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Culture of Transformed Horseradish Roots as a Source of Fusicoccin-Like Ligands

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Abstract. Endogenous fusicoccin (FC) or related substances were sought in horseradish (*Armoracia rusticana* P.) roots. An actively growing root culture was derived from plants transformed with *Agrobacterium rhizogenes*. The presence of FC-like substances in ethanolic extracts from roots was established in a radioreceptor binding assay with plasmalemmal FC receptors and in radioimmune analysis with an antiserum specific for FC A. FC-like ligands were found in the tissue and medium of aseptically grown culture.

The biosynthetic plant growth regulator fusicoccin (FC), produced by the phytopathogenic fungus *Fusicoccum amygdali* Del., has pronounced and versatile effects on plants. Analysis of the literature reveals that the typical tricyclic structure of the FC molecule is not unique, and related compounds are encountered widely not only among fungi but in plants as well (Muromtsev et al. 1994). Since 1980 Aducci and Ballio and co-workers have reported the presence of endogenous ligands of FC binding sites in plants (Aducci et al. 1980, Ballio and Aducci 1987; Marra et al. 1988). Indirect evidence for the presence of FC-like substances in plants has been obtained by Obrucheva and Antipova (1992) and Sultanbaev et al. (1993).

Since 1980 we have demonstrated more than once the existence of endogenous FC A in higher plants using GC/MS analysis (Muromtsev et al. 1986, 1987, 1989). Recently we showed that horseradish roots contain FC-like ligands capable of binding with antibodies and receptors specific for FC A (Babakov et al. 1994).

The stimulatory effect of FC on rhizogenesis (Sultonov and Muromtsev 1985) and its active involvement in transport processes (Aducci et al. 1988) give grounds for supposing that roots are an important site of localization of endogenous FC. However, the use of naturally obtained roots is open to the common objection about the possible extraneous (microbial) origin of FC-like substances in plant specimens. Hence, it is crucial that FC or related ligands be identified in sterile plant material.

In this connection we made use of cultured isolated roots, which have a number of properties important for such studies. Isolated roots are grown under controlled conditions, whereby the material is standardized. The culture is grown aseptically, thus precluding contamination with extraneous metabolites. Agrobacterial transformation enhances appreciably the root growth rate and biomass.

The choice of horseradish as the subject was coincidental since we already had at our disposal a transformed horseradish root culture as a potential source of peroxidase (Khadeeva et al. 1993a). However, there are reasons for believing that the FC-like ligands we found in horseradish roots would also be present in roots of other plants; at present we are expanding the range of subjects.

Abbreviations: FC, fusicoccin; GC/MS, gas chromatography/mass spectrometry; RIA, radioimmunoassay; RRA, radioreceptor analysis; BSA, bovine serum albumin; Mes, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.

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Materials and Methods

The transformation of horseradish roots was carried out using *Agrobacterium rhizogenes* (Khadeeva et al. 1993b). Mature horseradish leaves 15–20 cm long were sterilized first with 70% ethanol (5 min), then with chloramine B (20 min), and repeatedly

