the plasma membranes of protoplasts and the source tissue have been reported, early biochemical changes in the plasma membranes of cells during cell wall digestion have not been described previously. The results presented in this paper indicate that the plasma membranes of the wild carrot cells grown in suspension culture are very sensitive to exposure to cell wall digestion enzymes. Within 1.5 min after treatment of the cells with the enzymes, the pattern of ATP-dependent protein phosphorylation of the isolated membranes changed. The most dramatic changes were evident when calcium was added to the *in vitro* phosphorylation mixture. It is assumed that these represent altered/novel phosphorylation sites induced by the driselase treatment. These changes in the plasma membrane persisted and were evident in the fully isolated protoplasts (90 min treatment of the cells with driselase).

It has been suggested that both the proteases contaminating the cell wall digestion enzymes and the osmotic stress imposed during wall digestion could contibute to altering the plasma membrane of protoplasts (for review see Refs. 6 and 7). The responses described in this paper were rapid and did not result from osmotic stress alone. The responses were dependent on a heat-sensitive component of the driselase; however, they were probably not due to a nonspecific protease effect since neither *in vivo* nor *in vitro* treatment with driselase resulted in major loss of Coomassie blue-staining bands. Treatment with a protease such as trypsin would have caused major losses of membrane proteins (12). In addition, the driselase response could not be elicited by 1.5 min or 30 min treatment with trypsin *in vivo* and adding 1.0% BSA to the driselase to decrease nonspecific protease activity had no effect.

Carpita *et al.* (5) have shown that 3.8 to 4.0 nm pores exist in the cell walls of tissue culture cells and that these pores would allow large molecules to reach the plasma membrane. If some of the enzymes (*e.g*, glycosidases, phosphatases) in the driselase mixture reached the plasma membrane of the cells during the *in vivo* treatments, then they could render them more or less susceptable to calcium-dependent phosphorylation *in vitro*.

If the rapid response to driselase reported here was merely due to a direct enzymatic alteration of the membrane by driselase, then the *in vitro* treatment with driselase should have mimicked the *in vivo* treatment. Although some similarities were evident, increased calcium-dependent protein phosphorylation exemplified by the band of M_r 22,000 was only seen with the *in vivo* treatments. This suggested that the increased calcium-dependent phosphorylation either required cytosolic or cytoskeletal components which were lost during membrane isolation or that the stimulus was not the enzymes per se but rather components of the wall released as result of the enzyme treatment.

Small saccharides released from the cell wall did not appear to be involved since the response was not elicited with a 'once used' boiled enzyme solution. This solution, however, was re-

covered after 90 min exposure to the cells and any elicitors produced in the first few minutes of exposure may have been completely digested by the 90 min driselase treatment. The boiled enzyme solutions from shorter exposure times were not tested.

In summary, these experiments document a very rapid response of the plasma membrane to cell wall digestion. While a specific cause for the response to Driselase has not been identified, it is important to note that changes in *in vitro* and *in vivo* protein phosphorylation have been produced by very short time treatments with driselase *in vivo*. It would thus appear that, in some way, the plasma membrane is responding to initial cell wall damage. While the driselase/cultured cell system is artificial, the underlying mechanism of this response could operate in the intact plant in response to pathogen invasion.

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