

Lipid changes in tobacco cell suspensions following treatment with cellulase elicitor

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Tobacco (*Nicotiana tabacum*) KY14 cell cultures have previously been reported to produce capsidiol and other stress metabolites when treated with fungal elicitor or cellulase. Using a new high performance liquid chromatographic technique, we have measured the changes in sesquiterpene phytoalexins and membrane lipid classes that occur upon elicitation of tobacco cell cultures with cellulase. Measurable levels of capsidiol and debneyol were found in the tobacco cells and in the culture medium after 8 h of elicitor treatment, with levels continuing to increase for up to 24 h. For the duration of the experiments, the levels of most of the galactolipids and phospholipids were found to decrease in elicited cells and increase in control cells. The most striking change was a rapid decrease in the level of digalactosyldiacylglycerol in elicited cells, to less than 10% of the level in control cells. Among the sterol lipid classes, the most notable changes occurred in the levels of sterol esters and acylated sterol glycosides, which increased significantly in elicited cells within 2 to 4 h after addition of cellulase, but remained unchanged in control cells. Free sterols and sterol glycosides declined slightly, while free fatty acids dropped to low levels 24 h after treatment of cells with cellulase. The present results and those of previous studies indicate that esterification of phytosterols may be a widespread response to environmental or chemical stress.

Key words – Acylated sterol glycoside, capsidiol, cellulase, elicitor, esterification, *Nicotiana tabacum*, phytoalexin, phytosterol, sterol, sterol ester, sterol glycoside, tobacco.

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Introduction

Tobacco cell suspensions produce sesquiterpene phytoalexins (capsidiol, phytuberin, phytuberol and debneyol) when infected with pathogens such as *Pseudomonas lachrymans* or *Phytophthora parasitica* or when treated with cellulase or fungal elicitor (Chappell et al. 1987, Tanaka and Fujimori 1985, Threlfall and Whitehead 1988). It is hypothesized that sesquiterpene biosynthesis occurs as 15-carbon farnesyl pyrophosphate units are diverted from the sterol biosynthetic pathway into the sesquiterpene pathway (Brindle et al. 1988, Threlfall and Whitehead 1988, 1990). Results of experiments using radiolabelled precursors seem to support this the-

ory. In fungal elicitor-treated tobacco cells, Chappell et al. (1989) and Vogeli and Chappell (1988) found a net reduction of incorporation of radiolabel from ^{14}C acetate and ^3H mevalonate into the free sterols, isolated by precipitation with digitonin.

In addition to free sterols (St), the conjugated sterol derivatives normally found in plants include acylated sterol glycosides (ASG), sterol (fatty acid) esters (StE) and sterol glycosides (SG). The effects of environmental and chemical stresses on some of these plant sterol conjugates have been studied (Kesselmeier et al. 1987, Lynch and Steponkus 1987, Norcia et al. 1964, Tomlinson and Rich 1973, Whitaker et al. 1990, Yates et al. 1990). The results of the studies in which total free

sterols were measured support those with tobacco cell suspensions (Chappell et al. 1989, Vogeli and Chappell 1988): the levels of free sterols declined. However, levels of some of the sterol conjugates increased in response to treatments (Kesselmeier et al. 1987, Norcia et al. 1964, Tomlinson and Rich 1973, Whitaker et al. 1990). It would appear that reshuffling of the basic sterol unit is occurring from a "normal" configuration to a "stress" profile of sterol derivatives.

Besides these reported changes in sterol derivatization in plants, a decrease in glycerolipids, most notably phospholipids, has also been observed in response to environmental stress (Brown et al. 1991).

Using a new high performance liquid chromatographic (HPLC) technique (Moreau et al. 1990), which allows the measurement of the four sterol-containing lipid classes, as well as free fatty acids and individual glycerolipids, we undertook to investigate the effect of treatment of tobacco cell suspensions with the phytoalexin elicitor, cellulase, on the lipid classes in those cells.