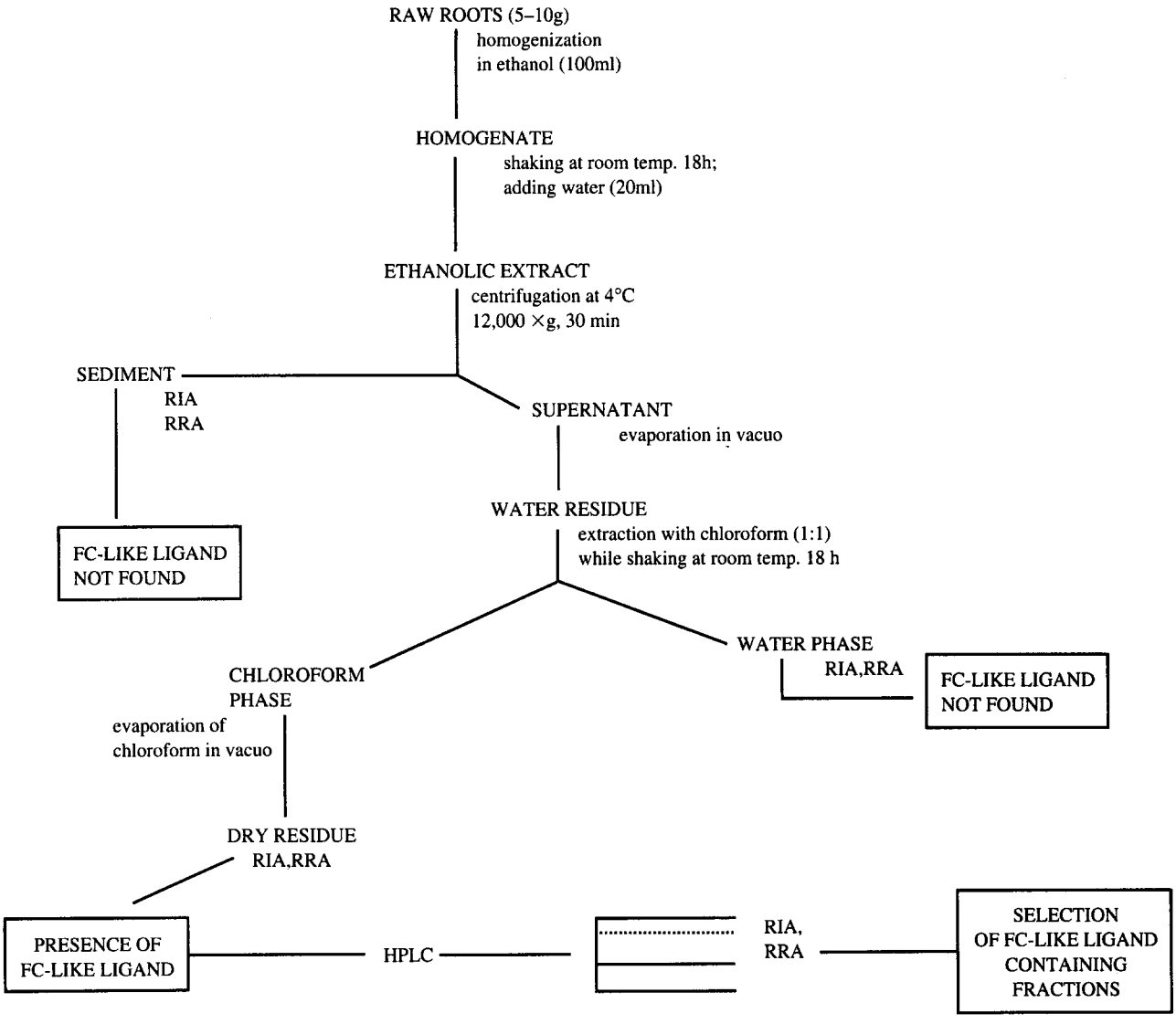

Fig. 1. Extraction of cultured roots.

with sterile distilled water. Leaves were then cut into pieces about 1 cm^2 and immersed for 10 min in a suspension of *A. rhizogenes* grown in LB medium for 18 h. Leaves were then dried on filter paper and placed in Petri dishes with agar MS medium without hormones (Murashige and Skoog 1962).

After 1–2 days, when explants were surrounded with a slight halo of bacterial growth, they were again dried and placed on the same medium supplemented with antibiotic cefotaxin (500 mg/liter). The developing roots were cut off, placed on the medium, and cultivated at 26°C for 4 weeks. Transfers were repeated four to six times until complete disappearance of the bacterial halo. Then the roots were placed on antibiotic-free MS medium. If there was no further bacterial growth the roots were passed into liquid nutritive medium B_5 without hormones (Gamborg et al. 1968). Roots were cultivated at 26°C in 0.5-liter flasks with 200 mL of medium and shaken in the dark (80 rpm). Transfers were repeated every 3–4 weeks. The rate of biomass accumulation was assessed by the growth index (the biomass at the end of passage relative to the biomass of the inoculum).

The work was carried out with a culture transferred over more than 3 years.

The presence of FC-like ligands was also examined in extracts from roots of natural soil-grown horseradish plants.

Extraction

Fresh roots (5–10 g) were homogenized in 100 mL of ethanol and shaken at room temperature for 18 h. Then 20 mL of water was added, and the mixture was centrifuged at 12,000 ×g (4°C, 30 min). After evaporation of ethanol, the ligand was extracted from the aqueous phase with an equal volume of chloroform by shaking the mixture at room temperature for 18 h. The chloroform phase was collected using a separatory funnel; the chloroform was dehydrated with sodium sulfate and evaporated. The dry material was stored at −20°C.

