Inhibition of Elicitor-Induced Phytoalexin Formation in Cotton and Soybean Cells by Citrate

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ABSTRACT

Addition of an elicitor preparation from Verticillium dahliae to soybean or cotton cell suspension cultures induces the formation of the phytoalexins, glycelollin or sesquiterpene aldehydes, respectively. Recent work (PS Low, PF Heinste 1986 Arch Biochem Biophys 249: 472–479) has shown that the induction of phytoalexin biosynthesis in these cells is preceded by rapid changes in the plant cell membrane which can be conveniently monitored by membrane associated fluorescent probes. Using this elicitation assay, we have found that citrate, a common metabolite of higher plants, acts as a potent inhibitor of elicitation when added prior to treatment with elicitor. The citrate concentration required to obtain a 50% inhibition of the elicitor-induced fluorescence transition in cultured cotton cells was found to be about 2 millimolar, while the concentration of citrate observed to inhibit elicitor-induced sesquiterpene aldehyde formation in the same cell suspensions was also 2 millimolar. Curiously, in the presence of elicitor, citrate at less than 1D10c concentrations increased cell mass accumulation significantly above control incubations without elicitor. A similar inhibition of glycelollin formation with an increase in cell mass accumulation was also observed upon addition of 1 to 5 millimolar citrate to soybean cell suspension cultures. The physiological significance of the inhibition by citrate of phytoalexin formation in plant cell suspensions was supported by the observation that a similar inhibition of sesquiterpene aldehyde formation occurs in cotton plantlets elicited by cold shock or F. dahliae stress. The specificity of citrate as an inhibitor of phytoalexin formation was demonstrated by data showing that other di- and tricarboxylic-hydroxy acids did not inhibit, with the exception of malate which inhibited phytoalexin formation in soybean cells with roughly half the potency of citrate. These experiments not only demonstrate that citrate can act as a specific inhibitor of elicitation, but they further confirm the validity of monitoring elicitation and its modulation with fluorescent probes.

To prevent successful invasion by phytopathogenic fungi, plants have developed elaborate defense mechanisms which include the formation of potentially toxic secondary metabolites called phytoalexins (1, 3, 18). The induction of phytoalexin formation is thought to be initiated by the interaction of the fungal conidia with the plant cell. On a molecular level this interaction requires a fungal elicitor molecule which is probably recognized by a receptor in the plant cell wall or membrane. Ultimately, the interaction leads to the activation of genes of the host plant which code for enzymes involved in the biosynthesis of phytoalexins (8, 19, 23). Once formed, the phytoalexins appear to be toxic to the invading pathogen and thereby retard the colonization of the host plant.

As with most other complex biochemical pathways, the defense response in plants is probably not free of biochemical regulation. Thus, there is a growing body of evidence to suggest that elicitation can be both enhanced and suppressed by endogenous and exogenous modulators. For example, xanthan (13) phaseolotoxin (14), and β-(1,3) and β-(1,6) glucans (mycolaminarins) (11, 20) have all been shown to be race specific suppressors of general defense reactions including phytoalexin biosynthesis (20). However, the molecular identities of the specific suppressors in most of these preparations have never been elucidated.

To probe the stimulus-response coupling associated with elicitation, an assay was developed (21) to monitor the initial events occurring in the plant cell upon interaction of the elicitor with the plant cell wall or membrane. This assay utilized membrane potential- and ion selective-fluorescent dyes to monitor the elicitor-induced changes in membrane permeability which occur within minutes of elicitor addition to the cultured plant cells. Since the fluorescence transitions were found to quantitatively correlate with phytoalexin formation, the fluorescence assays were concluded to represent an accurate means of monitoring elicitation.

Using the fluorescence assays described above, we decided to screen several plant metabolites for activity as suppressors or enhancers of elicitation. We report here that citrate, a common component of most plant cell extracts, is a potent inhibitor of elicitation.

MATERIALS AND METHODS

Chemicals. The fluorescent probes, 8-hydroxyppyrene-1,3,6-trisulfonic acid, trisodium salt (pyranine), and bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (oxonol VI) were purchased from Molecular Probes, Eugene, OR. Stock solutions of these dyes were prepared (1 mg/ml) in H2O (pyranine) or 50% ethanol (oxonol VI). Media ingredients were reagent grade chemicals and purchased from Sigma Chemical Co. and from Mallinckrodt, Inc. Glycelollin was a gift from Dr. David Kuhn, Purdue University. Cotton phytoalexins were previously isolated and characterized (2, 16). Sodium citrate solutions were adjusted to pH 6.0 and sterilized by membrane filtration (GS 0.2 μm, Millipore).

