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Modulation of Fusicoccin-Binding Protein Activity in Mung Bean (*Vigna radiata* L.) Hypocotyls by Tissue Maturation and by Fusicoccin[1]

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The phytotoxin fusicoccin (FC), after binding to a plasma membrane-localized receptor, causes higher plant cells to excrete protons. Ligand-binding analysis has been used to show that the plasma membrane of mung bean (*Vigna radiata* L.) hypocotyls contains both high-affinity (HA) and low-affinity (LA) binding sites for FC. The effect of tissue maturation on these sites was determined on isolated membrane vesicles from the meristematic region (hook) and the elongation zone and from mature hypocotyl tissues. In the meristematic region the HA:LA ratio was 1:20. As hypocotyl tissues matured, the site density of HA increased and there was no change in LA density, so that the HA:LA ratio increased to 1:2 in mature tissues. FC-induced proton excretion correlates with the HA density, not the LA density. When sections isolated from each region were incubated with FC prior to isolation of membranes, there was an apparent conversion of LA to HA sites during the first 90 min in all regions. During the next 1 to 3 h there was a further 2.5- to 3-fold increase in binding sites in all regions, accompanied by a slight decline in dissociation constant. The increase in binding sites, but not the apparent conversion of LA to HA, was partly blocked by cycloheximide. These data suggest that FC alters FC-binding protein activity in two ways: first, by causing an increase in affinity for FC of preexisting LA receptors, and second by inducing the synthesis of additional FC receptors. This apparent up-regulation of a phytotoxin receptor by its ligand in plants has not previously been reported.

The phytotoxin FC, produced by the fungus *Fusicoccum amygdali* Del., has the ability to cause proton excretion from all higher plants so far tested (Marré, 1979). After binding to a 30.5- to 32.5-kD receptor (FCBP) (de Boer et al., 1989; Feyerabend and Weiler, 1989) on the PM, it activates the PM H^+-ATPase, apparently by relieving the inhibition caused by the autoinhibitory C-terminal domain of the ATPase (Pugliarello et al., 1992; Johansson et al., 1993).

Our previous study (Basel and Cleland, 1992) of the developmental gradient of FCBP activity in mung bean (*Vigna radiata* L.) hypocotyls indicated that FCBP activity was affected by the maturity of the tissue. FCBP activity was barely detectable in the meristematic (hook) region but then increased steadily through the elongation zone and was highest in the most mature regions of the hypocotyl that were sampled. A number of reports have appeared stating that some tissues possess two classes of FC-binding sites: HA sites and LA sites (Aducci et al., 1982; Meyer et al., 1989; Schulz et al., 1990; Abramycheva et al., 1993). Because our previous FCBP assay would have measured only HA sites, this study was undertaken to determine whether mung bean hypocotyls possess both HA and LA FC-binding sites and, if so, whether the distributions of HA and LA sites were the same.

Preliminary experiments indicated that, when hook sections from mung bean hypocotyls were treated with FC, there was no detectable increase in proton excretion during the 1st h, as expected from the apparent lack of FCBP in this region. But after the 1st h, FC-enhanced H^+ excretion commenced, suggesting that FC might have up-regulated the ability of these cells to bind FC. To test these possibilities, ligand-binding analysis (Scatchard, 1949) has been used with microsomal and PM vesicles prepared from freshly cut sections of the mung bean hypocotyl or prepared after preincubation of sections for up to 6 h with or without FC. The Scatchard analysis has shown that FC-binding sites are under developmental control and are up-regulated by FC.