Specimen Processing

Chloroform and water phases were dried separately (Fig. 1), and the pellets were extracted with ethanol (1:10 w/v) at 4°C for 18 h. Extracts were centrifuged for 20 min at 10,000 ×g at 4°C. The supernatant was combined with 3–5 volumes of the assay medium (phosphate-buffered saline or 5 mM $MgSO_4$ in 20 mM HEPES/NaOH, pH 7.3, for radioimmunoassay (RIA) and radioreceptor analysis (RRA), respectively). Any precipitate was removed by centrifugation. Then serial triple dilutions (beginning with 1:50) of the supernatant were prepared, and a 10-mL aliquot of every dilution was used for competitive RRA or RIA.

Preparation of Membranes

Isolation of plant membranes was carried out in accordance with a previously described method (Abramycheva et al. 1991). Roots of *Zea mays* were homogenized in 50 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 2.5% polyvinylpyrrolidone, and 0.5% bovine serum albumin (BSA). Debris was removed by a 15-min centrifugation at 10,000 ×g, and the homogenate was centrifuged for 30 min at 80,000 ×g. The pellet was resuspended in solution S (0.33 M sucrose, 5 mM Tris-Mes, pH 7.3) and mixed with a two-phase polymer system: dextran T500, polyethylene glycol 4000 (6.3% final concentration), prepared in solution S with 3 mM KCl. After stirring and centrifugation, the upper phase was withdrawn, mixed with an equal volume of fresh lower phase, and centrifuged again. The upper phase was collected, combined with 5 volumes of solution S, and centrifuged for 30 min at 80,000 ×g. The pellet was suspended in solution S and stored at −20°C.

RRA Procedure

Synthesis of [^{3}H]dihydro-FC (3 TBq/mmol) and measurement of its binding to plasma membrane receptors were carried out as described previously (Abramycheva et al. 1991). Measurements were performed in a 200-mL final volume of medium containing 20 mM Tris-Mes, pH 7.2, 5 mM, $MgSO_4$, 1 nM [^{3}H]dihydro-FC, 15 mg of membranes, and various amounts of the tested extract. The amount of extract which inhibited [^{3}H]dihydro-FC binding by 50% was determined. We made an arbitrary presumption that the affinity of the endogenous ligand to the FC receptor is equal to that of FC A. Thus, various concentrations of endogenous ligand could be calculated simply and expressed in conditional FC units, using the calibration curve obtained for FC A. The extent of nonspecific binding of [^{3}H]dihydro-FC was assessed in the presence of 10 mM nonlabeled FC A.

Immunization Procedure

Conjugation of FC A with BSA was carried on according to Feyerabend and Weiler (1987). The conjugate contained 1.7 mol of fusicoccin/mol of BSA, as judged by spectrophotometry in concentrated H_2SO_4.

Rabbits were immunized with 1 mg of FC-BSA in complete Freund's adjuvant, injected subcutaneously in two popliteal lymph nodes. Booster injections were made four times at 30-day intervals. Rabbits were bled at the 7th and 9th days after the last injection. The titer of antifusicoccin antibodies was 1:2,000 in RIA.

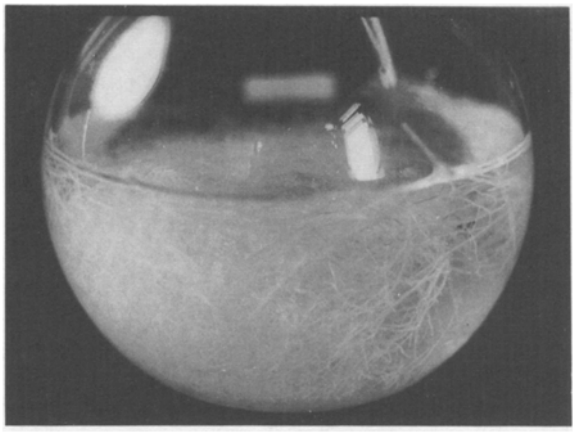

Fig. 2. Hairy roots.

RIA Procedure

Inhibition of [^{3}H]dihydro-FC binding to antibodies in the presence of various amounts of horseradish root ethanolic extract was measured in a 0.2-mL final volume of phosphate-buffered saline at room temperature. Each tube received 1 mL of rabbit antiserum and 1 nM [^{3}H]dihydro-FC. After a 1-h incubation, antigen-antibody complexes were precipitated by adding 0.2 mL of trichloracetic acid. Free labeled FC was removed by ultrafiltration (Synpor membrane filters, 0.85-mm pore diameter, Chemapol, Czech Republic). The extent of nonspecific binding of [^{3}H]dihydro-FC was assessed in the presence of 1 mM nonlabeled FC A. As in RRA, the amount of extract which inhibited [^{3}H]dihydro-FC binding by 50% was estimated. The concentration of endogenous ligand was calculated taking that 10^{-10} M reference FC A caused 50% inhibition of [^{3}H]dihydro-FC binding to antifusicoccin antibodies.

