Changes in Cucumber Cotyledon Membrane Lipid Fatty Acids During Paraquat Treatment and a Bacteria-Induced Hypersensitive Reaction

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ABSTRACT

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There is evidence that lipid peroxidation initiated by O_2^- radicals may be involved in altered plant cell membrane permeability in a bacteria-induced hypersensitive reaction. Such alterations have also been reported for paraquat-treated plants. Likely membrane targets for lipid peroxidation are unsaturated fatty acyl groups. We monitored levels of different fatty acyl groups in cucumber cotyledons during paraquat treatment and during a hypersensitive reaction induced by *Pseudomonas syringae* pv. *pisi*. Fatty acyl groups from galactolipids (a lipid found specifically in plastids) and polar lipids (found in all cell membranes) were analyzed in a total lipid extract. We also analyzed fatty acyl groups from polar lipids of an enriched plasma membrane fraction. The results verified that paraquat treatment reduces fatty acid unsaturation in plastid lipids. Fatty acid unsaturation decreased in the enriched plasma membrane fraction during both paraquat treatment and the hypersensitive reaction. These changes were concurrent with the onset of tissue collapse. We suggest that O_2^--initiated lipid peroxidation produced the altered plant cell membrane permeability observed in both paraquat treatment and bacteria-induced hypersensitive reaction.

Many reports (3,7,8,12,14) have indicated that alteration of the plant cell plasma membrane accompanies the hypersensitive reaction induced by pathogenic bacteria. It has been reported (10,11,13) that lipid peroxidation precedes membrane alteration (measured as electrolyte leakage) and that this membrane alteration is inhibited by addition of superoxide dismutase (11,13), a specific scavenger of superoxide radicals (O_2^-). Therefore, a possible sequence of events for the initiation of bacteria-induced hypersensitive reaction is: 1) O_2^- generation; 2) O_2^- initiation of lipid peroxidation; 3) lipid peroxidation-mediated alteration of the membrane lipid phase; and 4) altered membrane function.

A similar mechanism has been proposed for paraquat-induced tissue collapse. Treatment of leaf tissue with paraquat produces lipid peroxidation increased membrane permeability and resultant collapse of tissue (2). The lipid peroxidation is initiated by O_2^- radicals produced when the partially reduced dipyridyl paraquat cation is oxidized by O_2 in the chloroplast (6).

Unsaturated fatty acyl groups of membrane lipids are targets of free radical-induced lipid peroxidation (17). The resulting fatty acyl hydroperoxide molecules degenerate autocatalytically and release organic radicals, which can propagate a chain reaction of lipid peroxidation (17). Specific attack and loss of the unsaturated fatty acyl groups can produce decreased membrane fluidity (15,16) and resultant altered membrane permeability. Loss of unsaturation in discrete regions of membrane could produce "islands" of crystalline phase lipid in normal membrane gel phase at physiological temperatures (19). Theoretically, this could produce "cracks" between the two lipid phases that could alter normal membrane permeability. Also, it has been reported (18) that fatty acid hydroperoxide molecules (formed during lipid peroxidation) can transport Ca^{2+} across cell membranes. The presence of these molecules could allow the influx of Ca^{2+} from the cell wall into the cytoplasm and thus activate a Ca^{2+}/calmodulin-mediated initiation of electrolyte leakage and the onset of tissue collapse. Additionally, the function of membrane proteins (such as ion carriers) could be affected directly by alteration of their lipid environment.

The objectives of this work were to determine if any population changes occur in membrane lipid fatty acyl groups (specifically decreases in unsaturation) of cucumber cotyledons after treatment with a hypersensitive reaction inducing bacterium and if any observed changes correlate with the onset of the tissue collapse.

Fatty acids from membranes from cucumber cotyledons exposed to paraquat (where the mechanism of membrane alteration is known) were analyzed as controls.