MATERIALS AND METHODS

Plant Materials

Mung beans (*Vigna radiata* L.) seeds (Jungs' Seed Co., Madison, WI) were soaked in water with aeration overnight and then planted in vermiculite watered with 1 mM $CaSO_4$. After growth for 5 d at 25°C in the dark, hypocotyls were harvested and sectioned under dim green light. Three or five sections were then cut from each hypocotyl (Basel and Cleland, 1992). Section a is the region between the base of the cotyledons and the base of the hook and contains the meristematic tissue. Section b is the next 7.5 mm below the hook and makes up the bulk of the elongation zone. Sections c, d, and e were successive 7.5-mm sections below section b. Section c contained the end of the elongation zone and some mature tissue, and sections d and e were progressively more mature tissues. For the ligand-binding analysis, sections d and e were cut as a single section (d/e). Where indicated,

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Abbreviations: B_d, site density; CH, cycloheximide; FC, fusicoccin; FCBP, fusicoccin-binding protein; HA, high affinity; LA, low affinity; PM, plasma membrane.

sections were abraded before preincubation by placing them in a suspension of rottonstone (Empire White Products, Newark, NJ) and stirring the suspension for 15 min.

Incubations

All sections were pretreated for 1 h in 10 mM K-phosphate (pH 6.1) plus 0.1 mM $CaCl_2$. Sections were then incubated for up to 6 h in 1 mM K-phosphate, pH 6.1, with or without 1 μM FC. CH (20 μg mL^{-1}) was added to abraded sections during the final 20 min of preincubation and continued throughout the incubation period.

Membrane Preparation

After preincubation or incubation, sections were rinsed, blotted, and ground twice in a mortar and pestle with a freshly made extraction buffer (pH 7.8) composed of 10 mM Tris-HCl (pH 7.5), 250 mM Suc, and 1 mM EDTA (Sandstrom et al., 1987; Basel and Cleland, 1992). The slurry was filtered through four layers of cheesecloth and then centrifuged at 3000*g* for 20 min. The supernatant from that spin was respun at 80,000*g* for 30 min to form a microsomal pellet. Pellets were resuspended in a buffer of 5 mM K-phosphate (pH 7.8), 4 mM KCl, and 250 mM Suc (Sandstrom et al., 1987) and were stored in a freezer at −70°C.

To obtain PM-enriched membranes, microsomal membranes were extracted by aqueous two-phase partitioning with 6.3% (w/w) PEG and dextran. A single extraction gave a U_1 fraction, whereas three sequential extractions gave a U_3 fraction (Basel and Cleland, 1992). Phase-partitioned membranes were resuspended in a buffer composed of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 20% (v/v) glycerol and were then stored at −70°C.

FC-Binding Assay

FC binding was determined by a filter disc radiolabel assay as described by de Boer et al. (1989). Briefly, 30 μg of microsomal or U_1 proteins were suspended in a buffer of 25 mM Mes/Tris (pH 6.0), 1 mM $MgCl_2$, 0.5 mM EDTA, 25 mM KF, 10% (v/v) glycerol, 0.02% (w/v) NaN_3, and the protease inhibitors pepstatin (1 mg mL^{-1}), leupeptin (1 mg mL^{-1}), PMSF (0.2 mM), and aprotonin (5 μL mL^{-1}). [^{3}H]DihyroFC (130 TBq $mmol^{-1}$) was added to a final concentration of 0.1 to 40 nM (ligand-binding analysis) or 2 nM (FCBP activity); biological activity of dihydroFC is the same as that of FC (Ballio, 1977). The total volume of the assay was 1 mL. After incubation for 2 h at 30°C, labeled samples were passed through GF/B filters (Whatman, Hillsboro, OR) presoaked in 1% (v/v) polyethylenimine for 2 h. Filters were then soaked in Aquasol, and the counts were measured in a Beckman scintillation counter. Boiled samples were used as the control for nonspecific binding. Studies using 10 μM unlabeled FC instead gave the same values for specific binding (Basel, 1992). To test whether latency of FCBP might be a problem, some samples were solubilized with a 10:1 octyl-β-D-glucopyranoside:protein ratio. This gave nearly identical binding values as the intact vesicles, indicating that the FC-binding sites of the vesicles were accessible to FC (Basel, 1992).