Results and Discussion

Upon transformation, the horseradish root culture acquired some essential distinctive features. Phenotypically it was a conglomerate of rapidly growing and highly branching roots, known in the literature as *hairy roots*. Such roots generally display negative geotropism and hormone independence, and we also observed these properties in our culture (Fig. 2). Furthermore, the rate of biomass accumulation in this culture was about four times higher than with normal roots. These features permitted the material obtained to be classified as transgenic.

When the medium was inoculated with transformed roots, after a lag period of 1–5 days the roots started growing, producing a highly ramified tangle. Biomass accumulation peaked at days 10–18. In 21 days, the growth index reached 8–12, and the flask was filled with the root mass, which thereupon ceased to grow, as shown by measurements after 30, 40, and 60 days of culturing.

Table 1. Concentration of FC-like ligands in transformed horseradish root culture.

Passage	Culture age (days)	Root biomass (g wet wt)	Ligand content (nmol/kg)[a] RRA	RIA
A	14	6.3	17	15
B	14	6.8	150	117
A	21	9.9	10	3
C	30	9.6	75	58
A	40	7.5	4	2
D	60	10.1	90	70

[a] Calculated on the basis of arbitrary presumption that the dissociation constant of endogenous ligand is equal to that of FC A, in FC units.

Cultured roots were extracted as outlined in Fig. 1. Both RIA and RRA testified that fusicoccins (and/or FC-like ligands) go mainly to the chloroform phase, with only trace amounts found in the aqueous phase.

The FC-like ligands in transformed horseradish roots were assayed from 14 to 60 days of culturing. The relevant data for different passages are given in Table 1. As can be seen, FC-like ligands are detectable at any culture age. However, the quantitative variations are great and do not allow any definite conclusions concerning the kinetics of their production. It should be noted that the RIA and RRA estimates are in good agreement.

FC-like ligands were also found in the culture medium, presumably originating from the roots.

Quantification of FC-related substances in the roots of natural soil-grown plants yielded about 30 nmol/kg, which is comparable to the values for transformed cultured roots. This also argues in favor of the endogenous origin of FC in higher plants.

According to the preliminary data on the fractionation of the ethanolic extract by HPLC (in a methanol/water gradient), there are several fractions positive in a competitive RIA for FC; one of these coincides with FC A in chromatographic behavior. The latter fraction was examined by GC/MS analysis, which showed the presence of FC A as well as some other substances closely related to FC A.

Studies of endogenous fusicoccins are still scarce, and the conclusions are sometimes negative. There may be several reasons for such discrepancies. Apparently, if the content of the ligand in a sample is below the threshold of RIA or RRA sensitivity, then its detection and characterization become rather complicated.

Another important feature is the use of a highly sensitive modified RIA technique (the detection limit for FC A is about 2×10^{-11} M), which is more sensitive and feasible than the GC/MS approach we used earlier to detect FC A (Muromtsev et al. 1986). A serious shortcoming of GC/MS is that the entire identification procedure is aimed at a single compound (FC A), which may simply be absent from the plant material under study. In contrast, the immunochemical technique permits detection not only of FC A but also other FC-like ligands possessing similar antigenic determinants. Moreover, the sensitivity of RIA was found to be markedly higher than that of another previously used method, RRA.

On the other hand, FC-like ligands may have receptors different from those specific for FC A, or they may not react with antibodies against FC A. This phenomenon has been shown already for different gibberellins. Feyerabend and Weiler (1987) have reported widely divergent cross-reactivity of several FC-like substances with FC receptors and antifusicoccin antibodies.

The results of the research already carried out this way create the impression that FC A is only one representative of endogenous FC-related ligands in plants. Appropriate here is the consistently observed analogy between fusicoccins and gibberellins: the most commonly known GA_3 is not always present in plants, and the main endogenous gibberellins (phytohormones) are others, for example, GA_1, GA_5, GA_{20}, and GA_{32} (Pharis and King 1985). The major metabolites of the phytopathogenic fungi *Gibberella fujikuroi* and *Fusicoccum amygdali*, GA_3 and FC A, are perhaps closer to phytotoxins than to phytohormones, in view of their extremely high bioactivity and wide ranging action. Typical endogenous plant gibberellins and fusicoccins should be more specialized and should have more moderate activity. Probably the endogenous FC A we have found earlier in plants is just a single element of a multiple set of ligands or a hormonal complex. At present we are accumulating experimental data to support this suggestion.

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