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The activity of powdery-mildew haustoria after feeding the host cells with different sugars, as measured with a potentiometric cyanine dye

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Abstract. The biotrophic parasite *Erysiphe graminis f. sp. hordei* produces haustoria within the cells of its host *Hordeum vulgare*. To determine the physiological activity of these haustoria, the electric potential across the membranes in the mitochondria of the haustorium was studied. The membrane potential was estimated with the fluorescent potentiometric cyanine dye 3,3′-dibutyloxacarbocyanine iodide. The addition of depolarizing agents (carbonylcyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol or KCN) to infected cells resulted in an increase of fluorescence after the addition of low concentrations or a decrease of fluorescence after the addition of higher concentrations. When the infected host cell was fed with increasing concentrations of D-glucose (25, 50, 75 mM), corresponding decreases of fluorescence were measured immediately in the mitochondria of the fungal haustoria. Sucrose induced a similar reduction of fluorescence about 20 min later. D-Galactose and D-fructose induced a somewhat smaller reduction of fluorescence, L-glucose and D-glucitol had no effect. The results indicate that haustoria take up glucose from the host cells immediately. Sucrose, D-galactose and D-fructose seem to require time to be metabolized before their products reach the fungal haustorium or mitochondria.

Key words: *Hordeum* (*Erysiphe* infection) – *Erysiphe* – Mitochondrion (transmembrane potential) – Sugar transport (host-parasite).

Introduction

Haustoria are specialized structures of parasitic fungi that are differentiated within the host cell (Bushnell 1972). The haustoria of the rust fungi and the powdery mildew fungi generally consist of a slender haustorial neck and a haustorial body, which may have appendices. The whole structure is surrounded by the invaginated host-cell plasma membrane. Their structural qualities (see Littlefield and Heath 1979; Bushnell and Gay 1978) indicate that haustoria are the absorptive organs of these parasitic fungi. Studies with isotopes show that labelled material of the host cell accumulates within fungal structures (see Mendgen 1981; Hancock and Huisman 1981; Manners and Gay 1983). However, the role of haustoria in the host cells during uptake of metabolites is not known. Isolated haustorial complexes of *Erysiphe pisi* have been shown to have the capability to take up many metabolites (Gil and Gay 1977; Manners and Gay 1982). Also the accumulation of labelled material in the mycelium of *Erysiphe graminis* correlates quite well with the progress of the differentiation of fungal haustoria within the host epidermal cell (Mount and Ellingboe 1969; Slesinski and Ellingboe 1971).

Until recently, techniques have not been available to study the physiological responses to experimental treatment of haustoria within host cells. Consequently, we have no understanding of how haustoria respond shortly after application of sugars or other nutrients to host cells.

Recent progress in the use of cyanine dyes, which can be an indicator of membrane potential, indicates that they can be used in the study of host-parasite interaction. Bushnell et al. (1987) showed that certain cyanine dyes stain filamentous particles that are mitochondria in cells of host and parasite. The bright fluorescence can be reduced with depolarizing agents or inhibitors like carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP) and KCN. Here, we show that the fluorescence in the mitochondria of the

Abbreviations: CCCP = carbonylcyanide *m*-chlorophenylhydrazone; $DiOC_4(3)$ = 3,3′-dibutyloxacarbocyanine iodide; DNP = 2,4-dinitrophenol

parasitic fungus *Erysiphe graminis f. sp. hordei* can be correlated with the amount and the type of different sugars fed to the host cells. Some preliminary results have already been published (Mendgen et al. 1988).

Material and methods

Plant and fungus. Barley, *Hordeum vulgare* L. cv. Proctor, was raised in compost soil (Einheitserde W 75) at 20° C and illuminated 16 h per day (~120 μmol·m^{-2}·s^{-1}; HQL-lamps; Osram, München, FRG). Eight-day-old plants were used to excise the inner epidermis from the coleoptile, as described by Bushnell et al. (1967). The monolayer of cells was mounted as shown by Mendgen and Dressler (1983) except that the adaxial surface of the monolayer was positioned uppermost. The tissue was floated on 0.01 M $CaCl_2$ solution and both ends of the coleoptile were kept in place by cover glasses. The microscope slide with the epidermal mount was stored in a plastic box except when it was examined with the microscope. In the plastic box, relative humidity was kept at 80% with a 20% solution of glycerine and the box was stored under laboratory conditions at 20° C.