Abbreviations – ASG, acylated sterol glycosides; DGDG, digalactosyldiacylglycerol; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; MGDG, monogalactosyldiacylglycerol; SG, sterol glycosides; StE, sterol esters; St, free sterols.

Materials and methods

Growth and treatment of cells

Cell suspension cultures of *Nicotiana tabacum* KY 14, were obtained from Dr Joseph Chappell, Univ. of Kentucky, and were maintained on a modified Murashige-Skoog medium as previously described (Chappell et al. 1987). Cells were grown at 25°C and subcultured every 7 days. Three-day-old cells were treated with fungal glucan elicitor from *Phytophthora parasitica* (Vogeli and Chappell 1988) (5 µg glucose equivalents ml⁻¹ cell suspension), cellulase (0.1 µg ml⁻¹ cell suspension) from *Trichoderma viride*, ethephon (1 mM), or CuCl₂ (0.5 mM).

Extraction and chromatography

Cells were separated from the culture filtrate by gentle vacuum filtration through nylon cloth (5 µm mesh) in a Buchner funnel. After the fresh weight of the cells was measured, chloroform and methanol were added, the cells were homogenized with a Polytron homogenizer (Brinkman Co., Westbury, NY, USA), and the lipids were extracted according to the method of Bligh and Dyer (1959). Separate cell samples were dried by lyophilization and weighed. The culture filtrate (7–10 ml) was extracted two times with 10 ml diethyl ether. The solvents from both the cellular and media extracts were

evaporated under a stream of nitrogen and the mass of each was measured. The residue (containing a mixture of lipids and phytoalexins) was redissolved in hexane/isopropanol (90:10, v/v) and the total lipid mixture was used directly for HPLC analyses. HPLC-flame ionization detector analyses of lipid classes were performed as previously described (Moreau et al. 1990). Authentic standards of each lipid class were used to identify retention times and to correlate peak area with lipid mass. Sesquiterpene phytoalexin analyses were performed with a column of 5 µm Spherisorb CN (3 × 100 mm) (Chrompack Inc., Raritan, NJ, USA) under isocratic conditions with hexane/isopropanol (97:3, v/v) as previously described (Moreau et al. 1992). The identities of these phytoalexins were confirmed by GC-MS (Moreau et al. 1992). The retention times of the phytoalexins were 3.3, 3.9 and 11.9 min for phytuberol, debneyol and capsidiol, respectively. Each experiment was performed at least 3 times with two independent replicates for each treatment.

