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Perception of Fungal Sterols in Plants[1]

Subnanomolar Concentrations of Ergosterol Elicit Extracellular Alkalinization in Tomato Cells

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Suspension-cultured cells of tomato (*Lycopersicon esculentum* Mill.) reacted to spores and spore exudates of the pathogen *Cladosporium fulvum* with a rapid, transient alkalinization of their growth medium that resembled the previously described alkalinization response elicited by chitin fragments (G. Felix, M. Regenass, T. Boller [1993] Plant J 4: 307–316) and was likewise inhibited by the protein kinase inhibitor K-252a. However, the spore factor recognized by the cells differed from chitin fragments in that it was butanol soluble and active in cells refractory to stimulation by chitin fragments. The spore factor was purified and identified as ergosterol, the main sterol of most higher fungi. With pure ergosterol, half-maximal induction was reached at about 10 pM. After treatment with ergosterol, tomato cells became refractory to a subsequent stimulation by *C. fulvum* and vice versa, indicating that ergosterol was the principal component of the spores recognized by the plant cells. Most other sterols were inactive, including cholesterol, a range of animal steroid hormones, and all natural plant sterols tested, except for stigmasterol, which was about 10^6 times less active than ergosterol. Our data demonstrate that tomato cells perceive ergosterol with a selectivity and sensitivity that resembles the perception of steroid hormones in animals.

Chemosensory perception of microbial substances plays a major role in the interaction of plants with microbes. Thus, many plants have sensitive and selective perception systems for elicitors derived from pathogenic fungi, which cause the induction of a variety of defense responses (Dixon and Lamb, 1990; Ryan and Farmer, 1991). Oligosaccharides, glycopeptides/glycoproteins, peptides/proteins, and the fatty acid arachidonic acid have been identified as fungal compounds with elicitor activity in various plant systems (Ebel and Scheel, 1992). In addition, plants may also recognize their symbionts by chemical signals, as exemplified by the recognition system of leguminous plants for Nod factors, specific lipochitooligosaccharides produced by symbiotic rhizobia (Fisher and Long, 1992).

Changes in plasma membrane properties, including depolarization, efflux of K^+, and alkalinization of the growth medium, are among the earliest responses of plant cells to microbial elicitors (Dixon and Lamb, 1990; Scheel and Parker, 1990; Wei et al., 1992) and endogenous elicitors (Mathieu et al., 1991) as well as to Nod factors (Ehrhardt et al., 1992). In recent work in our laboratory it was observed that washed yeast cell walls induced a rapid alkalinization of the growth medium in suspension-cultured tomato (*Lycopersicon esculentum* Mill.) cells (Felix et al., 1993). This occurred because enzymes present in the culture medium released chitin fragments from the yeast cell walls, and because the tomato cells had a highly sensitive, specific perception system for the chitin oligosaccharides responsible for the rapid, transient induction of alkalinization. After a first stimulation with chitin fragments, the tomato cells did not respond to a second application of the same stimulus; however, throughout this refractory period they remained responsive to a different stimulus, xylanase (Felix et al., 1993). It was subsequently observed that the lipochitooligosaccharides (Nod factors) secreted by rhizobia also induced alkalinization in the tomato cells, and that the Nod factors were recognized by the same perception system as the chitin fragments, based on the criterion of the mutual establishment of a refractory state (Staehelin et al., 1994).

We became interested to find out whether factors similar to the chitin fragments were also released when intact spores of a fungal pathogen, *Cladosporium fulvum*, were added to the tomato cells. We found that tomato cells indeed reacted to small amounts of spores with a characteristic alkalinization response. However, the factor released from the spores to which the plant cells reacted was unrelated to chitooligosaccharides and turned out to be the main sterol of the higher fungi, ergosterol. Here we describe the characteristics of the perception system of tomato cells for ergosterol and discuss the possibility that plants have receptors for fungal sterols resembling animal steroid receptors in selectivity and sensitivity.