The powdery-mildew fungus, *Erysiphe graminis f. sp. hordei,* Race A1-1, was propagated on 10- to 30-d-old barley plants raised as described above. For inoculation of the host cells, spores were blown into a settling tower and allowed to fall onto the epidermal cell layer.

Dyes, inhibitors and sugars. 3,3'-Dibutyloxacarbocyanine iodide [$DiOC_4(3)$]; $1 \cdot 10^{-6}$ M, from Molecular Probes, Eugene, Oregon, USA, was dissolved in potassium-phosphate (Merck, Darmstadt, FRG) buffer, 0.01 M, pH 6.2.

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP; Sigma, St. Louis, Mo., USA) and potassium cyanide (KCN; Merck) were dissolved in 0.01 M phosphate buffer as above.

D-Glucose, L-glucose, sucrose, D-galactose, β-D-fructose and D-sorbitol (Sigma) were also dissolved in the same phosphate buffer as above.

Infection and measurements of fluorescence. The surface of the mounted coleoptile epidermis was inoculated in a settling tower with spores of *E. graminis* so that about 10% of the epidermal cells became infected. By 48 h after inoculation, spores had formed small colonies, each with one haustorium. For measurements of fluorescence, haustoria with well developed "fingers" and no sign of vacuolation were selected under low-intensity bright-field illumination. In the host cells, particles in the cytoplasm streamed evenly at normal speed. A fine Pasteur pipette was used to replace the $CaCl_2$ solution under the epidermal cell layer with dye solutions, inhibitor solutions or the control buffer. This procedure was performed with the whole mount positioned under the microscope objective lens. Compared to our earlier measurements (Bushnell et al. 1987), the method was improved to perform an exchange of the fluids within 30 s. For measurements of fluorescence intensity, a microscope photometer (MPV 2; Leitz, Wetzlar, FRG) equipped with an incident-illumination system (Ploemopak with a 100-W, DC, high-pressure mercury lamp) and a filter block I2 (BP 450–490, RKP 510, LP 515) was used with a Leitz fluorescence objective 40/0.75. The measuring diaphragm had an opening of 9·3 μm and was focused exactly over the center of the haustorial body with the mitochondria. A shutter system allowed a measuring time (and a similar time of exposure to the UV light) of 0.25 s.

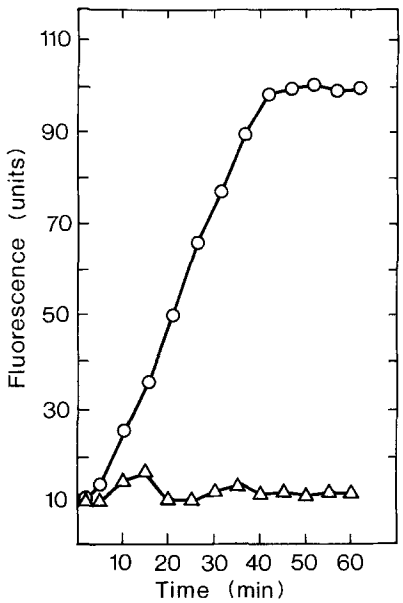

Fig. 1. Fluorescence (relative units) of mitochondria in the haustorial body (o—o) of *Erysiphe graminis f. sp. hordei* and fluorescence of the host (Δ—Δ) after incubating the epidermal cells of *Hordeum vulgare* with a solution of 10^{-6} M $DiOC_4(3)$

In order to avoid photobleaching, only one measurement was made every 3–5 min. Thus, our measurements cannot follow rapid changes of fluorescence. The lines in the figures are drawn only to guide the eye. Each experiment was repeated at least three times with different coleoptile mounts.

Results

Incubating the monolayer of host cells with a solution of 10^{-6} M $DiOC_4(3)$ resulted in a gradual and steady increase in the fluorescence of mitochondrial particles in the haustorial body of *E. graminis f. sp. hordei* as reported earlier (Bushnell et al. 1987). There was only a small increase in fluorescence of the host cytoplasm including mitochondria (Fig. 1). Fluorescence intensity reached a plateau after 40 min and remained constant for at least 60 min.

The addition of a 10^{-6} M solution of CCCP resulted almost immediately in a sharp increase of fluorescence and was followed, about 2 min later, by a fast decrease of fluorescence which reached its minimum after about 40–50 min (Fig. 2). A similar result was obtained with a solution of 10^{-4} M DNP and 10^{-3} M KCN (data not shown).