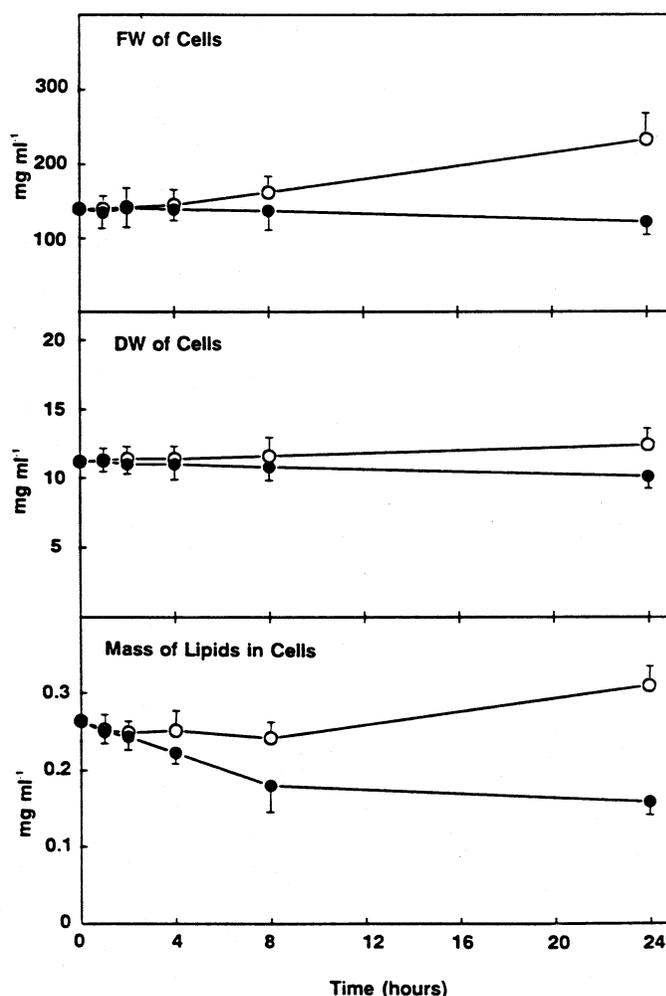


Fig. 1. Changes in FW and DW of cells and in total lipid mass following treatment of tobacco cell suspensions by cellulase. Cells were treated as described in Materials and methods. Lipids were extracted with chloroform-methanol (Bligh and Dyer 1959), dried under a stream of nitrogen and weighed. Data presented are the mean of 4 experiments \pm SE. Control, ○; elicitor-treated cells, ●.

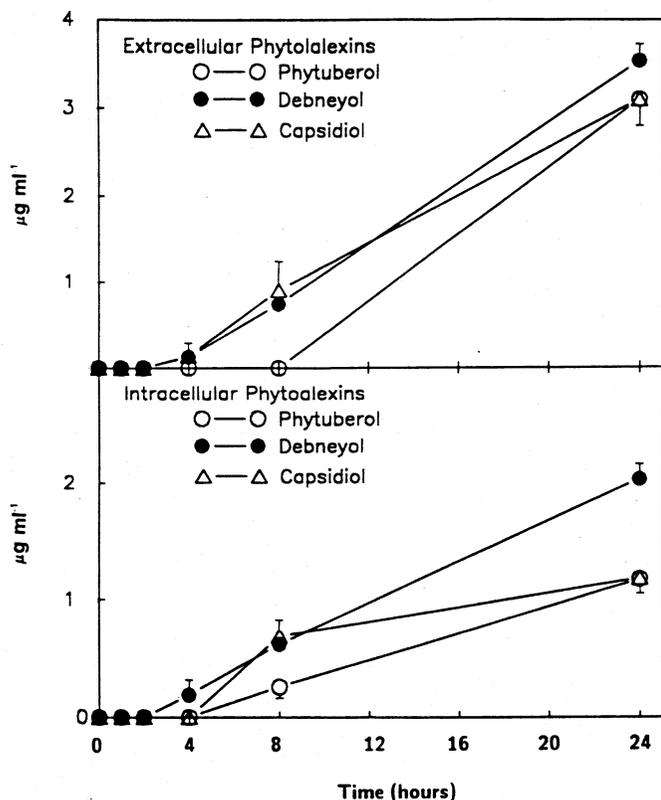


Fig. 2. Intracellular and extracellular levels of capsidiol and other sesquiterpene phytoalexins during the elicitation of tobacco cell suspensions by cellulase. The cells were homogenized in chloroform-methanol (Bligh and Dyer 1959) to obtain intracellularly located phytoalexins. The medium was extracted two times with one volume of diethyl ether to determine the extracellular levels of phytoalexins. Data presented are the mean of 4 experiments \pm SE.

Results

During a 24 h experiment, there were small increases in the dry and wet weights of the cells serving as controls (Fig. 1). Treatment of the cells with cellulase caused small decreases in the dry and wet weights. The mass of the total lipids increased slightly (about 10%) in control cells but decreased substantially (about 40%) in elicitor-treated cells.

Cellulase treatment induced the accumulation of phytuberol, debneyol, and capsidiol in the cells and in the culture filtrates (Fig. 2). The extracellular accumulation of phytuberol lagged behind that of capsidiol and debneyol. Intracellular levels ranged from 1/3 to 2/3 of the extracellular levels. No phytoalexins were found in the untreated control cells or their culture filtrates (data not shown).