MATERIALS AND METHODS

Plant and Fungal Material

A cell-suspension culture of tomato (*Lycopersicon esculentum* Mill., line Msk8), originally provided by Dr. Maarten Koornneef (Agricultural University, Wageningen, The

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Abbreviation: chitotetraose, *N,N',N',N'*-tetraacetylchitotetraose.

Netherlands) was grown and subcultured as described (Felix et al., 1991a). Cells were used for experiments 7 to 12 d after subculture.

Cladosporium fulvum, race 5, kindly supplied by Drs. M. Joosten and P. De Wit (Agricultural University, Wageningen, The Netherlands), was subcultured on potato dextrose agar at room temperature. After 2 weeks of growth, spores were harvested from the agar plates with a brush and weighed. For quantitation, spores were suspended in water and counted in a hematocytometer under the microscope. From these counts a weight of about 1.7 ng/spore was estimated.

Chemicals

Ergosterol, dehydroergosterol, vitamins D2 and D3, β-sitosterol, campesterol, lanosterol, desmosterol, cholesterol, and cholestanol were purchased from Sigma. β-Estradiol, hydrocortisone, testosterone, and dexamethasone-21-acetate were kindly provided by Dr. J.-P. Jost (Friedrich-Miescher-Institute, Basel, Switzerland). Stigmasterol, 7-dehydrocholesterol, and K-252a were supplied by Fluka (Buchs, Switzerland). Sterols were dissolved and diluted in methanol or DMSO. A stock solution of K-252a (1 mM) was prepared in DMSO.

Assay of the Alkalinization Response

The alkalinization response was assayed as described previously (Felix et al., 1993) by continually registering the pH in the growth medium of aliquots (2–5 mL) of the tomato cell-suspension culture. Spores of *C. fulvum* were added suspended in water. For treatment with sterols and K-252a, ≤1 μL of the respective stock solutions were added per mL of cell suspension. Addition of equivalent amounts of the corresponding solvent, either methanol or DMSO, did not cause measurable alterations in the extracellular pH or in the responsiveness of the cells.

Preparation and Purification of a Lipophilic Spore Factor Recognized by the Tomato Cells

Spores of *C. fulvum* (1.5 g, harvested from 40 Petri dishes) were suspended in 40 mL of 1-butanol and shaken for 2 h. Afterward, the butanol supernatant was collected and the spores were reextracted with 40 mL of 1-butanol. The supernatants were combined and dried in a rotary evaporator. The residue was dissolved in 5 mL of 1-butanol, extracted twice with an equal volume of water, and evaporated to dryness. In different extractions about 30 mg of dried, crude lipophilic spore extract was obtained per g of spores extracted.

Portions (about 500 μg) of the crude lipophilic spore extract were dissolved under sonication in 90% (v/v) methanol (50 μL), centrifuged, passed through a 0.2-μm filter, and subjected to HPLC on a C_8 column (Nucleosil [Macherey-Nagel, Düren, Germany], 250 mm × 5 mm, 5 μm). The column was eluted with a linear gradient from 90 to 95% (v/v) of methanol in water within 20 min, followed by 10 min with 100% methanol. The flow rate was 1 mL/min, and the absorption of the eluate was monitored at 275 nm. Fractions of 250 μL were collected. Aliquots (2 μL) of the fractions were added directly to the cells for testing alkalinization-inducing activity. Activity eluted as a single peak with a retention time of approximately 10 min. Fractions containing activity were pooled, dried, resuspended in 200 μL of *n*-hexane, and extracted twice with water (1:1, v/v). The hexane phase containing the spore factor was further purified by HPLC on a silica gel column (Nova-Pak Silica column [Waters-Millipore, Milford, MA], 150 mm × 3.9 mm, 4 μm). The column was eluted with *n*-hexane: butanol (99:1, v/v) and the eluate was monitored for alkalinization-inducing activity. Activity, eluting as a single peak, was pooled, dried, and stored at −20°C.

Analysis of the Purified Spore Factor by IR Spectroscopy

IR spectroscopy of the purified spore factor and of reference samples of ergosterol was performed by Dr. K. Alt and P. Acker (Ciba, Basel, Switzerland) according to standard procedures (KBr).