The increase or the decrease of the fluorescence was dependent on the concentration of the inhibitor. Further dilution of CCCP to 10^{-8} M resulted in an increase of fluorescence that remained stable over a period of at least 30–40 min (Fig. 3). Similar

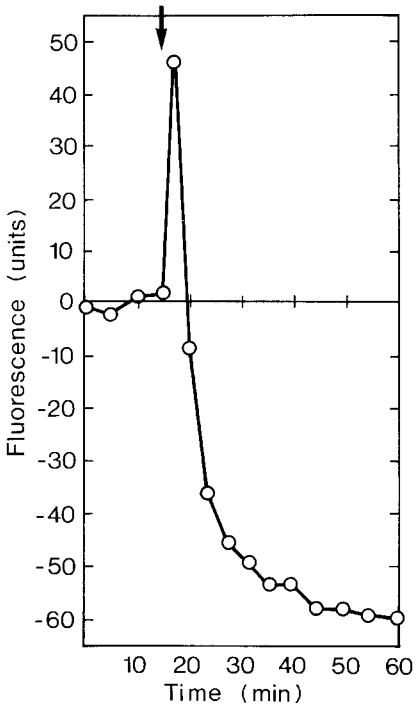

Fig. 2. Fluorescence of mitochondria (relative units) in haustoria after incubating the host with $DiOC_4(3)$ for 50 min and subsequent addition (*arrow*) of CCCP (10^{-6} M)

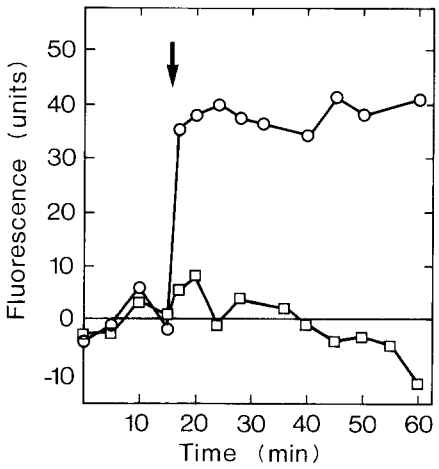

Fig. 3. Fluorescence of mitochondria in haustoria after feeding the host with $DiOC_4(3)$ for 50 min and subsequent addition of a $1 \cdot 10^{-8}$ M solution of CCCP (*arrow*). □—□ = control buffer

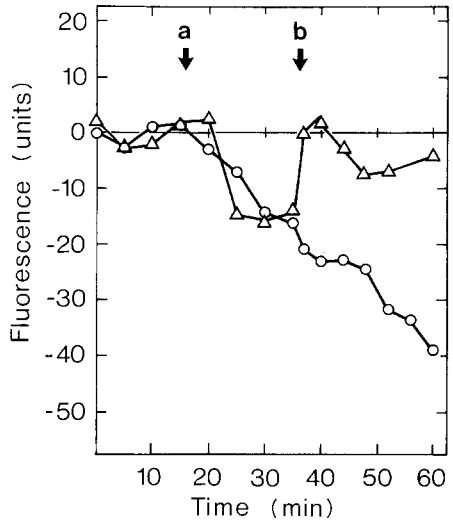

Fig. 4. Influence of D-glucose (○—○) or D-glucose and KCN (△—△) on fluorescence of mitochondria in haustoria after the host was fed with $DiOC_4(3)$ for 50 min. *Arrows:* addition of 50 mM glucose (*a*) and addition of 10^{-5} M KCN to the glucose solution (*b*)

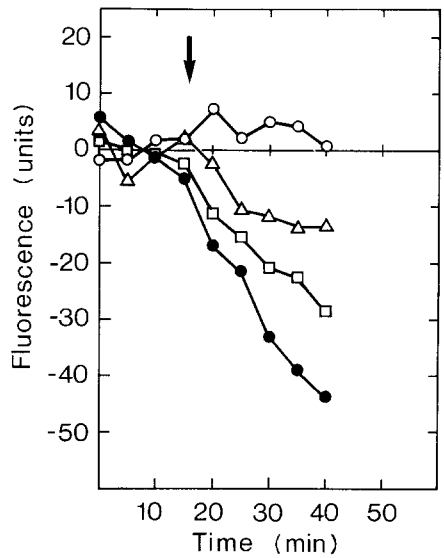

Fig. 5. Change in fluorescence of haustorial mitochondria after the host was fed with $DiOC_4(3)$ for 50 min and then fed (*arrow*) with different concentrations of D-glucose. ●—● = 75 mM; □—□ = 50 mM; △—△ = 25 mM; ○—○ = buffer control

results were obtained with a 10^{-6} M solution of DNP and a 10^{-5} M solution of KCN in buffer.