Scatchard Plot Analysis

To determine the binding parameters of the FCBP, the K_d and the B_d, ligand-binding data were analyzed according to the Scatchard transformation (Scatchard, 1949). In the Scatchard transformation, the inverse negative slope of the best-fit line represents the K_d, and the x axis intercept represents B_d/K_d.

Ligand binding was measured as described in "FC-Binding Assay," by varying the concentrations of [^{3}H]dihyroFC between 0.1 and 40 nM while keeping protein amounts constant. Binding was saturated at 40 nM (data not shown). Binding constants were calculated, assuming either one or two binding sites, and Scatchard transformations were plotted using the computer program Enzfitter 1.03 (R.J. Leatherbarrow, Elsevier-Biosoft, Cambridge, UK).

FC-Induced Acidification

Abraded sections (0.4 g fresh weight) were incubated in 2 mL of 1 mM K-phosphate (pH 6.1) with or without 1 μM FC. The pH change was monitored with a pH meter.

Chemicals

[^{3}H]DihydroFC was prepared by Amersham by catalytic hydrogenation of FC. FC, pepstatin, leupeptin, PMSF, aprotonin, and polyethylenimine were obtained from Sigma. All other chemicals were reagent grade.

Replication

Experiments were duplicated using triplicate readings for each concentration of [^{3}H]dihyroFC unless otherwise specified.

RESULTS

Modulation of FC-Binding Sites during Tissue Maturation

The types and amounts of FC-binding sites in meristematic tissues (section a), the elongation zone (section b), and mature tissues (section d/e) were determined by ligand-binding assay and subsequent Scatchard analysis (Scatchard, 1949). The Scatchard plots for FC binding to membrane from all three sections were biphasic, indicating the existence of both HA and LA FC-binding sites (Fig. 1). For the HA sites, the K_d values for all three sections were similar (0.18–0.31 nM), but the B_d increased >10-fold between the meristematic and the mature tissues (Table I). By contrast, the B_d of the LA sites was nearly constant (approximately 2 pmol mg^{-1}), but the K_d appeared to decrease with increasing age of the tissue, suggesting that a modification of the LA sites occurred during tissue maturation. The percentage of FC-binding sites that were HA sites increased from <5% in section a to >35% in section d/e.

FC-Induced Proton Excretion and Tissue Age

The effect of tissue age on the capacity of mung bean hypocotyl sections to excrete protons in response to FC was determined (Figs. 2 and 3). When the FC-induced pH change

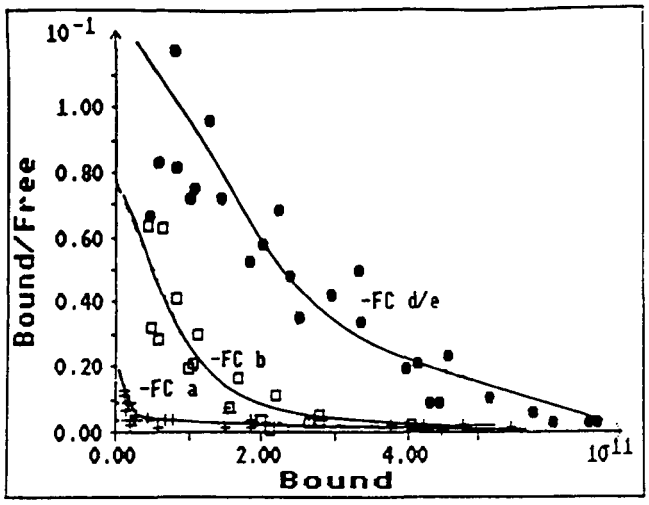

Figure 1. Scatchard analysis of the binding of 0.1 to 20 nM [^{3}H]-dihydroFC to microsomal membranes from the meristematic (section a), elongating (section b), and mature (section d/e) regions of the mung bean hypocotyl. Sections were cut, membranes were isolated, and binding was assayed as described in "Materials and Methods." Each Scatchard plots could be resolved into two straight lines (not shown), indicating the presence of both HA- and LA-binding sites, by the use of the Enzfitter 1.03 computer program. Units of binding are mol of FC bound.

during the 1st h was compared with the HA B_d, measured on U_3 fractions from these sections, a good correlation between the two activities was noted (Fig. 2). In contrast, there was no correlation between the FC-induced pH change and the number of LA FC-binding sites (Table I, Fig. 2).