RESULTS

Alkalinization of the Growth Medium of Tomato Cells in Response to Intact Spores of *C. fulvum*

Addition of intact spores of *C. fulvum* to suspension-cultured tomato cells induced a rapid increase of the extracellular pH (Fig. 1, A and B). When treated with about 32,000 spores per mL of suspension, alkalinization started after a lag of 2.5 min and reached a transient maximum, 0.6 unit above the initial pH ($\Delta pH_{max} = 0.6$), after about 7 min. Although doses of >32,000 spores per mL did not induce a stronger alkalinization response (data not shown), lower doses of spores were found to cause alkalinization after a longer lag phase and the pH increase (ΔpH_{max}) was lower (Fig. 1A). Doses of 100 spores per mL or less did not lead to a significant alkalinization response. When spores were incubated for some minutes in water and subsequently removed by centrifugation, a large part of the alkalinization-inducing activity remained in the supernatant (data not shown), indicating that alkalinization was not due to metabolism of the spores themselves. The drug K-252a, known to inhibit protein kinase activity in tomato microsomes in vitro (Grosskopf et al., 1990) and to block the responses to various microbial signals in tomato cells in vivo (Grosskopf et al., 1990; Felix et al., 1991b, 1993; Staehelin et al., 1994), completely abolished the alkalinization response induced by the spores of *C. fulvum* (Fig. 1B). This indicates that the observed medium alkalinization is not due to a direct perturbation of the plant plasma membrane, but rather is the result of an active response of the plant cells dependent on changes in protein phosphorylation.

Purification of the Spore Factor Inducing Alkalinization and Its Identification as Ergosterol

Since the alkalinization-inducing factor was released from the spores during incubation with tomato cell suspensions, we tried to purify and characterize this factor in

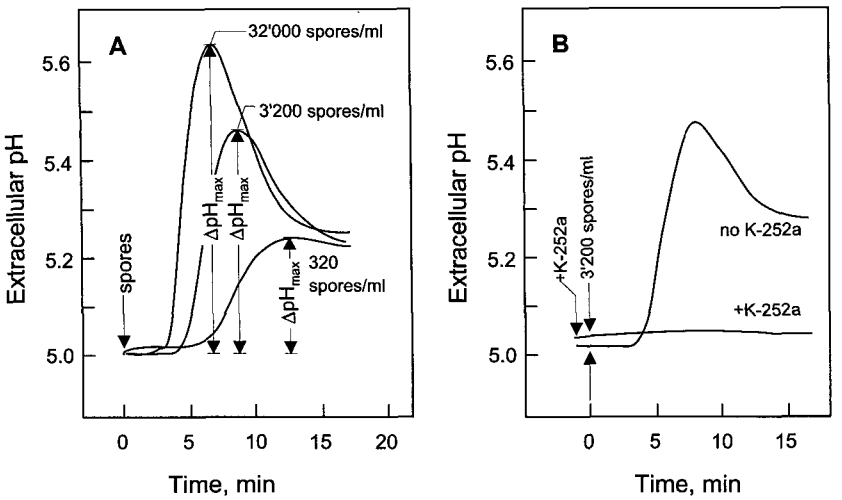

Figure 1. Alkalinization of the culture medium of tomato cells in response to spores of *C. fulvum*. A, Effect of different doses of *C. fulvum* spores. B, Inhibition of the alkalinization response induced by *C. fulvum* spores in cells pretreated for 1 min with 1 μM K-252a.

soluble extracts. Upon mixing water extracts from spores with 1-butanol, most of the activity (>95%) partitioned into the butanol phase, demonstrating that it is a lipophilic compound. To prepare the alkalinization-inducing spore factor in high yield, spores were directly extracted with 1-butanol. The butanol extract was chromatographed by reverse-phase HPLC on a C_8 column. Fractions with alkalinization-inducing activity eluted as a single, broad peak with a retention time of about 10 min (Fig. 2B). All other fractions tested were devoid of alkalinization-inducing activity. For further purification, the pooled fractions were subjected to normal-phase HPLC under apolar conditions. Again, activity eluted as a single peak and the preparation of purified spore factor obtained exhibited an absorption maximum at 275 nm (data not shown). When compared to