The fluorescence of haustorial mitochondria also changed after host cells were fed with glucose. Compared with the controls, which exhibited a steady level of fluorescence (Figs. 1, 3) after the uptake of $DiOC_4(3)$, addition of a 50 mM D-glucose solution resulted in a gradual reduction of fluorescence (Fig. 4). The reduction of fluorescence could be abolished and the original fluorescence was retained by the simultaneous addition of KCN with the sugar (Fig. 4).

The reduction in fluorescence intensity was dependent on the concentration of glucose fed to the host cells (Fig. 5). It also depended on the type of sugar supplied (Fig. 6). D-Glucose (50 mM) induced an immediate reduction of fluorescence. Sucrose, (which was used only at a concentration of

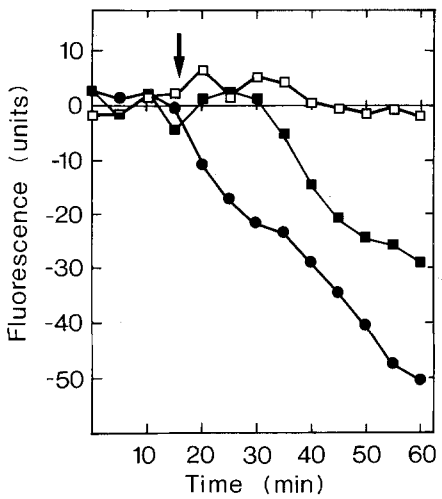

Fig. 6. The effect of 25 mM sucrose (■——■) and 50 mM D-glucose (●——●) on the fluorescence of haustorial mitochondria. □——□ = buffer control. *Arrow* = addition of sugars or change of buffer

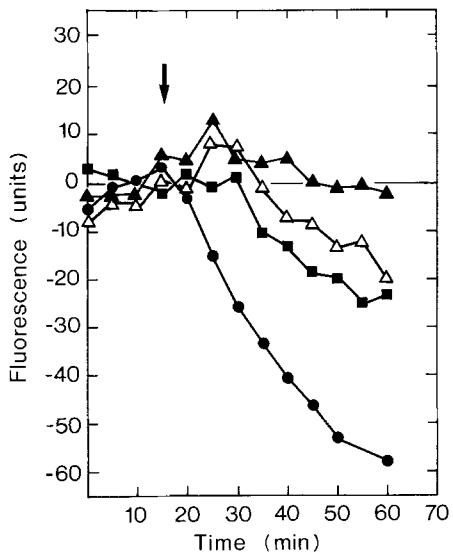

Fig. 7. The influence of 50 mM D-glucose (●——●), 50 mM D-fructose (■——■) and 50 mM D-galactose (△——△) on the fluorescence of haustorial mitochondria in host cells, previously fed with $DiOC_4(3)$. ▲——▲ = buffer control; *arrow* = addition of sugars or change of buffer

25 mM) induced a comparable reduction in fluorescence about 20 min later.

Compared with D-glucose, which had a very fast and marked influence on the fluorescence of particles in the haustoria, other monosaccharides were much less effective. Floating the epidermal cell layer on solutions of L-glucose (50 mM) or D-sorbitol (50 mM) did not influence the fluorescence in the haustoria of the parasite (data not shown). D-Fructose (50 mM) or D-galactose (50 mM) showed a small effect which became obvious only about 20 min after the host was fed with the sugars (Fig. 7).

Discussion

The potentiometric dye used in this study is transported electrophoretically into the mitochondrial membranes of the cells where it accumulates at internal negative membrane potentials (Waggoner 1979). The dye molecules are thought to bind noncovalently to hydrophobic sites (Smith et al. 1981; Guillet and Kimmich 1981). The redistribution of the dye molecules correlates with a change of the fluorescence. The amount of fluorescence depends on the concentration of the dye in the membrane (Sims et al. 1974). At low concentrations (10^{-8}–10^{-7} M in the aqueous solution), uptake of dye results in an increase of fluorescence. At high concentrations, generally in a range of 10^{-4} M, further uptake of dye into the membranes results in a quenching of fluorescence. This reduction of fluorescence can be explained by the formation of nonfluorescent di- or oligomers of the dye or by hydrophobic interaction between monomers of the dyes (Freedman and Hoffman 1979; Guillet and Kimmich 1981; Emaus et al. 1986). Therefore, the interpretation of an increase or decrease of fluorescence should be made with care.