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Plant Cell Cultures. Cell suspension cultures of *Gossypium arboreum* L. Nanking were initiated and maintained as previously described (17). *Glycine max* Merr var Kent cultures were obtained from Dr. Mike Hasegawa, Purdue University and were grown in W-38 medium (15). Cultures were transferred to fresh medium every 7 d.

Phytoalexin Assays. Phytoalexins of cotton cell suspension cultures were determined as described (16). Briefly 1 cm³ of loosely packed cells of a 6-d-old culture was inoculated into 20 ml of medium. After 36 h of incubation, experiments were started with the addition of elicitor preparation and citrate, as indicated. The cells were then cultured as above for an additional 48 h, after which the column was reversed, and the filtrate was used to determine dry weight by the phloroglucinol procedure (4). The residue from the methanol extraction was used to determine dry weight (16).

The formation of glyceollin in the medium of soybean cell suspension cultures was measured according to the procedure of Ebel et al. (12). Three cm³ of loosely packed cells of a 6-d-old soybean culture were inoculated into 100 ml of fresh medium. After an incubation period of 30 h, experiments were started with the addition of elicitor preparation and citrate, as indicated. Cells were harvested after an additional 40 h incubation and glyceollin was determined (12) in the medium by extraction with 15 ml of ethyl acetate. After separation of the two layers the ethyl acetate was removed under a stream of N₂ and the residue taken up in 100 μl of ethanol (95%). The ethanol solution was quantitatively applied onto a 2 mm thick silica gel thin layer plate. The plate was developed with toluene-chloroform-acetone (40:25:35, v/v) and the spot corresponding to glyceollin, identified by comparison of its R₅ value with the migration of authentic glyceollin, extracted with 2 ml ethanol. Glyceollin was measured spectrophotometrically at 285 nm (E₂₈₅ = 10,300, ethanol) after purification on silica gel plates and detection at 254 nm (12). The cell mass residue was used for dry weight determination.

Formation of Phytoalexins in Cotton Seedlings. *G. arboreum* seeds (500) were warmed at 80°C water for 105 s and quickly chilled in ice water. Floating seeds were discarded and the remaining seeds were allowed to germinate in damp paper towels in the presence of 1 g benomyl/L H₂O. The seeds were maintained for 4 d at 28°C, and each cold shocked for 4 d at 10°C or injected with live *Verticillium dahliae* conidia. The germinated seedlings were severed at the transition zone and the rants were divided into groups of 2.2 g each and placed into 50 ml of 0.02 M potassium phosphate buffer (pH 6.5), containing 0.5% sucrose and 0.1% sodium acetate. Varying amounts of citrate were then added. The pH of the incubation solution was unaffected by the addition of citrate. The subsequent incubations were carried out in 22 × 12 × 7 cm glass pans. The seedlings were then covered with a plastic wrap. After 48 h at 28°C, the seedlings were removed and allowed to stand in 50 ml of absolute ethanol for 15 min. The ethanol was decanted and the seedlings homogenized 3 times in 10 ml of absolute ethanol. The combined ethanol extracts were filtered consecutively through Whatman No. 50 filter paper and a sintered glass, medium porosity filter. The filtrate was evaporated to dryness and taken up in HPLC grade methanol. The solution was filtered (1.2 μm) and diluted to 2 ml. Aliquots were injected onto a 5 m Spherisorb Hexyl column (25 cm × 4.6 mm) in a Hewlett Packard 1090 high pressure liquid chromatograph, equipped with a divide array detector. The column was maintained at 40°C. Elution was accomplished with a gradient of methanol and H₂O each containing 0.1% H₃PO₄. From 0 to 5 min a methanol/H₂O gradient of 20 to 60% was used and from 5 to 30 min a methanol/H₂O gradient of 60 to 100% was employed. The following retention times were recorded, in min: deoxy hemigossypol (dDHG), 10.4; hemigossypol (HG), 10.8; deoxy-6-methoxy hemigossypol (dMHG), 11.7; 6-methoxy hemigossypol (MHG), 12.1; gossypol (G), 16.7; 6-methoxy gossypol (MG), 17.2; 6,6’-dimethoxy gossypol (DMG), 17.7; G, MG, and DMG were not adequately resolved and are reported as one peak.

Elicitor. The crude elicitor used throughout these experiments was an autoclaved cell wall-membrane preparation of *V. dahliae* 277, as described (21). A typical elicitor preparation contained 70 μg of protein and 134 μg of glucose equivalents per ml (21). Elicitor was stored frozen under sterile conditions. Since the rate of fluorescence decay upon addition of different concentrations of elicitor showed a biphasic response (21), care was taken to use optimum elicitor concentration for all incubations.