Cellulase treatment of the tobacco cell suspensions induced changes in the levels of each of the steryl lipid classes (Fig. 3), while their levels were essentially unchanged in control cells. In elicited cells, the levels of StE and ASG were slightly elevated in the 1 h sample and were increased about 2-fold by 8 h. The levels of free sterols dropped about 10% by 2 h and remained at

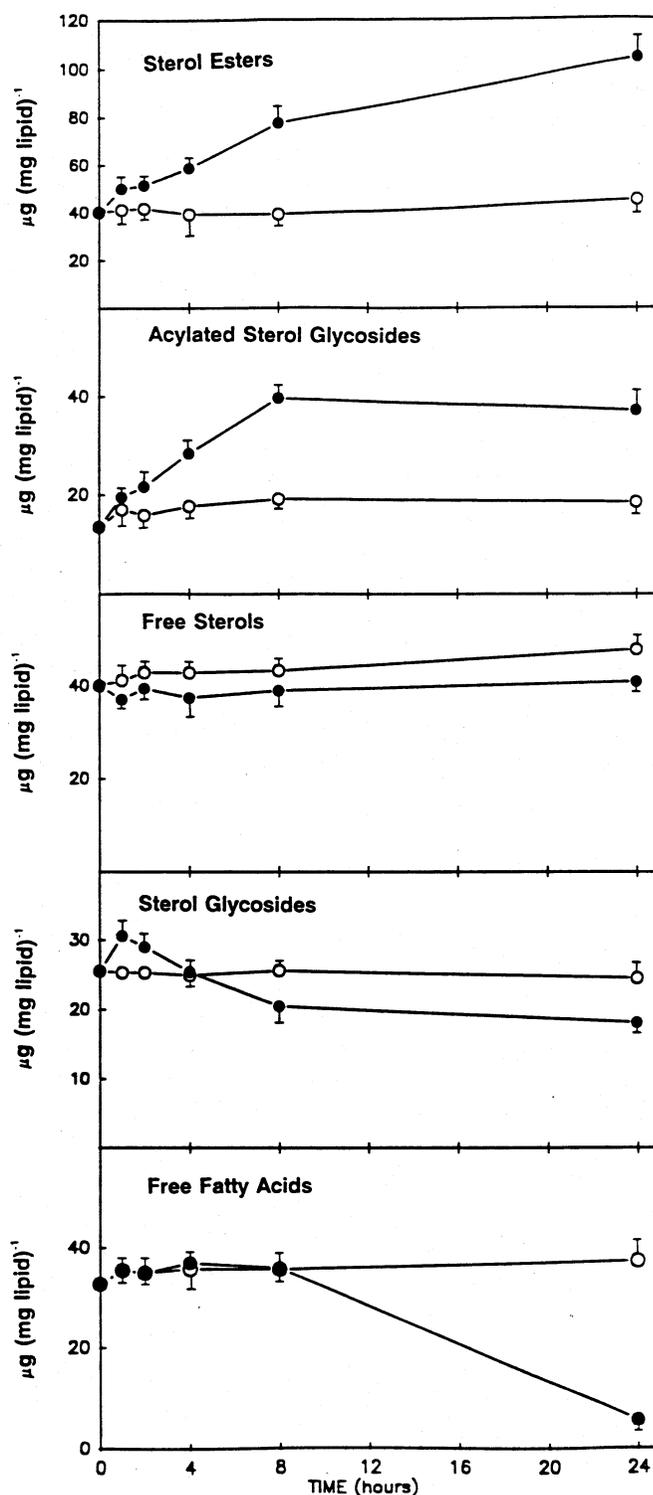


Fig. 3. Changes in the levels of intracellular steryl lipid classes and free fatty acids during elicitation of tobacco cell suspension by cellulase. Data presented are the mean of 3 experiments \pm SE. Control. \circ ; elicitor-treated cells. \bullet .

this level for the duration of the experiment. The levels of SG showed a transient increase of about 20% at 1–2 h and then decreased to below control values at 8 to 24 h. There was no detectable effect of cellulase on the levels of free fatty acids for up to 8 h, but at 24 h after cellulase treatment their levels were decreased by 90%. When

Tab. 1. Changes in the levels of steryl lipids during elicitation of tobacco suspension cells by cellulase. Number in parantheses is the mol % of total sterols. The μmol values were calculated from HPLC analyses using the average molecular weights of StE = 670; ASG = 830; SG = 570; and St = 410.

