TRANSFER OF TOXIN SUSCEPTIBILITY TO PLANT PROTOPLASTS VIA THE HELMINTHOSPOROSIDE BINDING PROTEIN OF SUGARCANE

Gary A. Strobel and Kenneth D. Hapner

Department of Plant Pathology
Montana State University
Bozeman, Montana 59715

Received March 7,1975

SUMMARY:

The eyespot disease of sugarcane is caused by Helminthosporium sacchari. Helminthosporoside, a host-specific toxin produced by H. sacchari, is essential for the pathogenicity of this fungus. The presence of the helminthosporoside-binding protein in sugarcane likewise appears to be essential for susceptibility to the toxin. The results of this report show that leaf cell protoplasts of tobacco and toxin resistant sugarcane effectively adsorbed the toxin-binding protein derived from membranes of susceptible sugarcane. These protoplasts then became susceptible to the helminthosporoside. They also functioned to takeup raffinose, a trisaccharide structurally related to the toxin. Tobacco protoplasts were treated with [^{14}C] - binding protein, ruptured, and fractionated on a sucrose density gradient column. A peak of radioactivity was associated with the enriched plasma membrane fraction. The results support the hypothesis that the binding protein is the primary recognition site governing susceptibility of sugarcane to helminthosporoside.

INTRODUCTION:

The helminthosporoside-binding protein is associated with the plasma membrane in sugarcane and possesses α-galactoside binding activity; presumably, this protein functions in α-galactoside transport (1). Helminthsporoside, a host-specific toxin produced by Helminthosporium sacchari, is an α-galactoside of cyclopropanediol and binds to this protein (2,3). Only clones of sugarcane possessing a functional binding protein are susceptible to the fungus and likewise to the toxin (4). The protein, being located primarily on the plasma membrane, is accessible to the toxin which readily diffuses from the fungal spores and mycelium (5). Upon complexing with the toxin, a conformational change presumably occurs in the binding protein which in turn activates other membrane proteins including the K, Mg ATPase (1). Presumably, this activation results in an imbalance of electrolytes resulting in cell death.

A question concerning the role of the binding protein in disease susceptibility is whether or not it is the only gene product in a susceptible sugarcane plant that governs specificity. This question would be answered if the protein were incorporated into the plasma membrane of a resistant sugarcane cell or indeed into a cell of an unrelated species rendering them susceptible to the effects of the toxin. This report attempts to verify the successful uptake of the binding protein of susceptible sugarcane by protoplasts of both resistant sugarcane and tobacco with the concomitant transfer of toxin susceptibility to heretofore resistant protoplasts.

MATERIALS AND METHODS:

The methods of preparing the toxin-binding protein varied from that previously reported in that the pelleted membrane fraction was treated for 4 hr in 1.0 M trichloroacetate - Na salt buffered with 0.05 M Tris · HCl, pH 7.2 at 4° C (3). The supernatant liquid obtained after centrifugation at 48,000 xg was chromatographed on a 98 x 1.5 cm column of Bio gel P-100 as previously described (3). The protein eluting at the volume containing toxin-binding activity was collected and concentrated. The rationale for using trichloroacetic acid, a chaotropic agent, rather than Triton X-100 was that it could be completely removed from the protein. Small amounts of detergent associated with the protein would likely be detrimental to the plasma membranes of protoplasts treated with the protein. Subsequently, the protein was placed on a 2.5 x 5 cm affinity column of aminoethyl Bio gel P-150 to which melibionate was covalently linked (7). The column was rinsed with 30 ml of 0.05 M Tris · HCl buffer, pH 7.2 and the protein eluted from the column with 10 ml of 0.1 M melibiose. It was dialyzed against 0.01 M MES buffer, pH 5.6 and concentrated with a UM 10 Amicon filter. It possessed a specific activity of 103 nmoles raffinose bound/mg protein (1). Gel electrophoresis of the protein preparation revealed the presence of a single band (3).

The binding protein was labeled with ^{14}C by treating 128 µg of the protein preparation obtained from the Bio gel P-100 step in 2.0 ml of 0.5 saturated sodium acetate in the presence of 250 µCi of [^{14}C]acetic anhydride with a specific activity of 30 mCi/mM at 4°C for 3 hr (6). The preparation was exhaustively dialyzed in 0.01 M Tris, pH 7.2 and was subsequently purified by affinity column chromatography. The protein eluted from the column with buffered melibiose had a specific radioactivity of 31,900 dpm/21 µg.