A time course for FC-induced proton excretion (Fig. 3) confirmed our preliminary indications that in section a, FC caused no proton excretion during the first 1 to 2 h but that thereafter FC-induced proton excretion occurred (Fig. 3, open circles). After section a had been incubated for 5 h in FC, the solution pH was greatly reduced but still greater than that of the solution containing section d. With section b there was some delay before maximum FC-induced acidification occurred, but the lag was less than with section a, and the final solution pH was intermediate between sections a and d/e. These data are consistent with the possibility that the density

Table I. *Effect of tissue age on the K_d and B_d of LA and HA FC-binding sites*

Scatchard analysis was performed on the binding of 0.1 to 20 nM [^{3}H]FC to microsomes from meristematic (section a), elongating (section b), and mature tissues (section d/e) to yield K_d and B_d values for HA and LA FC-binding sites.

Section	HA Site		LA Site	
	K_d	B_d	K_d	B_d
	nM	pmol mg^{-1} protein	nM	pmol mg^{-1} protein
a	0.19	0.11	16	2.2
b	0.18	0.48	5	1.5
d/e	0.31	1.2	1.2	2.2

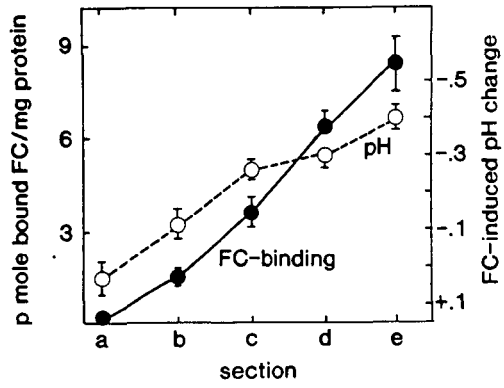

Figure 2. Comparison of the initial rate of FC-induced proton excretion and the number of HA FC-binding sites from five sequential sections along the mung bean hypocotyl that differ in tissue age. Section a contains the youngest tissues, and section e contains the most mature. Procedures for measuring proton excretion and HA-binding sites are described in "Materials and Methods." Binding sites were measured on U_3 fractions, using 2 nM ^{3}[H]dihydroFC, which accounts for the greater B_d values obtained here compared with microsomal values in Table I.

of HA FC receptors might increase when tissues are subjected to FC.

FC-Induced Modulation of the FCBP Activity

Mung bean hypocotyl sections were incubated for 0 to 6 h with or without 1 μM FC, a PM-enriched (U_1) membrane fraction was prepared from these sections, and the numbers of HA FC-binding sites were compared. The results are shown in Figure 4 for section a (meristematic region) and section b (elongation zone). Similar results were obtained with section d/e (data not shown). In the absence of FC the FCBP activity was low and remained constant during the 6 h, showing that excision of the section from the hypocotyl

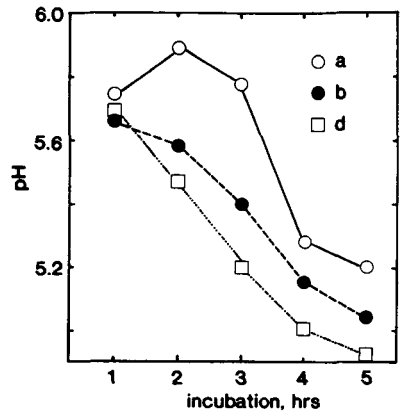

Figure 3. Time course of FC-induced proton excretion from sections cut from the meristematic (a), elongating (b), and mature regions (d) of the mung bean hypocotyl. Sections were incubated with 1 μM FC, and the pH of the solution was measured as described in "Materials and Methods." Data are from one representative experiment.