	Sterol per culture flask (μmol)				
	StE	ASG	St	SG	Total
0 h Control	0.158 (27)	0.044 (8)	0.259 (44)	0.121 (21)	0.582 (100)
24 h Control	0.207 (26)	0.070 (9)	0.366 (47)	0.138 (18)	0.781 (+33)
24 h Cellulase	0.244 (47)	0.071 (14)	0.147 (29)	0.051 (10)	0.513 (-12)

expressed on a molar basis (Tab. 1), it is obvious that a substantial increase in total sterol occurred in control cultures over 24 h, with very little change in the proportions of the four steryl lipid classes, whereas in cultures treated with cellulase for 24 h, there was a small net loss of total sterol and large increases in the proportions of StE and ASG at the apparent expense of St and SG.

Examination of the membrane glycerolipids revealed that cellulase treatment induced a substantial reduction in the levels of galactolipids and a small reduction in the levels of phospholipids (Fig. 4). The levels of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) decreased by about 40% and 90%, respectively, during the 24 h following cellulase treatment.

Other potential elicitors of sesquiterpene phytoalexins were tested for their effects on the steryl lipid classes and glycerolipids (Fig. 5). Although treatment of the cells with fungal glucan elicitor elicited the production of phytoalexins, it did not cause significant changes in the levels of StE or ASG. Ethephon, an ethylene-generating growth regulator and inducer of senescence in many plant tissues, did not elicit phytoalexins or have any effect on the lipids. Copper, an effective elicitor of isoflavanoid phytoalexins in legumes, did not elicit sesquiterpene phytoalexins in tobacco cells. Like cellulase, however, CuCl_2 induced a large increase in the level of ASG (about a 3-fold stimulation) and a decrease in the levels of St, SG, DGDG and every other lipid class that was analyzed (data not shown). Unlike cellulase treatment, which produced an increase in StE, treatment of the cells with CuCl_2 caused a decrease in the levels of StE (Fig. 5). For each lipid, the effect of CuCl_2 treatment was greater in magnitude than the effect of cellulase treatment.

Discussion

Many treatments induce changes in the sterol-containing lipid classes of plant tissues. During the treatment of oat leaves with cell wall degrading enzymes, Kesselmeier et al. (1987) observed that the levels of SG and ASG increased, and the level of St declined. Similarly, Tomlinson and Rich (1973) and Whitaker et al. (1990) reported that treatment of leaves with ozone caused an increase in the levels of SG and ASG and a decrease in the level of St. Lynch and Steponkus (1987) reported

that cold acclimation of rye seedlings decreased the levels of ASG and SG in the plasma membranes. Norcia et al. (1964) reported that treatment of pea plants with gibberellic acid caused a 2- to 3-fold increase in the level of StE in roots and shoots. Also, Yates et al. (1990) reported that treatment of celery cell suspension cells with allylamine terbinafine (an antifungal agent) decreased the levels of StE and St.

In the present study, we noted marked increases in the levels of both StE and ASG during elicitation with cellulase, starting at 1 h and continuing for up to 24 h. The level of SG increased only transiently from 1-2 h and then dropped to a level below that of the controls. The level of St was consistently lower in elicitor-treated cells. Most of the previous studies with the exception of those of Norcia et al. (1964) and Yates et al. (1990) did not measure the levels of StE; this is the first report of the induction of sterol esterification by a chemical elicitor of phytoalexins.