Protoplasts of tobacco (Nicotiana tabacum L. 'Xanthi-NC') were prepared according to the procedure of Shepard and Totten, (8). Protoplasts of resistant sugarcane clone H50-7209 were obtained as outlined by Strobel and Hess (5) according to Shepard and Totten (8).

Approximately 10^5 protoplasts of tobacco in 0.7 ml of 0.2 M sucrose containing 0.01 M MES buffer, pH 4.5, were incubated for 3 hr at 23°C with 1.8 µg of the toxin-binding protein. Approximately 4 x 10^3 protoplasts (20 µl) were placed on a sunken slide and 1 µl of helminthosporoside (0.02 µmole) was added. The cells were incubated at 23°C and hourly, for 3 hr, the number of turgid rounded cells (usually floating on the surface) and the number of cells that appeared dead (having a ruptured outer membrane,

usually settled to the bottom) were determined. Similar experiments were conducted with protoplasts of resistant sugarcane clone H50-7209.

Protoplasts (10^5) of sugarcane H50-7209 were incubated in 0.2 ml of a solution containing 2 µg of poly L-ornithine, 0.01 NaCl, 0.3 M sucrose, and 0.1 M K citrate for 3 hr at 23°C. Subsequently, about 2 x 10^3 protoplasts (20 µl) were treated with helminthosporoside as described above. Protoplasts affected by the treatments and appropriate controls were determined over a 3 hr incubation period.

[^{14}C] - Raffinose uptake was measured in sugarcane protoplasts (10^5) that had been treated with either the binding protein or poly L-ornithine under the conditions already described. The technique for [^{14}C] - raffinose preparation and the methods of measuring uptake are described elsewhere (1).

Protoplasts were ruptured in a 5 ml glass homogenizer in the presence of 0.25 M sucrose, 2.5 mM mercaptoethanol, 3 mM EDTA and 0.05 M Tris · HCl, buffer, pH 7.2 according to Leonard and Hodges (9). The suspension (0.5 ml) was layered on to a sucrose density gradient made up of 3.5 ml of 45% sucrose and 1.0 ml of 34% sucrose and centrifuged at 95,000 xg for 2 hr. The fraction enriched in plasma membranes concentrates at the 35-45% sucrose interface as previously demonstrated (5,9).

Radioactivity associated with membranes was determined after digesting the membranes in 0.1 ml of NCS (Nuclear Chicago Corp.). All aqueous samples were dissolved in 12 ml of Aquasol and cpm corrected to dpm by a linear quench correction curve.

RESULTS AND DISCUSSION:

Protoplasts of tobacco and resistant sugarcane incubated with the toxin-binding protein, followed by a treatment with helminthosporoside, took on the properties of susceptible cane protoplasts treated with the toxin (swelling, protuberances). Such protoplasts also showed an increased mortality over the appropriate controls (Fig. 1).

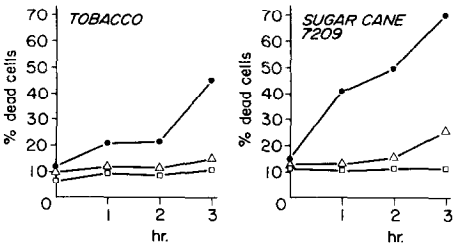

Fig. 1. The protoplasts of both tobacco and resistant sugarcane were treated with 0.18 ng purified binding protein per cell. Helminthosporoside was added to yield a final concentration of 1 mM. Each total cell mortality calculation is based on an average of 3 separate determinations. Protoplasts treated with protein and toxin (● - ●), protoplasts treated with protein (△ - △), protoplasts with toxin (□ - □).

If the treated sugarcane protoplasts had actually incorporated functional binding protein into their outer membrane, then the physiological characteristic of active α-galactoside transport should be demonstrable. Fig. 2 shows that protoplasts pre-treated with the binding protein took up

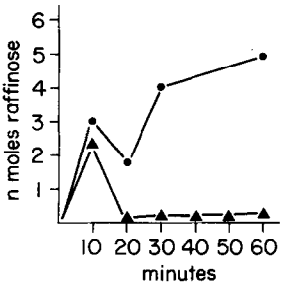

Fig. 2. The uptake of [^{14}C] - raffinose by 10^5 sugarcane protoplasts that were preincubated with 1.8 µg of the purified toxin-binding protein and the administered 0.12 µmoles of [^{14}C] - raffinose (57,700 dpm). An equal number of non-treated protoplasts served as a control. Treatment (● - ●), Control (▲ - ▲).

[^{14}C] - raffinose to a much greater extent than a comparable amount of protoplasts not treated with the binding protein. When these protoplasts were washed with 20 ml of 0.4 M mannitol solution and subsequently ruptured to release cellular contents there was 0.52 nmole of raffinose bound to the insoluble protoplast material from the treated protoplasts and 0.19 nmole associate with the control. This suggests the association of the binding protein with the membranous cellular contents.