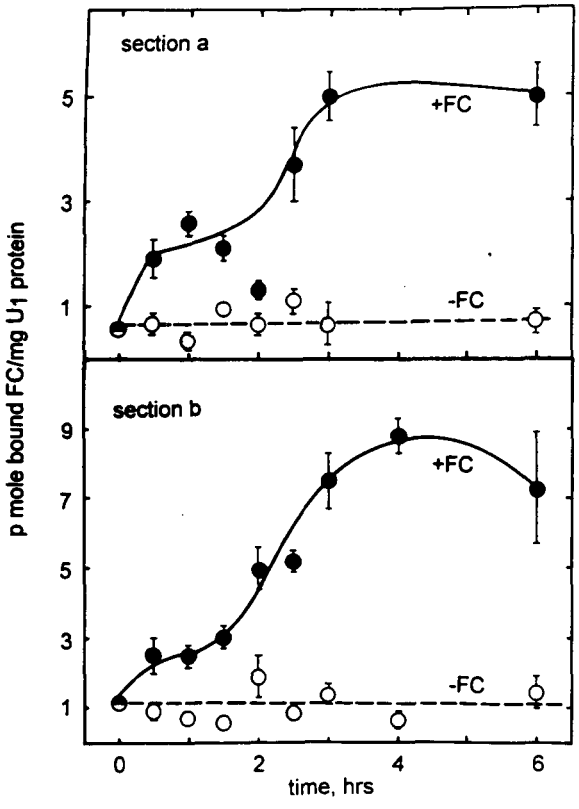

Figure 4. Time course of change in HA binding of FC to PM-enriched membranes (U_1) from the meristematic (a) or elongating (b) region of the hypocotyl, as influenced by the presence of FC. Sections were incubated with (+FC) or without (−FC) 1 μM FC for the times indicated, then a U_1 PM-enriched membrane fraction was isolated, and HA binding was assayed using 2 nM [^{3}H]dihydroFC.

did not alter the number of HA FC-binding sites. In the presence of 1 μM FC, there was a rapid 2- to 3-fold increase in HA FC-binding sites in the 1st h. After a lag of another hour, FCBP activity again increased an additional 2- to 3-fold, reaching a maximum at 3 to 4 h. The rapidity of this increase in FCBP activity in the first 30 min was examined in more detail for sections a and b (Fig. 5). HA FC-binding activity increased without any apparent lag in section a, whereas in section b there appeared to be a 10-min lag.

To characterize further the changes in both HA and LA sites, Scatchard analyses were performed on FC binding to microsomal fractions from sections a, b, and d/e after pretreatment of sections with or without FC. Because of the amount of material needed, microsomal fractions were pooled from the early FC-induced phase (0.5- to 2-h incubation) and later FC-induced phase (3- to 6-h incubation). Scatchard plots are shown for section a (Fig. 6); similar results were obtained for sections b and d/e (data not shown). The calculated B_d and binding constants from all treatments are shown in Table II.

A similar pattern of FC-induced change occurred in all three sections. The Scatchard plots for both the early and late phases of FC preincubation each showed only a single FC-binding site. During the early phase, the disappearance of the LA sites was nearly matched by the increase in HA sites, although the total B_d decreased slightly and the K_d increased about 2-fold. This is consistent with a conversion of LA to HA FC-binding sites. During the latter phase of induction, on the other hand, there was a nearly 3-fold increase in FC-binding sites in all tissues. The K_d for these binding sites was about 5-fold greater than that of the original HA sites. These sites appeared to be comparable in binding affinity to the lower-affinity class of binding sites that exist in vivo in the mature tissues (section d/e).