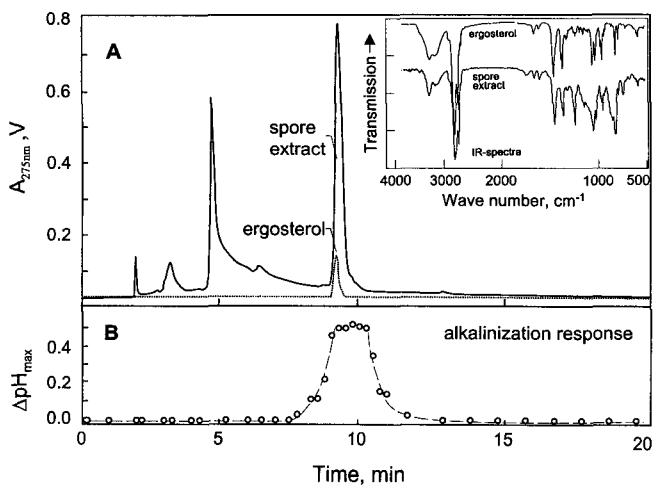

Figure 2. Reverse-phase HPLC of butanol extract of *C. fulvum* spores. Spore extract (about 500 μg dry weight) was chromatographed on a C_8 column. A, Absorption monitored at 275 nm (solid line). Dotted line, Chromatogram of 2 μg of pure ergosterol. Inset, IR spectra of pure ergosterol and of the purified spore factor (from the silica gel column). B, Eluate was fractionated (250 μL) and assayed for alkalinization-inducing activity on tomato cells (1 μL eluate/mL cell suspension).

known lipophilic substances of fungi, these characteristics of the spore factor were reminiscent of the main fungal sterol, ergosterol. Indeed, the activity of the spore extract co-chromatographed with pure ergosterol on the C_8 reverse-phase column (Fig. 2A); the IR spectrum of the purified spore factor exhibited the characteristic absorption bands of ergosterol, with similar relative intensities, at 2955, 2870, 1659, 1458, 1381, 1369, 1069, 1040, 984, 968, 947, 802, and 604 cm^{-1} (Fig. 2A, inset; additional absorption bands represent an impurity, most likely silica from the column). Most importantly, pure ergosterol exhibited alkalinization-inducing activity when applied to the suspension-cultured tomato cells even at subnanomolar concentrations (Fig. 3; see below). The pattern of the alkalinization response induced by ergosterol matched precisely the one observed after addition of spores, and it was also completely blocked in cells pretreated with K-252a (data not shown). These results identify the purified spore factor as ergosterol.

Establishment of a Refractory State after Perception of Ergosterol

Previous work had established that tomato cells stimulated once with chitin fragments entered a refractory state and did not react to a second application of chitin fragments for at least 8 h, whereas they remained fully responsive to different stimuli such as fungal xylanase (Felix et al., 1993). To test whether a similar refractory state was established after ergosterol treatment, cells were sequentially treated with ergosterol, spore suspension, and chitotetraose in various combinations (Fig. 3). After a first stimulation with 10 nM ergosterol, the cells did not react to a second dose of ergosterol, but they were still fully responsive to chitotetraose (Fig. 3A). Conversely, cells treated once with chitotetraose did not react to a second dose of chitotetraose but still reacted to ergosterol (Fig. 3A).