Potentiometric dyes have been shown to be a useful tool to visualize mitochondria in plants (Matzke and Matzke 1986; Liu et al. 1988; Wu 1987) or to study the membrane potential of mitochondria of different organisms (e.g. Johnson et al. 1981; Gallo et al. 1984; Pena et al. 1984) by measuring their fluorescence. Also, Bushnell et al. (1987) recently showed that the dyes stain filamentous particles in the cells of host and parasite, including the haustoria of *Erysiphe graminis f. sp. hordei*. It was assumed from their typical filamentous structure, quantity and movement that these particles were mitochondria. The fluorescence of these particles could be reduced after the application of protonophores or inhibitors as a consequence of the vanishing membrane potential. We likewise found that certain concentrations of CCCP, DNP or KCN reduced mitochondrial fluorescence. In addition, fluorescence increased after application of lower concentrations of the same depolarizing agents. The short peak of fluorescence after the application of higher concentrations is obviously a response to low concentrations as the substances diffuse into the haustorium at the beginning of the experiment. Since we apply dye only to the lower side of the host cell from where it

penetrates into the cell, the dye solutions have no direct contact with the fungus which penetrated from the upper surface, through the cuticle, into the host cell. Therefore, we have no control on the dye concentration in the host and especially in the parasite.

The increase of fluorescence with low concentrations of CCCP, DNP or KCN indicates a small reduction of the membrane potential. Obviously, dye molecules are redistributed, their concentration is reduced to some extent and the quenching effects are abolished. Only at high concentrations (e.g. 10^{-5}) of these substances is the membrane depolarized sufficiently for most of the dye to leave the mitochondria and the haustoria. Our experiments give no hints on the value of the membrane potential in the mitochondria. We do not know the dye concentrations there, the internal pH and the concentrations of other ions. Also, we know nothing about the permeability of the membranes surrounding the haustorium. Therefore, calculations on the membrane potential are not possible (Waggoner 1979). Also, we do not know why fungal mitochondria exhibit brighter fluorescence with $DiOC_4(3)$ than do host mitochondria. Either fungal membranes have different qualities concerning their ability to bind this dye or their membrane potential is different. Because of the low intensity of light emitted by host mitochondria with $DiOC_4(3)$, we were not able to measure increases or decreases of host fluorescence in response to experimental treatments.

In spite of these difficulties, we tried to correlate the fluorescence of haustorial mitochondria with different concentrations of glucose fed to the host cells. The experiments were performed when fluorescence had reached the highest level possible and remained stable for at least 60 min. Although we know nothing about the real sugar concentrations within haustoria, reduction of fluorescence correlated well with increasing concentrations of glucose fed to host cells and seems to indicate an increase of membrane potential (hyperpolarisation) of mitochondria. As a result, dye concentration increases in the membranes, di- and oligomers form and the fluorescence is reduced. The effect of the sugars can be abolished after careful application of KCN. Obviously, we can demonstrate the reaction and the activity of the fungal mitochondria in the haustoria after feeding the host cell.

The different sugars had a typical influence on the reaction of fungal mitochondria: D-glucose is taken up immediately by the host cells and may be transported as glucose or one of its metabolites into the haustorium. It seems to stimulate fungal respiration and consequently increases the mitochondrial membrane potential. L-Glucose had no effect and may not be metabolized at all. The same holds for D-sorbitol.

Sucrose needs to be metabolized for about 20 min until its products reach the fungal mitochondria. This could be the result of low invertase activity in the walls of host and parasite, although high invertase activities have been reported in infected tissues (Manners and Gay 1983; Farrar 1985). Likewise, fructose or its metabolites reach the fungal haustorium only after a delay about of 10–20 min. D-Galactose is even less effective. This indicates that D-glucose is preferred to other sugars by the host-parasite complex or by the fungal haustorium. We assume that D-glucose is taken up actively and therefore reaches the haustorium immediately since there is much evidence now for an active transport of glucose across the plasma membrane (Getz et al. 1987). This transport is energized by coupling the flux of the substrates to the influx or efflux of H^+ (Sze 1985). A proton-translocating ATPase is responsible for this active transport (Taiz 1986).

Although we cannot measure the membrane potential quantitatively in the mitochondria of haustoria, our results indicate that the measurement of $DiOC_4(3)$ fluorescence is a useful approach to study the activity of haustoria. It may be useful in further studies on the efficiency of haustoria in incompatible host-parasite combinations or after the application of fungicides to host cells.

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