The increase in total number of HA-binding sites, especially during the later phase of FC incubation, raises the possibility that FC has induced the synthesis of additional FC receptors. To test this idea, sections were incubated with FC in the presence or absence of CH, and the differences in binding B_d was compared using Scatchard analysis. During the early phase (0.5–2 h), there was no difference in HA-binding B_d with or without CH. However, the further FC-induced increase in HA density during the later phase (3–6 h) was inhibited 40 to 75% by CH (Table III). CH had no significant effect on the K_d of the HA sites, nor did it alter the apparent conversion of LA to HA sites during the early phase of FC induction (data not shown).

DISCUSSION

The existence of two types of FC-binding sites has been reported previously for maize coleoptile (Aducci et al., 1986), maize root (Aducci et al., 1982; Abramycheva et al., 1993), spinach leaves (Aducci et al., 1982), *Vicia faba* leaves (Abramycheva et al., 1991), *Arabidopsis* shoots (Meyer et al., 1989), and *Corydalis* suspension-culture cells (Schulz et al., 1990). In general, these HA sites have K_d values of 0.5 to 3 nM, whereas the LA sites have values of 10 to 70 nM. In *Commelina* leaf cells, on the other hand, only one class of intermediate-affinity binding sites (K_d = 5.2 nM) was found (Oecking and Weiler, 1991). Mung bean hypocotyls are shown here to have two classes of FC-binding sites in meristematic, elongating, and mature tissues. The K_d values for the HA sites are lower (0.18–0.31 nM) than any previously recorded and appear to

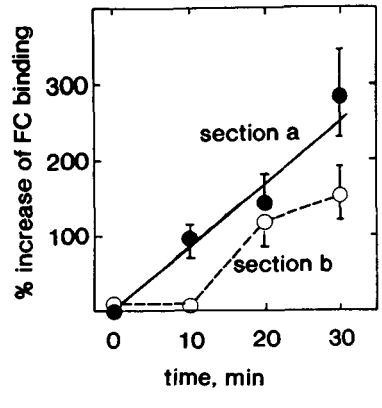

Figure 5. Time course of change in HA binding of FC to PM-enriched membranes (U_1) in response to incubation of sections for 0 to 30 min with 1 μM FC. Conditions were the same as in Figure 4.

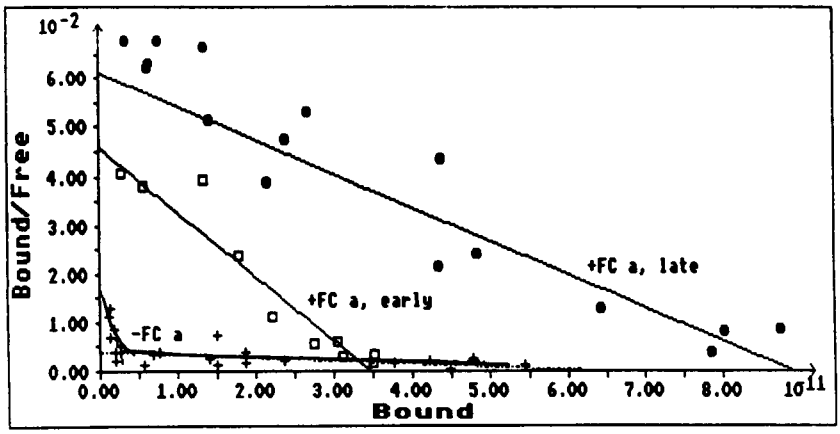

Figure 6. Effect of FC preincubation of meristematic tissue (section a) on the binding constants and B_d of FC binding, as analyzed by a Scatchard plot. Sections used as initially cut (−FC), after 0.5 to 2.0 h of incubation with 1 μM FC (early), or after 3 to 6 h with FC (late). Binding of 0.5 to 40 nM [^{3}H]dihydroFC to microsomal membranes was determined. Units of binding are mol of FC bound.

be independent of tissue age. In contrast, the K_d values for the LA sites continuously decline as the tissues age (from 16 to 1.2 nM) but remain at least 4-fold higher than the values for the HA sites. Abramycheva et al. (1993) have reported that maize roots possess a very LA FC-binding site (K_d = 1300 nM) with a B_d at least 10-fold greater than any previously reported. We can find no evidence for such a site in mung bean hypocotyls. Their use of a technique involving dilution of radioactive FC with unlabeled FC makes it possible that these sites are actually an artifact caused by nonspecific FC binding.