MATERIALS AND METHODS

Plants and bacteria. Cucumber seedlings (*Cucumis sativa* L. 'Straight Eight,' from Royal Seeds, Kansas City, MO) were grown in soil under fluorescent light for 8 days (16-hr photoperiod at 22–28 C). The bacterium used was *Pseudomonas syringae* pv. *pisi*. Bacteria were obtained from colonies derived from single cells and maintained on nutrient agar slants. The bacteria were transferred to nutrient broth and incubated at 25 C on a rotary shaker (150 revolutions min^{-1}) for 18 hr to obtain cells in the log phase of growth. Bacteria were collected by centrifugation at 12,000 *g* for 10 min and then resuspended in sterile deionized water. The cell concentration was adjusted with a spectrophotometer and verified by plate count. Heat-killed bacteria were obtained by autoclaving living bacteria (15 min, 121 C, 15 psi).

Membrane lipid fatty acid analysis. Initially, we looked for changes in fatty acids from a total lipid extract. But, because of possible masking of fatty acid changes in the plasma membrane by a greater quantity of lipids from unaffected membranes, we also obtained an enriched plasma membrane fraction and analyzed possible fatty acid changes in this fraction.

By use of a hypodermic syringe, the entire intercellular space of cucumber cotyledons (8 days old) was infiltrated with: 1) water; 2) live *P. s. pisi* (10^8 cells ml^{-1}); heat-killed *P. s. pisi* (10^8 cells ml^{-1}); or 4) paraquat (10 $\mu g\ ml^{-1}$). After incubation samples were collected, the lipids were extracted and purified and the fatty acids quantified. To prevent lipid oxidation during extraction, all organic solvents contained butylated hydroxytoluene (~50 $\mu g\ ml^{-1}$). This general procedure was performed for total lipids and the enriched plasma membrane lipid fraction.

Total lipids. After 4 or 6 hr of incubation 20 cotyledon disks (0.6 cm diameter) were collected from four cotyledons (two plants) for each treatment and transferred to Corex centrifuge tubes. Liquid N_2 was added, and the disks were ground with a glass rod. Two milliliters of isopropyl alcohol was added, and samples were heated at 65 C for 30 min. After heating, 1 ml of chloroform was added, and samples were capped with aluminum foil and incubated in the dark for 2 hr. After incubation, samples were centrifuged for 10 min at 10,000 *g*. The pellet was maintained at 0 C for subsequent extraction. Two milliliters of water was added to the supernatant solution and then agitated with a Vortex mixer. As much as possible of the bottom lipid containing layer was collected with a Pasteur pipet. An additional lipid extraction of the pellet was facilitated by adding liquid N_2 and grinding the pellet with a glass rod. Three milliliters of chloroform:methanol (1:2) plus 0.8 ml H_2O was added and the sample agitated with a Vortex mixer. As much as possible of the bottom lipid containing layer was collected with a Pasteur pipet. The lipid extractions in chloroform from the supernatant solution and reextraction of the pellet were combined and chloroform evaporated under N_2. One milliliter of chloroform was added, and samples were maintained at 0 C for later lipid purification.

Lipids were purified by thin-layer chromatography. The samples were concentrated by evaporating the chloroform under N_2 and then adding drops of chloroform. All of the concentrated samples were applied to activated, 0.25-mm thick silica gel G thin layers on 20- × 20-cm glass plates. The lipids were separated by an acetone:acetic acid:water (100:1:1) solvent system. The solvent was allowed to migrate to within 3 cm of the top of the plate. The polar lipid region (origin) and galactolipid (determined from standards) regions were scraped into tubes for methylation. Fatty acids were released and methylated by adding 2 ml of 5% (v/v) acetylchloride in dry methanol and heating at 80 C for 1 hr. The methylation reaction was stopped by adding 1 ml of H_2O. Fatty acid methyl esters were collected by adding 2 ml of petroleum ether, agitating it, and removing all of the top layer with a Pasteur pipet.

Fatty acid methyl esters were quantitated by gas chromatography by use of a 6-ft × 1/4-inch (i.d.) glass column packed with 10% EGSS-X chromosorb WHP (Supelco). The column temperature was maintained at 185 C. A Perkin Elmer 3920B gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard 3390A electronic integrator was employed.