In summary, an increase in ASG and a decrease in St accompanies the treatment of plant tissues with a wide variety of chemicals, including ozone (Tomlinson and Rich 1973, Whitaker and Rowland 1990), cellulase (Fig. 3) and other cell wall degrading enzymes (Kesselmeier et al. 1987), gibberellic acid (Norcia et al. 1964), CuCl_2 (Fig. 5) and allylamine terbinafine (Yates et al. 1990). Changes in the other sterol conjugates varied depending upon the particular chemical treatment. StE decreased in response to allylamine terbinafine (an antifungal agent) (Yates et al. 1990) and CuCl_2 (Fig. 5), but increased in response to cellulase (Figs 3 and 5) and gibberellic acid (Norcia et al. 1964). SG increased within several hours after treatment with ozone (Tomlinson and Rich 1973, Whitaker et al. 1990) or cell wall degrading enzymes (Kesselmeier et al. 1987). In contrast, cellulase treatment induced an initial increase in SG (Fig. 3), which was transient and ended as a decrease relative to the control at 24 h after treatment.

Total intracellular sterols remained approximately the same in control (378 μg per flask) and in elicited cells (315 μg per flask). The lack of incorporation of radiolabel into St and inhibition of squalene synthase indicate that synthesis of phytosterols stops upon elicitation of sesquiterpene synthesis (Chappell et al. 1989, Vogeli and Chappell 1988). Thus, it appears that the changes in levels of free and conjugated sterols result from increased fatty acyl esterification of St and SG and per-