Neither the affinities nor the number of FC-binding sites is invariant in plant tissues. Aducci et al. (1986) showed that treatment of maize coleoptiles with 10 μM IAA for 1.5 h resulted in an increase in B_d of HA sites by as much as 50%. Because auxins have also been reported to increase the amount of PM H^+-ATPase protein in maize coleoptiles (Hager et al., 1991), it is possible that both ATPase and FCBP are incorporated into the PM together. Auxin does not cause any change in FC binding in mung bean hypocotyls, however (data not shown), and in this organ there is no correspondence between the developmentally induced changes in FCBP and in ATPase (Basel and Cleland, 1992). Aducci et al. (1980) and Ballio and Aducci (1987) found that prolonged washing of maize roots in large volumes of distilled water resulted in a 2-fold increase in FC-binding sites (probably HA sites). This increase might be due to displacement of a natural ligand from the FCBP, opening up sites for FC binding. No such washing effect seems to occur with mung bean hypocotyls, however (Fig. 4).

Aducci et al. (1984) have reported that treatment of solubilized FCBP from spinach leaves with either phosphatase or α-mannosidase results in a loss of FC binding. This suggests that the FCBP must be phosphorylated and glycosylated for maximum activity. Whether the decrease in binding was due to a decrease in B_d or an increase in K_d was not determined. It is of interest that Tognoli and Columbo (1986) have shown that a 33-kD protein in sycamore cell cultures is phosphorylated in response to FC; whether this protein is an FCBP is unknown.

In this study it was shown that as mung bean hypocotyl tissues mature, there is a 10-fold increase in B_d for the HA

Table II. *Change in densities and binding constants of FC-binding sites in response to treatment of tissues with FC*

Scatchard analysis was performed on the binding of 0.1 to 20 nM [^{3}H]dihydroFC to microsomal membranes from meristematic (section a), elongating (section b), and mature (section d/e) regions of the mung bean hypocotyl after treatment with FC for 0.5 to 2.0 h (early) or 3 to 6 h (later). B_d and K_d values were calculated by a computer program.

Section	Period	B_d		K_d	
		HA	LA	HA	LA
		pmol mg^{-1} protein		nM	
a	Initial	0.1	2.2	0.19	16
	Early FC	1.16	0	0.67	
	Later FC	3.0	0	1.5	
b	Initial	0.48	1.5	0.18	5
	Early FC	1.6	0	0.47	
	Later FC	4.8	0	1.05	
d/e	Initial	1.2	2.2	0.31	1.2
	Early FC	2.7	0	0.68	
	Later FC	7.9	0	1.6	

Table III. *Effect of CH on the FC-induced increase in HA FC-binding sites*

Scatchard analysis was performed on the binding of 0.1 to 20 nM [^{3}H]dihydroFC to microsomal membranes from meristematic (section a), elongating (section b), and mature (section d/e) regions of the mung bean hypocotyl after treatment with 1 μM FC, and with or without 20 μg mL^{-1} CH. Membranes were isolated after 0.5 to 2.0 h (early) or 3 to 6 h (late) of incubation. B_d values were determined with a computer program.