When the tomato cells were first exposed to a suspension of intact *C. fulvum* spores, they became refractory to ergosterol but still reacted to chitotetraose (Fig. 3B). Correspondingly, tomato cells rendered refractory to ergosterol after

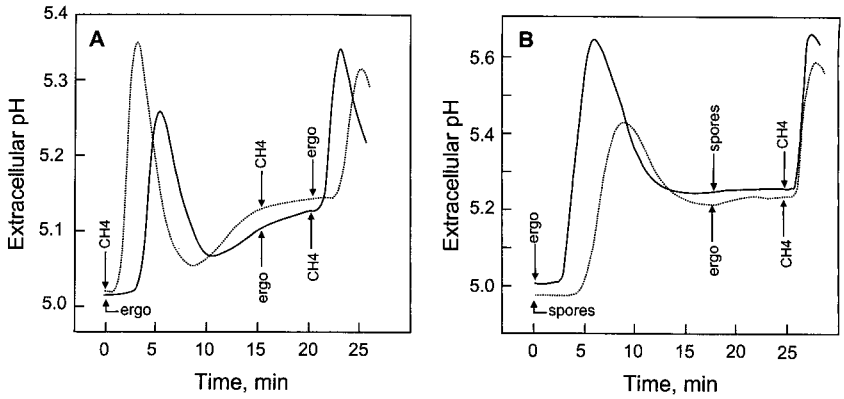

Figure 3. Establishment of a refractory state in tomato cells after treatment with spores of *C. fulvum*, ergosterol, and chitotetraose. A, Cells treated with either ergosterol (10 nM) or chitotetraose (10 nM) at 0 min were subsequently treated with ergosterol (10 nM) and chitotetraose (10 nM) as indicated by the arrows. B, Cells treated with either ergosterol (10 nM) or spores of *C. fulvum* (3200 spores/mL) at 0 min were subsequently treated with ergosterol (10 nM), spores (3200 spores/mL), and chitotetraose (10 nM) as indicated by the arrows.

application of this compound were unable to react to *C. fulvum* spores but still reacted to chitotetraose (Fig. 3B). Thus, ergosterol appears to be the only determinant in the spore suspensions that leads to medium alkalinization in tomato cells.

Sensitivity and Specificity of the Ergosterol Perception System of Tomato Cells

To examine the sensitivity of the perception system of tomato cells for ergosterol, different doses were assayed for induction of extracellular alkalinization. From the initial pH and the transient pH maximum reached within 10 min of treatment, the pH increase (ΔpH_{max}) was deduced and plotted against the concentration of sterol applied (Fig. 4). In a set of similar, independent experiments, the pH increase observed with saturating doses of ergosterol varied between 0.3 and 0.6 pH units, and the minimal concentration required to induce a significant induction of alkalinization (threshold concentration) varied between 0.1 and 1 pM. Three representative dose-response curves are shown in Figure 4. Reproducibly, however, half-maximal induction was observed between 3 and 10 pM and saturation of the response was reached at a concentration of about 0.1 nM.

To test the specificity of the perception system, ergosterol-derived substances, as well as several other sterols and steroids of plant and animal origin, were assayed for their capacity to stimulate an alkalinization response. The chemical structures of some of the sterols tested are depicted in Figure 5. Among the substances tested, only dehydroergosterol was active to the same degree as ergosterol (Fig. 4; Table I). Vitamin D_2, another direct derivative of ergosterol, displayed significant alkalinization-inducing

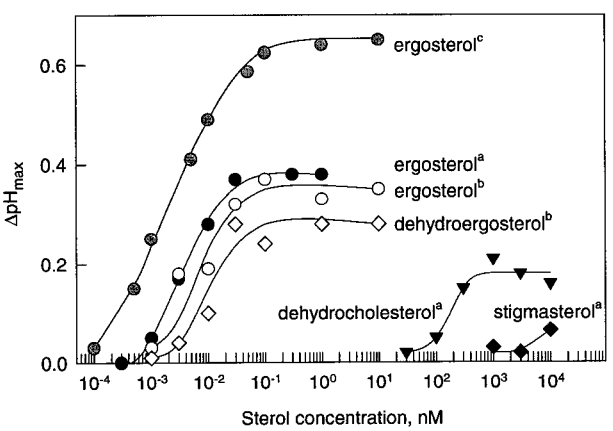

Figure 4. Dose-response curves for extracellular alkalinization of tomato cells induced by ergosterol (○, ●), dehydroergosterol (◇), dehydrocholesterol (▼), and stigmasterol (◆). Maximal pH increase above baseline (ΔpH_{max}), occurring 5 to 10 min after the addition of the stimulus, was read from continuous tracings of the extracellular pH values. Three batches of cells were used as indicated by superior letters a, b, and c.