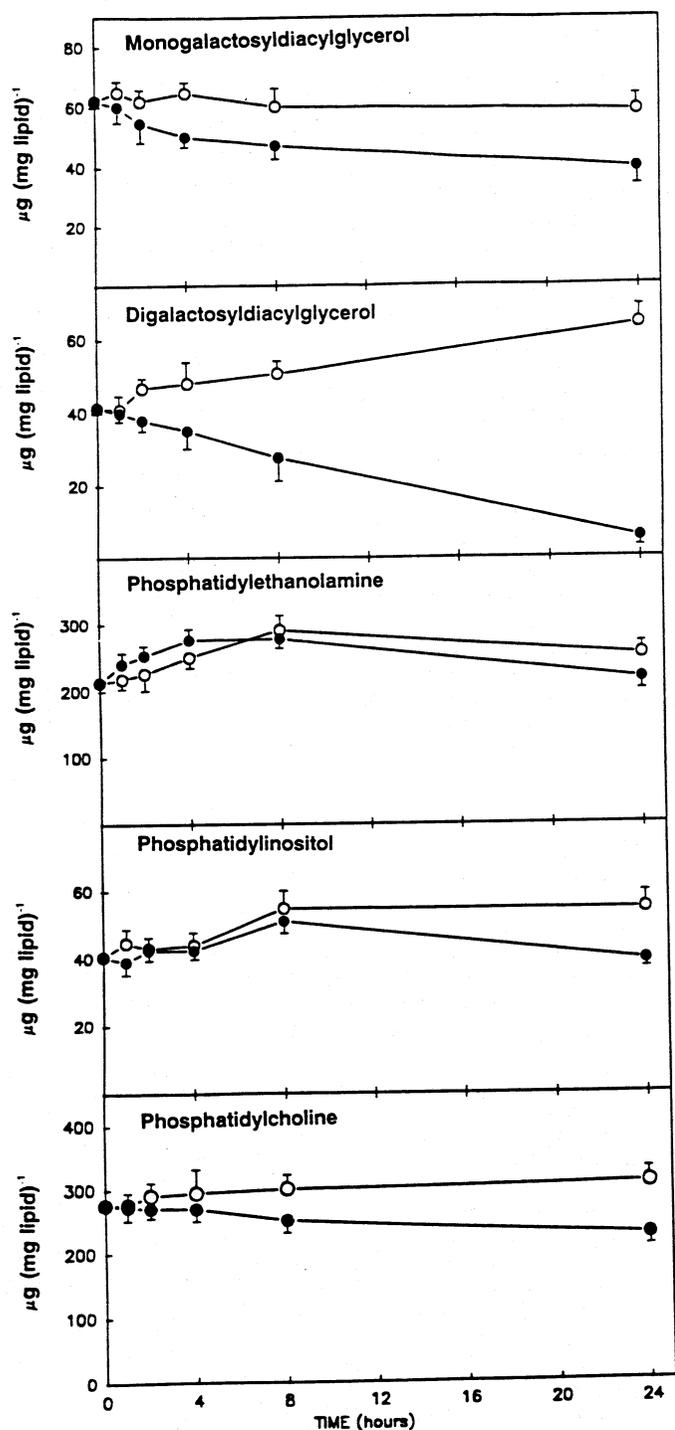


Fig. 4. Changes in the levels of intracellular galactolipid and phospholipid classes during the elicitation of tobacco suspension cells by cellulase. Data presented are the mean of 3 experiments \pm SE. Control, \circ ; elicitor-treated cells, \bullet .

haps some deglycosylation followed by esterification of preexisting SG. The probable pathway is diagrammed in Fig. 6. Of these three enzymatic steps, the only one which has been well characterized is the UDP-sterol glucosyl transferase (Ury et al. 1989).

Several questions are raised by these results: are the same sterols present and equally interchangeable in the conjugation process? What are the sources of fatty acids

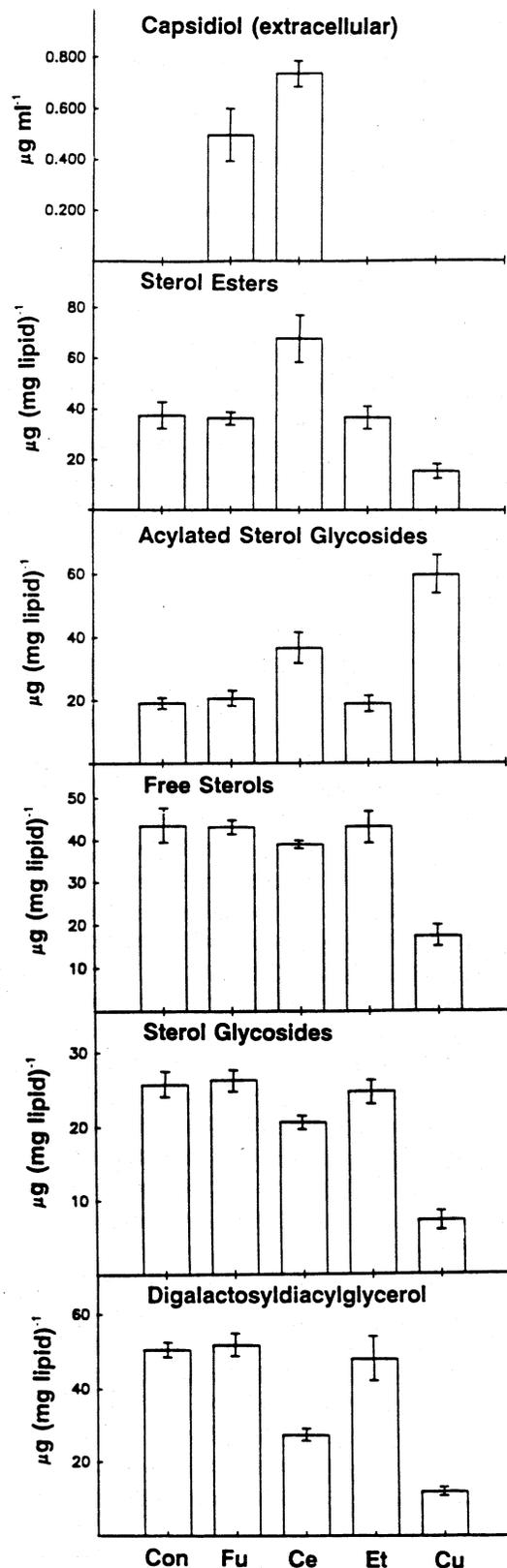


Fig. 5. The effects of various potential elicitors on the levels of extracellular phytoalexins and levels of intracellular sterol lipid classes and digalactosyldiacylglycerol. Cell suspensions were treated for 6 h, the cells and media were separated by filtration, and each was extracted as described in Materials and methods. The treatments consisted of a water control (Con), fungal glucan elicitor (Fu) ($5 \mu\text{g}$ glucose equivalents ml^{-1} cell suspension), cellulase (Ce) ($0.1 \mu\text{g ml}^{-1}$ cell suspension) from *Trichoderma viride*, ethephon (Et) (1 mM), or CuCl_2 (Cu) (0.5 mM).