Fluorescence Assay. The rate of fluorescence change upon addition of elicitor was measured in a Perkin Elmer MPF 44A spectrophotometer as previously described (21) with a few modifications. To 1.2 ml of cells in a 4 ml quartz cuvette was added 2 μl of dye stock solution. When citrate was used in the experiment, the addition of citrate preceded elicitor addition by about 15 s. Changes in fluorescence were recorded continuously and the rate of fluorescence change was calculated from the slope over a region of the scale where a linear decrease in fluorescence was observed (21). Linearity was usually obtained between 80 and 50% of the original fluorescence.

**RESULTS**

The induction of phytoalexin biosynthesis by elicitor molecules and the associated membrane changes can be conveniently assayed with fluorescent molecular probes (21). The elicitor-induced changes of the probe’s fluorescence occur within minutes after addition of elicitor and presumably correspond to early events in the signal transduction pathway (21). Using two such probes, pyranine, a membrane-impermeant, pH-sensitive fluorescent dye and oxonol VI, a membrane potential-sensitive dye which partitions into the membrane, elicitation of cotton and soybean cells was monitored. As previously noted, a rapid decrease in fluorescence was observed after a lage period of 5 to 7 min following addition of an elicitor preparation from *V. dahliae* (Fig. 1A). Prior addition of 6 mm sodium citrate partially inhibited the response and 30 mm citrate completely eliminated the pyranine transition. A similar experiment using oxonol VI as the molecular probe showed that 6 mm sodium citrate had no effect and 30 mm citrate only partially inhibited the elicitor-induced fluorescence change (Fig. 1B). The sodium citrate concentration required to obtain 50% inhibition (IC₅₀) of the elicitor-induced pyranine transition appeared to be about 2 mm for both *G. arboreum* and *G. max* cells (Fig. 2).

Since the elicitor-induced fluorescence changes of pyranine have been shown to correlate with phytoalexin formation in cotton cell suspension cultures (21), the effect of citrate on phytoalexin formation in *V. dahliae* elicitor-induced cotton and soybean cell suspension cultures was investigated. When sodium citrate was added to *G. arboreum* cells prior to addition of elicitor, a concentration dependent decrease in phytoalexin formation was observed (Fig. 3), confirming the prediction of the fluorescence assay (Fig. 1A). The concentration required for 50% inhibition (about 2 mm) was also similar to that seen above (Fig. 2). However, when the order of elicitor and citrate addition was reversed, *i.e.* elicitor first and citrate 0.5 to 30 min later, only a minor diminution of the defense response was measured, suggesting the sequence of elicitor and citrate addition is critical to the inhibitory mechanism. Curiously, citrate at IC₅₀ concentrations or less, stimulated, in cell mass accumulation in the same cell suspension cultures (Fig. 4).

A similar effect of citrate on glyceollin formation in soybean cell suspension cultures was observed. Addition of 660 μM (46.2 μg protein, 88.4 μg glucose equivalent) of an elicitor preparation from *V. dahliae* to a 100 ml culture of *G. max* caused the
Fig. 1. Effect of citrate on elicitor induced pyranine (A) and oxonol VI (B) fluorescence transitions in soybean cell suspension cultures. Soybean cell suspensions (1.2 ml) were treated with 2 μl of pyranine (A) (pH-sensitive dye) or 2 μl of oxonol VI (B) (membrane potential sensitive dye). Thereafter, the following additions were made: (1) 10 μl of an elicitor preparation from V. dahliae; (2) 6 mM citrate followed about 15 s later by 10 μl of elicitor; (3) 30 mM citrate followed about 15 s later by 10 μl of elicitor; or (4) no additions. Fluorescence was continuously monitored and recorded as described in “Materials and Methods.”

Fig. 2. Effect of increasing concentrations of citrate on the elicitor-induced fluorescence transitions of pyranine in cultured cotton (●) and soybean (X–X) cells. Experimental details as in Figure 1. Arrows indicate the inhibitory dose required for approximately 50% inhibition (ID50).

accumulation of 11.4 μmol glycine/g dry weight in the medium compared to 0.38 μmol/g dry weight in unstimulated cultures. However, as before, addition of 5 mM citrate prior to elicitation inhibited glycine formation by 70%. Furthermore, citrate also increased cell growth in soybean cultures (Fig. 5); however, this increase was not as pronounced as in the cotton cell cultures. At the highest citrate concentration tested (5 mM), 0.38 g dry weight of cell mass accumulated in 100 ml compared to 0.3 g dry weight in 100 ml of untreated cell suspensions.