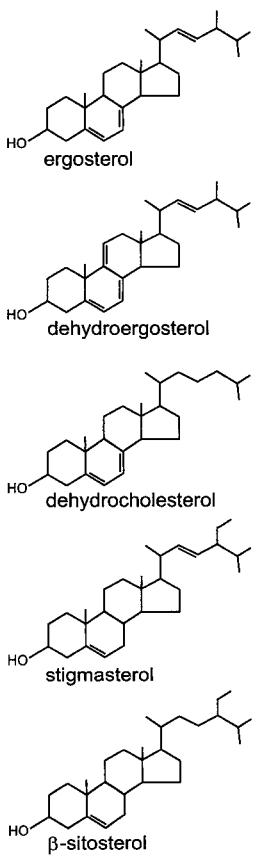

Figure 5. Structures of sterols used to determine specificity of the alkalinization response in tomato cells.

Table I. *Activity of sterols and steroids of different origin in inducing extracellular alkalinization in tomato cells*

Compound	Occurrence	Threshold Concentration in Bioassay[a]
Ergosterol	Fungi	0.1–1 pM
Dehydroergosterol	(Derived from ergosterol)	3 pM
Vitamin D_2	(Derived from ergosterol)	100,000 pM
7-Dehydrocholesterol	Animals	100,000 pM
Stigmasterol	Plants	1,000,000 pM
β-Sitosterol	Plants	None[b]
Campesterol	Plants	None[b]
Lanosterol	Fungi, animals, plants	None[b]
Desmosterol	Animals, red algae	None[b]
Cholesterol	Animals	None[b]
Cholestanol	Animals	None[b]
Vitamin D_3	Animals	None[b]
β-Estradiol	Animals	None[b]
Hydrocortisone	Animals	None[b]
Testosterone	Animals	None[b]
Dexamethasone-21-acetate	Synthetic	None[b]

[a] Minimal dose that causes significant alkalinization ($\Delta pH_{max} \geq 0.04$). [b] Tested at concentrations up to 1,000,000 pM.

activity when applied at concentrations ≥100 nM. Commercial preparations of dehydrocholesterol and the plant sterol stigmasterol stimulated medium alkalinization down to concentrations of approximately 100 nM and 1 μM, respectively (Fig. 4). Compared to ergosterol, 1 μM of stigmasterol provoked an alkalinization response equivalent to the one observed with 1 pM ergosterol. All other sterols and steroids tested were completely inactive when applied at concentrations up to 1 μM (Table I). When any one of the inactive sterols and steroids was given first to tomato cells at concentrations up to 1 μM, the cells were still fully responsive to 10 nM ergosterol, indicating that none of them interfered with ergosterol perception.

DISCUSSION

The present work was initiated on the basis of the observation that tomato cells recognize the presence of intact spores of the tomato pathogen *C. fulvum* and react with an alkalinization response in a manner resembling the perception of chitin fragments described earlier (Felix et al., 1993). We initially suspected that recognition of fungal spores was based on the liberation of chitin fragments. However, the analysis presented here clearly demonstrates that the alkalinization response induced by intact spores has nothing to do with chitin fragments but is solely due to ergosterol released by the fungal spores, and that the tomato cells have an exceedingly sensitive perception system for ergosterol, the main sterol of most higher fungi.

The response of the tomato cells to ergosterol is similar to their response to chitin fragments (Felix et al., 1993) in several respects. In both cases the cells react to subnanomolar amounts of the stimuli, and in both cases the alkalinization observed is transient and cannot be reinstated by a second stimulation with the same stimulus, indicating a desensitization phenomenon similar to sensory adaptation in animal systems. In both cases the alkalinization response was prevented by the addition of the protein kinase inhibitor K-252a, indicating an involvement of protein phosphorylation (Felix et al., 1991b, 1994). A characteristic difference of the alkalinization response to saturating doses of these two stimuli was found in the lag phase, which was considerably shorter for chitin fragments (≤1 min) than for ergosterol (2–3 min).