Enriched plasma membrane. A modification of the procedure of Whitman and Travis (21) was used to obtain an enriched plasma membrane fraction. They reported (1,20) that the purity of this plasma membrane fraction was greater than 75%. After 2, 4, or 6 hr of incubation, the cotyledons from 10 cucumber plants per treatment were collected and placed in a mortar. Liquid N_2 was added, and cotyledons were ground to a fine powder with a pestle. The powdered tissue was then extracted with 7 ml of 0.01 M Tris buffer containing 0.25 M sucrose, pH 7.8. The brei was filtered through four layers of cheesecloth and centrifuged for 5 min at 500 *g*. The pellet was discarded, and the supernatant solution was centrifuged for 10 min at 10,000 *g*. The enriched plasma membrane fraction was collected by centrifuging the supernatant solution for 35 min at 80,000 *g*. The pellet containing the enriched plasma membrane fraction was resuspended in 0.5 ml of the Tris buffer/sucrose solution. Two milliliters of isopropyl alcohol was added to samples, and lipids were extracted as described above.

Procedures for purification of lipids, methylation, and quantitation of fatty acid methyl esters were the same as those performed for the total lipid extract with the exception that only the polar lipids were collected.

Analysis of changes in fatty acid unsaturation. The methyl esters of the following fatty acids were quantitated (for all samples): myristic acid (14:0, first number is number of carbons, second number is number of double bonds); palmitic acid (16:0); palmitoleic acid (16:1); stearic acid (18:0); oleic acid (18:1); linoleic acid (18:2); and linolenic acid (18:3). Levels of fatty acid unsaturation were calculated as the total percent unsaturation (sum of the percents of the unsaturated fatty acids: %16:1 + %18:1 + %18:2 + %18:3).

We looked for correlation between decreases in fatty acid unsaturation and onset of tissue collapse observed during bacteria-induced hypersensitive reaction and paraquat treatment. Decreases in fatty acid unsaturation were identified by comparing the level of unsaturation (total percent unsaturation) calculated for appropriate control treatments with *P. s. pisi* and paraquat treatments.

RESULTS

The visible symptom of the bacteria-induced hypersensitive reaction, tissue collapse, was observable by 6 hr after infiltration of cucumber cotyledons with *P. s. pisi*. By 12 hr, greater than 80% of the tissue had collapsed. Infiltration of cotyledons with paraquat (10 $\mu g\ ml^{-1}$) also induced the onset of visible tissue collapse by 6 hr and greater than 80% of the tissue had collapsed by 12 hr. No tissue collapse was observed in cotyledons infiltrated with water or heat-killed *P. s. pisi* through 14 hr.

In control treatments (infiltration with water or heat-killed *P. s. pisi*), there were only minor changes in fatty acid composition of lipids from the total lipid extract and the enriched plasma membrane fraction. These data are not presented here but are detailed elsewhere (9). Representative fatty acid profiles of a control treatment (4 hr after infiltration with water) are presented (Table 1).

In control-treated cotyledons, the fatty acid composition of galactolipids and polar lipids of the total lipid extract were quite different (Table 1). Linolenic acid (18:3) made up greater than 80% of the total galactolipid fatty acids, and each of the other fatty acids was less than 6%. Whereas, in the polar lipids, palmitic acid (16:0), linoleic acid (18:2), and linolenic acid (18:3) each exceeded 20% of the total fatty acids. Fatty acid composition of polar lipids from the enriched plasma membrane fraction and the total lipid extract were similar; although myristic acid (14:0) and palmitic acid (16:0) were approximately 5% higher and linolenic acid (18:3) was approximately 9% lower in the plasma membrane enriched fraction (Table 1).