Section	CH	B_d		Increase in B_d in Later Phase
		Early phase	Later	
		pmol mg^{-1} protein		
a	−	0.90	2.3	1.4
	+	0.90	1.27	0.37
b	−	1.2	3.2	2.0
	+	1.1	2.1	1.0
d/e	−	1.8	4.9	3.1
	+	1.7	3.6	1.9

sites with no major change in K_d and a 10-fold decrease in K_d of the LA site with no change in B_d. It would appear that these developmental shifts in HA and LA sites are separate and unconnected changes. The increase in density of HA sites might involve synthesis of new receptors and/or activation of inactive precursors.

When hypocotyl sections are treated with 1 μM FC, a two-phase increase in FCBP activity is induced, regardless of the maturity of the tissue. During the first phase, which occurs within the first 60 to 90 min, there is a total loss of the LA sites, compensated in part by an increase in HA sites. The affinity of the HA sites for FC also declines somewhat. Because these changes are not affected by CH, it would appear that the LA sites have been converted into HA sites, in part, and in part have been degraded. The conversion of LA to HA sites might involve phosphorylation or glycosylation of the receptors (Aducci et al., 1984). Alternatively, a GTP-mediated disaggregation-coupling change in the receptor, similar to that of the epinephrine receptor (Rodbell, 1980), might occur. During the second phase (2–3 h after addition of FC), there is a further change in the HA sites; the B_d increases about 3-fold, whereas the affinity decreases about 2-fold. Because at least part of the change in B_d is inhibited by CH, it appears that there has been some de novo synthesis of new HA FC-binding sites. Scott (1992) reported that treatment of pea epicotyl sections with FC for 6 h resulted in an increased abundance of several mRNAs, including several giving rise to proteins of about 30 kD. One or more of these transcripts might be the FCBP.

The magnitude of the FC-induced increase in FC-binding sites may actually be greater than indicated by the data presented here. Because FC binds tightly to the FCBP (Dohrmann et al., 1977; Meyer et al., 1989; Oecking and Weiler, 1991), some binding sites may still be occupied by FC that had bound during the preincubation of live sections with FC. However, because of the duration of time needed to prepare the membrane fractions, most of the FC will probably have been dissociated from the FCBP prior to the onset of the binding assay.

Up-regulation of animal receptors by their ligands has been demonstrated for the prolactin receptor (Posner et al., 1975), the estrogen receptors in rat uterus (Mester et al., 1974), the τ-aminobutyric acid receptor (Maksay and Ticku, 1984), and the interleukin 2 receptors in response to lectins (Heckford et al., 1986). Trewavas (1980) reported that auxin receptors of Jerusalem artichoke tissue were up-regulated by the auxin 2,4-D. In each of these cases the up-regulation occurred over time spans of 1 d or more; by contrast, substantial up-regulation of the FCBP occurs within 3 h. This up-regulation occurs in response to an exogenous phytotoxin rather than to an endogenous ligand, although an endogenous FC-like ligand may exist in plants (Aducci et al., 1980; Ballio and Aducci, 1987; Muromtsev et al., 1989). No similar up-regulation of any other phytotoxin receptor has been reported.

Since the discovery of FC receptors it has been assumed that the HA sites mediated the physiological action, namely the enhanced proton excretion. Abramycheva et al. (1991, 1993) have argued that the physiological receptors are the LA sites. This is based on the fact that the K_m for FC-induced maize root elongation and K^+ uptake is similar to the K_d for the LA site and 100-fold greater than the K_d for the HA site. In mung bean hypocotyls, however, the initial rate of FC-induced proton excretion correlates with the density of HA sites as both change during tissue maturation and does not correlate with the LA B_d. In addition, after incubation of sections with FC the LA sites disappear, to be replaced by additional HA sites. Why, then, is there such a difference between the K_d values for FC binding and the K_m for proton excretion? In part this is due to the cuticle, which restricts entry of FC. When the cuticle was removed the K_m for FC-induced proton excretion from *Avena* coleoptiles was reduced 10-fold (R.E. Cleland, unpublished data). Another factor is that the physiological responses require FC to penetrate to all cells in a tissue, and this, too, increases the amount of FC needed to obtain a saturated physiological response.

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