Perception of ergosterol in tomato cells resembles the perception of steroid hormones in animals with regard to sensitivity and selectivity. However, in contrast to the animal receptors, which are specific for endogenous steroids that act as hormonal signals, the plant perception system recognizes ergosterol, an exogenous, "nonself" sterol that is produced only by fungi and not by the plants themselves. In fact, all plant (and animal) sterols tested had no activity in the bioassay, except for a commercial preparation of stigmasterol that was about 10^6 times less potent than ergosterol. It is possible that this minute activity is due to a trace of ergosterol (at the 0.0001% level) present in the stigmasterol preparation, e.g. stemming from fungi growing on the plant material used for isolation. It is equally possible that the reaction to vitamin D_2 is due to a minor contamination by ergosterol, which serves as precursor in its preparation. The only sterol with an activity approaching that of ergosterol was dehydroergosterol, a synthetic derivative of ergosterol with a closely similar structure (Fig. 5).

Based on these data we hypothesize that plants possess ergosterol receptors for the detection of invading fungi. Binding sites for vitamin D_3 and the endogenous plant sterols β-sitosterol and stigmasterol have been observed in French bean roots (Vega and Boland, 1988), but the biological role of these sterol-binding sites and their specificity with respect to "self" and "nonself" sterols remain to be examined. Sterol- or steroid-binding proteins are also present in different yeasts (Loose et al., 1981; Feldman et al., 1982) and in some water molds (Riehl and Toft, 1984), and their biological significance remains an open question, too.

Ergosterol is the main sterol of most higher fungi, with an essential role in membrane stabilization (Burden et al., 1989). Plants do not form ergosterol but possess a number of other sterols. Since sterol biosynthesis in plants and fungi is divergent, the fungal-specific enzymes for ergosterol biosynthesis are well-known targets for agricultural fungicides (Bossche, 1988; Burden et al., 1989). Inhibition of ergosterol biosynthesis in plant-colonizing fungi may not only reduce fungal growth but also change their sterol composition and therefore interfere with the plant's potential to recognize the fungi.

Ergosterol occurs in many plant pathogens, such as the *C. fulvum* described here, as well as in saprophytes and in fungi establishing a mutualistic symbiosis with plants (Burden et al., 1989). The same is true for chitin, the main cell-wall constituent of both groups of fungi. It is a matter of speculation whether the perception system described

here is relevant for plant-pathogen or plant-symbiont interaction. However, it is interesting to note that some of the biotrophic pathogens such as powdery mildew fungi (*Erysiphe* spp., Loeffler et al., 1984) and rust fungi (*Uromyces* spp., Burden et al., 1989) do not contain ergosterol but possess derivatives as membrane sterol components. We found that the tomato cells do not react to butanol extracts of *Erysiphe* and *Uromyces* spores (data not shown).

It is worth noting that scientists have quantitated chitin and ergosterol in plant tissues in an effort to measure fungal colonization, both by pathogens and by symbiotic mycorrhizal fungi (e.g. Johnson and McGill, 1990), with detection limits on the order of 10 to 100 ng/mL for the compounds in question. The data presented here demonstrate that the plant cells themselves have detection systems for the same highly characteristic compounds of fungi, and that their detection limits in the bioassays described here and by Felix et al. (1993) are on the order of 1 to 10 pg/mL. Both symbiotic and potentially pathogenic fungi possess ergosterol and chitin, and it remains to be seen how the perception systems of plants for these fungal components are connected to symbiosis and/or pathogenesis.

In conclusion, the tomato cells examined in this study have exceedingly sensitive perception systems for two highly characteristic components of higher fungi, namely for fragments of chitin, a principal constituent of their cell walls, and for ergosterol, the main sterol of their membranes.

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