TABLE 1. Fatty acid composition[a] of cucumber cotyledons 4 hr after infiltration with H_2O

Fatty[b] acid	Total lipid extract		Enriched plasma membrane extract
	Galacto lipids	Polar lipids	Polar lipids
14:0	2.4 ± 0.6	0.8 ± 0.2	6.0 ± 1.0
16:0	5.9 ± 0.1	28.5 ± 0.8	33.9 ± 1.4
16:1	0.7 ± 0.1	6.5 ± 0.4	4.5 ± 0.4
18:0	1.7 ± 0.1	6.3 ± 0.2	6.8 ± 0.1
18:1	1.5 ± 0.1	4.8 ± 0.3	4.7 ± 0.2
18:2	3.1 ± 0.1	20.2 ± 0.4	19.1 ± 0.1
18:3	84.7 ± 0.5	34.0 ± 0.5	25.1 ± 0.8

[a]Composition = mean ± SE percent of total fatty acids from four experiments with two replications per experiment.
[b]First number indicates number of carbons, second number indicates number of double bonds.

TABLE 2. Total percent unsaturation[a] of fatty acids from a total lipid extract from infiltrated cucumber cotyledons

	Total % unsaturation			
	Galacto lipids		Polar lipids	
	4 hr	6 hr	4 hr	6 hr
Control[b]	88.7 ± 1.0	87.6 ± 0.7	64.8 ± 0.7	65.5 ± 1.1
Pseudomonas syringae pv. *pisi*	89.2 ± 0.8	88.6 ± 1.2	65.6 ± 0.6	65.6 ± 0.8
% Change[c]	+0.5	+1.0	+0.8	+0.1
Control[b]	90.0 ± 0.9	88.5 ± 0.7	65.5 ± 0.7	65.2 ± 0.5
Paraquat	89.5 ± 1.1	86.1 ± 0.9*[d]	64.8 ± 0.9	63.8 ± 0.6
% Change[c]	−0.5	−2.4	−0.7	−1.4

[a]Mean ± SE of the sum of percents of fatty acids with double bonds from a minimum of three experiments with two replications per treatment.
[b]Control treatment for live *P. s. pisi* was heat-killed *P. s. pisi*. Control treatment for paraquat was water.
[c]% change = difference of total percent unsaturation of control treatments and *P.s. pisi* and paraquat treatments.
[d]An asterisk indicates that the means of *P. s. pisi* or paraquat treatments were significantly different ($p = 0.05$ using a Student's t test) than their respective controls.

In cotyledons infiltrated with live *P. s. pisi*, fatty acid unsaturation was not significantly ($p = 0.05$) different from the control treatment (heat-killed *P. s. pisi*) through 6 hr of incubation, in either the galactolipid or polar lipid fractions of the total lipid extract (Table 2). In cotyledons treated with *P. s. pisi*, polar-lipid fatty acid unsaturation was significantly ($p = 0.05$) lower after 4 hr of incubation from the enriched plasma membrane fraction (Table 3).

In paraquat-treated cotyledons, fatty acid unsaturation was significantly ($p = 0.05$) lower than the control, after 6 hr of incubation, in the galactolipid fraction of the total lipid extract (Table 2). Fatty acid unsaturation was significantly ($p = 0.05$) lower than the control in paraquat-treated cotyledons after 4 and 6 hr of incubation in the polar lipid from the enriched plasma membrane fraction (Table 3).

DISCUSSION

Analysis of fatty acids from galactolipids (specific plastid membrane lipids) indicated that significant decreases in unsaturation of the plastid membranes occurred in paraquat-treated cotyledons. This decreased unsaturation was concurrent with the onset of increased tissue collapse. In tissue undergoing the bacteria-induced hypersensitive reaction there were no changes in unsaturation (through 6 hr of incubation) of the galactolipid fatty acids.

No changes in unsaturation were indicated in polar lipid fatty acids from the total lipid extract in either paraquat or live bacterial treatments. To determine if there were changes in fatty acid unsaturation of the plasma membrane that were masked by a greater quantity of lipids from unaffected membranes, we obtained an enriched plasma membrane fraction. Fatty acid analysis of this fraction indicated that decreased unsaturation, which was concurrent with the onset of tissue collapse, occurred in both paraquat and live bacterial treatments.