Poly L-ornithine induces endocytosis and membrane damage and hence permits the uptake by protoplasts of substances that are normally excluded (10). Poly L-ornithine-treated resistant sugarcane protoplasts took up [^{14}C] - raffinose to the extent of 2.6 nmole over 3 hr, which was about half that of protoplasts treated with the binding protein (Fig. 2). Nevertheless, poly L-ornithine treated protoplasts were not more susceptible to the effects of helminthosporoside than the controls over 3.5 hr. This suggests that poly

L-ornithine and the binding protein enhance raffinose uptake via different mechanisms. Presumably this difference may represent a specific α-galactoside uptake in the case of the binding protein, and a non-specific mechanism (pinocytosis or passive diffusion) in the case of poly L-ornithine (10).

[^{14}C] - binding protein (5.9 µg) was added to approximately 3 x 10^6 tobacco protoplasts in 0.5 ml of 0.2 M sucrose-buffer. The cells were incubated 2 hr at 23°C, after which they were disrupted and the suspension was layered on to a sucrose density gradient column. Fig. 3 illustrates the layering pattern observed after centrifugation. A peak of radioactivity appeared at the

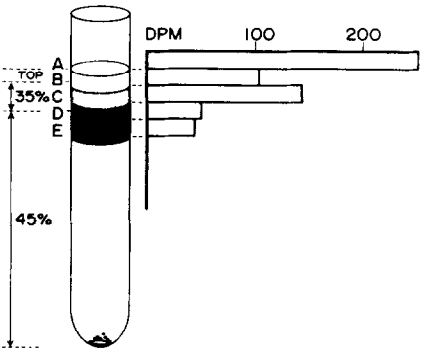

Fig. 3. Density gradient centrifugation of a membrane suspension of 3 x 10^6 protoplasts that had been incubated with ^{14}C binding protein for 2 hr at 23°C. The radioactivity occurring in each layer is shown to the right of the centrifuge tube. The various layers of membranes are labeled B⟶E with A as the meniscus. To the left of the tube, the concentrations of sucrose are shown as 35% and 45%. Layer C is the plasma membrane rich fraction (5,9). Details of the experiments are in the Materials and Methods section.

meniscus of the column, and another was associated with the enriched plasma membrane fraction. The amount of radioactivity associated with the enriched plasma membrane fraction was adequate to account for the association of approximately 10^5 molecules of the toxin-binding protein per protoplast.

The experimental data support the hypothesis that the toxin-binding protein of sugarcane becomes associated with the plasma membrane of plant

protoplasts, rendering them susceptible to helminthosporoside and imparting to them the ability to actively take up the α-galactoside, raffinose. Although not strictly comparable, experiments of this type have been reported by Anraku who demonstrated that reduced galactose transport activity in shocked cells of E. coli can be partially restored by prior incubation of the cells with shock fluid containing galactose binding protein (11). The data further implicate the plasma membrane toxin-binding protein in susceptible sugarcane as the primary recognition site for the host-specific toxin, helminthosporoside.

ACKNOWLEDGMENTS:

This work was supported by NSF grant GB 43192 and is paper No. 576 of the Montana Agricultural Experiment Station. The authors appreciate the suggestions and help of Dr. J.F. Shepard.

REFERENCES

1. Strobel, G.A. (1974). Proc. Nat. Acad. Sci. USA 71,4232-4236.
2. Steiner, G.W., and Strobel, G.A. (1971). J. Biol. Chem. 246,4350-4357.
3. Strobel, G.A.(1973). J. Biol. Chem. 248,1321-1328.
4. Strobel, G.A. (1973). Proc. Nat. Acad. Sci. USA 70,1693-1696.
5. Strobel, G.A. and Hess, W.M. (1974). Proc. Nat. Acad. Sci. USA 71,1413-1417.
6. Riordan, J.F., Vallee, B.L. (1972). Methods in Enzymology 25, part B, 494-499.
7. Hayes, Colleen E., and Goldstein, I.J. (1974). J. Biol. Chem. 249,1904-1914.
8. Shepard, J. and Totten, R. (1975). Plant Physiol. (in press).
9. Leonard, R.T., and Hodges, T.K. (1973). Plant Physiol. 52,6-12.
10. Burgess, J., Motoyoshi, F., and Fleming, E.N. (1973). Planta 112,323-332.
11. Anraku, Y. (1967). J. Biol. Chem. 242,793-800.