The inhibitory effect of citrate was specific. Other di- and tricarboxylic hydroxy acids, with the possible exception of malate, did not appreciably inhibit the elicitor-induced fluorescence changes. However, malate at a concentration of 25 mM inhibited elicitation with 57% the potency of citrate (Table I). Addition of

Fig. 3. Elicitor-induced sesquiterpene aldehyde phytoalexin formation in cotton cells as a function of time at varying citrate concentrations. G. arboreum cells (1 cm² in 20 ml of medium) were treated with 0.15 ml of elicitor preparation (X–X), 0.15 ml elicitor and 180 μM citrate (●), 0.15 ml elicitor and 1.8 mM citrate (A–A), or 0.15 ml elicitor and 18 mM citrate (■–■). Citrate was added 5 min before elicitor addition. Control incubations without elicitor or citrate addition were obtained at zero time and after 120 h (O). Sesquiterpene aldehydes were determined in the cell mass by the phloroglucinol method (see “Materials and Methods”).

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was further by in Ca\textsuperscript{2+} dry weight citrate fluorescence transition. medium after the dahliae 277 increasing amounts on and after 120 phytoalexin incubations without growth of cultured (x-x) 0.40 FIG. 4. The effect of Ca\textsuperscript{2+} chelator, EGTA, at a concentration where all Ca\textsuperscript{2+} in the medium would have been chelated, did not inhibit fluorescence transition. The effect of citrate on the induction of phytoalexin formation was further investigated in 4-d-old G. arboreum seedlings elicited either by cold shock or treatment with V. dahliae. The results obtained (Table II) show that the fungal pathogen-elicited formation of the major cotton phytoalexins, (HG, G, MG, DMG) was reduced at citrate concentrations of 0.18 to 1.44 mm. At higher concentrations of citrate and in cold-shocked seedlings the inhibitory effect was considerably more pronounced (Table II).

DISCUSSION

The elicitation of phytoalexin formation in plants has been attributed to a variety of substances. Some of these have been found to be highly specific pathogen- or plant cell wall-derived molecules, while others may be simply general cellular perturbers (9). Specific inhibition of the plant cell response to elicitor molecules has not been reported although a number of biological extracts and buffers have been found to partially attenuate elicitation (10, 20, 22, 24). We have found that phys-
iological concentrations of citrate, a normal plant metabolite, can effectively inhibit elicitation.

The selectivity of the citrate effect is demonstrated by the relative impotence of similar di- and tricarboxylic hydroxy acids with the possible exception of malate (Table I). Furthermore, as shown in Figure 1, citrate was found to block only the oxonine fluorescence transition and not the transition of oxonol, suggesting proton fluxes are specifically inhibited without significantly altering the changes in membrane potential deriving from other ion fluxes. Thus, the oxonol transition was only altered at very high, toxic citrate concentrations where cotton cell growth was inhibited (Fig. 4). Such toxicity can be excluded as the cause of the observed suppression of elicitation at lower citrate concentrations, since 1.8 mm citrate actually increased cotton cell growth over and above control values (Fig. 4) even though it inhibited both the elicitor-induced fluorescence changes as well as phytoalexin formation. Similarly, soybean cell growth in culture was increased by low concentrations of citrate (Fig. 5). Finally, the specificity of the citrate inhibition can be inferred by the observation that addition of citrate 30 s to 15 min after addition of elicitor nearly obliterates the inhibitory effect, i.e. the order of elicitor and suppressor (citrate) addition is apparently very important.

Although the molecular mechanism by which citrate inhibits elicitor-induced phytoalexin formation is at present unclear, certain observations are noteworthy. First, the inhibition most likely occurs at the cell membrane, since (a) the molecular probes which first detect the inhibition reside at this site (5-7, 25) and (b) as pointed out above, addition of citrate after treatment with elicitor has a diminished inhibitory effect on both the pyranine fluorescence transition and on phytoalexin formation. Second, since the oxonol-detected, elicitor-dependent events in the membrane are largely unaffected by citrate, it can be suggested that citrate blocks only one of several elicitor stimulated changes. At the same argument, it is clear that oxonol and pyranine report on different elicitor-stimulated events. Whether these citrate-resistant events involve elements of a signal transduction sequence leading to the biosynthesis of other defense products cannot be concluded from the data. However, citrate apparently does not act as a Ca²⁺ chelator since incubations with 0 to 5 mm EGTA or EDTA showed no inhibition of fluorescence transition. Similarly, simple Ca²⁺ chelation by citrate as the inhibitory mechanism is not likely from the observation that malate showed 57% inhibition of elicitor induced fluorescence transition yet is equally effective in binding Ca²⁺ as tartrate and succinate, which showed no inhibition, and is a poorer Ca²⁺ chelator than maleate, which did not inhibit (Table I). Finally, since citrate was effective in inhibiting elicitation in whole seedlings by both V. dahliae and cold-shock, it is probable that both stimuli, biotic and abiotic, depend on a common, citrate sensitive pathway in promoting the defense response in plants.