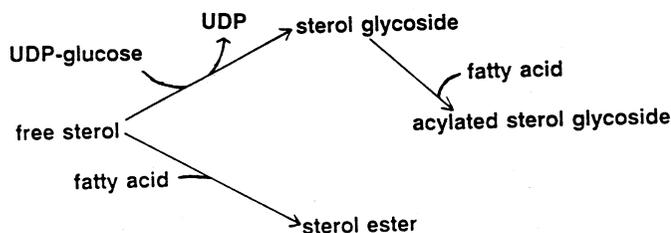


Fig. 6. Proposed pathway of sterol glycosylation and fatty acylation in plant cells.

and hexoses for derivatization? In future studies we intend to determine which phytosterols and which fatty acid combinations are accumulated during this stress-induced process. Whitaker et al. (1990) reported that stigmasterol and sitosterol were the major sterols in snapbean leaves and that ozone treatment increased the ratio of stigmasterol to sitosterol in St and SG, but had no effect on the ratio in ASG.

The cellulase-induced reduction in the levels of all glycerolipids suggests that fatty acids may be donated from all glycerolipids for the esterification of St and SG. In addition, we believe that the more rapid decline in DGDG deserves further study. If one considers these observations from the perspective of cellular compartments, it is notable that St, ASG and SG (of which only ASG increases while the others decrease) are thought to occur mainly in the plasma membranes (Goat et al. 1991). Unlike the other sterol lipid classes, StE are not thought to partition into the bilayer portion of membranes, but rather are sequestered in lipid droplets (similar to triacylglycerols) (Kwong et al. 1971). The possible effects of these changes in the proportions of plasma membrane sterols on the physical structure and permeability of that membrane might be an important aspect of adaptation to environmental and chemical stresses. Interestingly, MGDG and DGDG are localized mainly if not exclusively in the plastids (Douce et al. 1990), which are one of the proposed sites of synthesis of isoprenoids such as sesquiterpene phytoalexins (Kleinig 1989).

Several labs have reported that elicitation of phytoalexins induced the biosynthetic enzymes for sesquiterpene phytoalexin biosynthesis and reduced the level of enzymes for sterol biosynthesis in cell suspension cultures of tobacco (Chappell et al. 1989, Threlfall and Whitehead 1988, Vogeli and Chappell 1988) and potato (Brindle et al. 1988). In two of these studies (Chappell et al. 1989, Vogeli and Chappell 1988), the incorporation of ^{14}C -acetate or ^3H -mevalonate into St (measured by precipitating St with digitonin) was completely inhibited within 6–8 h after elicitor treatment. Other sterol lipid classes (StE, ASG and SG) were not quantified. In light of our current results, further work is needed to help us understand the possible effects of other chemical and environmental stresses on the levels of sterol lipid classes.

The levels of other types of plant lipids (not detec-

table by our HPLC analysis of lipid classes) have also been reported to respond to various types of stimuli. Kurosaki et al. (1987) reported that when carrot suspension cells were elicited with pectic fragments, there was a rapid breakdown in phosphatidylinositol. Chen and Boss (1990) reported that treatment of carrot cells with driselase or hemicellulase caused a rapid increase in the levels of phosphatidylinositol diphosphate. Rogers et al. (1988) reported that treatment of bean suspension cells with fungal cell wall elicitor caused the induction of phytoalexins and peroxidation of cellular lipids. It is not known whether there are any interrelationships between changes in sterol lipid classes, phosphoinositides or peroxidized lipids. Rather than focusing on changes in one class of lipids, further studies need to determine how various types of physical and chemical stresses affect all of the major types of membrane lipids.

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