TABLE 3. Total percent unsaturation[a] of fatty acids purified from an enriched plasma membrane fraction from infiltrated cucumber cotyledons

	Total % unsaturation		
	2 hr	4 hr	6 hr
Control[b]	57.9 ± 1.1	51.0 ± 0.6	52.8 ± 1.1
Pseudomonas syringae pv. *pisi*	56.9 ± 0.9	43.5 ± 0.8*[d]	49.1 ± 1.8
% Change[c]	−1.0	−7.5	−3.7
Control[b]	56.6 ± 0.7	53.4 ± 0.9	56.6 ± 0.7
Paraquat	58.2 ± 1.4	48.8 ± 1.2*	52.9 ± 0.9*
% Change[c]	+1.6	−4.6	−3.7

[a]Mean ± SE of the sum of percents of fatty acids with double bonds from a minimum of three experiments with two replications per treatment were performed.
[b]Control treatment for live *P. s. pisi* was heat-killed *P. s. pisi*. Control treatment for paraquat was water.
[c]% change = difference of total percent unsaturation of control treatments and *P. s. pisi* and paraquat treatments.
[d]An asterisk indicates that the means of *P. s. pisi* or paraquat treatments were significantly different ($p = 0.05$ using a Student's t test) than their respective controls.

The decrease in fatty acid unsaturation was transient in cotyledons exposed to live bacteria but not in cotyledons exposed to paraquat. This transient decrease in fatty acid unsaturation agrees with a report (13) that both O_2^- production and lipid peroxidation increased, transiently, during the onset of a bacteria-induced hypersensitive reaction. Thus, in the bacteria-induced hypersensitive reaction electrolyte leakage and the resultant tissue collapse may not be affected directly via a damaged plasma membrane. Rather, the onset of electrolyte leakage and tissue collapse may be triggered by transient alteration of the plasma membrane.

It has been reported (18) that fatty acid hydroperoxide molecules (formed during lipid peroxidation) can transport Ca^{2+} through cellular membranes. Because Ca^{2+} concentration is much higher in the cell wall than in the cytoplasm, the presence of fatty acid hydroperoxide molecules in the plasma membrane could allow an influx of Ca^{2+} into the cytoplasm. Increased cytoplasmic Ca^{2+} concentration could then trigger efflux of electrolytes via a Ca^{2+}/calmodulin-mediated signal transduction pathway. This is supported by experiments of M. Atkinson (unpublished) that a rapid influx of Ca^{2+} into plant cells precedes tissue collapse in a bacteria-induced hypersensitive reaction in tobacco tissue. In addition, this cellular uptake of Ca^{2+} is required for subsequent electrolyte leakage and tissue collapse.

The results support involvement of O_2^--initiated lipid peroxidation in membrane alteration, which produces a resultant tissue collapse in tissue treated with paraquat or a hypersensitive reaction-inducing bacterium. In paraquat-treated tissue, decreased levels of fatty acid unsaturation were observed in lipids from both the plastid and plasma membrane. Light microscopy indicated that the parenchyma cells in the cucumber cotyledons contained numerous chloroplasts, which are in intimate contact with the plasma membrane. Therefore, in paraquat-treated tissue, O_2^--initiated lipid peroxidation could begin, as proposed (2,6), in the chloroplast and be propagated to the plasma membrane.

During the hypersensitive reaction in cucumber cotyledons induced by *P. s. pisi*, lower fatty acid unsaturation was observed only in lipids from the enriched plasma membrane fraction. Because no decrease in fatty acid unsaturation was observed in plastid membrane lipids, O_2^--initiation of lipid peroxidation at the plasma membrane is suggested. This is supported by reports of O_2^- generation by plasma membrane vesicles and the surface of protoplasts from potato, when exposed to an elicitor from an incompatible race of the fungal pathogen, *Phytophthora infestans* (4,5).

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