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Endogenous fusicoccin-like ligand revealed in higher plants by radioreceptor and radioimmunoassays

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Abstract Radioimmuno- and radioreceptor assays were developed for quantitating fusicoccin-like substances in plants. FC-like ligands were found in cultured horseradish roots (70–90 pmol/g) and headed cabbage leaves (9–11 pmol/g). Detection of FC-like ligands in sterile root culture further argues in favour of endogenous fusicoccin representing a new type of phytohormone.

Key words: Fusicoccin; Receptor; Endogenous ligand; Phytohormone

1. Introduction

Fusicoccin, a metabolite of the fungus *Fusicoccum amygdali* Del., exhibits pronounced effects on plant growth and development: it speeds up seed germination, promotes plant growth [1], and enhances their resistance to stress [2]. At the cell level, FC activates proton extrusion from cells and blocks outward-rectifying K^+ channels in plasma membranes [1,3]. The primary target of FC are plasma membranes carrying FC receptors [4].

Since the late seventies we have been engaged in the search for endogenous fusicoccins in plants. The premises for this endeavour are as follows. (i) There is a striking analogy between fusicoccins and the well-known phytohormones gibberellins. Both are diterpenoids comprising groups of closely related compounds; both have a broad spectrum of physiological activity; both have been discovered as metabolites of phytopathogenic fungi. Gibberellins were detected in higher plants and thus acquired the status of phytohormones about a quarter of a century after their identification as metabolites of the fungus *Gibberella fujikuroi*. Fusicoccin was discovered by Ballio in 1964 [5]. (ii) Analysis of the literature [6] shows that the typical core of the FC molecule is not at all unique, but is widely encountered among both fungal and plant metabolites. Hence the enzymic systems making this peculiar group of diterpenoids are rather common. (iii) Receptors for FC are widespread in plants [4].

In 1980 we obtained the first evidence for the existence of endogenous FC in higher plants [7]. Then, combining HPLC and GC/MS analysis, we managed to demonstrate the presence of endogenous FC A (1–10 pmol/kg) in headed cabbage leaves and in maize roots and cobs [8,9]. Later, the presence of an endogenous FC-like ligand in plants was confirmed by Ballio et al. [10]. However, we still lack the data necessary for characterizing any photyhormone: how widely the endogenous FC is encountered in plants, where it is located in the plant, how its content changes during plant ontogeny, in what forms it is present, etc. To this end, we have developed highly sensitive quantitative assays for FC-like ligands based on interaction with FC receptors and anti-FC antibodies; this paper reports the first data obtained.

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Abbreviations: FC, fusicoccin; RIA, radioimmunoassay; RRA, radioreceptor assay; GC/MS, gas chromatography/mass spectrometry.

2. Experimental

2.1. Objects

Plasma membranes were isolated from the roots of maize cv. Nadnepryanskaya 4-day seedlings [11], suspended in 5 mM Tris-HCl, pH 7.3, 1 mM EDTA, 40% (v/v) glycerol, and stored at −20°C. Transgenic horseradish root culture was derived by transforming *Armoracia rusticana* leaf disks with wild-type *Agrobacterium rhizogenes* [12]. The culture was used at the age of 45 days.

2.2. Radioreceptor and radioimmunoassays

[^{3}H]DihydroFC (sp.act. 3 TBq/mmol) was synthesized as described earlier [11].

For immunization, FC was activated by converting it to an aldehyde with periodate, and conjugated to BSA with $NaBH_4$ [13]. The product contained 1.7 mol FC per mol BSA, as assayed spectrophotometrically in conc. H_2SO_4 [13].

Antibodies against FC were raised by immunizing rabbits with the FC–BSA conjugate (1 mg in complete Freund's adjuvant) into popliteal lymph nodes and subcutaneously into 4 points on the back, followed by 4 booster injections after 30 days. Blood was taken on the 7th and 9th day after the last booster. In RIA the sera had a titre of 1:2,000.

[^{3}H]DihydroFC binding with plasma membranes was assayed as described earlier [11]. One sample (final volume 200 μl, pH 7.2) contained 15 μg plasma membrane protein, 1 nM [^{3}H]dihydroFC, and different amounts of the tested extract. The amount of extract inhibiting [^{3}H]dihydroFC binding by 50% was used to calculate the concentration of the endogenous ligand, using a calibration curve on the assumption that the ligand affinity for the FC receptor is the same as that of reference FC.

Binding of [^{3}H]dihydroFC (1 nM) with antibodies was carried out in 200 μl PBS for 60 min at room temperature, using 1–2 μl of serum and varying amounts of the extract. After the incubation, an equal volume of 15% TCA was added, and non-bound label was removed by vacuum filtration through 0.85-μm nitrocellulose filters. Non-specific [^{3}H]dihydroFC binding was determined by adding 1 μM free FC or using an equal volume of control serum. Similarly to RRA, the amount of the ethanolic extract inhibiting [^{3}H]dihydroFC binding by 50% was used to calculate the concentration of the endogenous ligand.

2.3. Extracts

Horseradish roots (10–50 g) or cabbage leaves (50 g) were homogenized in 200 ml ethanol with a Waring Blender. The homogenate was shaken for 18 h at room temperature, filtered through Miracloth, and centrifuged for 30 min at 12,000 × *g*. The supernatant was combined with 20 ml of water, and ethanol was removed in a rotary evaporator.

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The aqueous residue was mixed with an equal volume of chloroform and shaken for 16 h. The aqueous phase was lyophilized, and the chloroform phase was vacuum-dried; the dry residues were stored at −20°C. Then, 10–20 mg of the residue was extracted with ethanol (1:10, w/v) for 16 h at 4°C in an Eppendorf 5432 mixer and centrifuged for 20 min at 14,000 rpm, 4°C in an Eppendorf 5415 centrifuge. The supernatant was combined with 3–5 vol. of the [^{3}H]dihydroFC binding assay medium, and the precipitate formed was removed in an Eppendorf centrifuge. After making appropriate dilutions, 10 μl-aliquots were taken for [^{3}H]dihydroFC binding competition assays.

2.4. Partition coefficient

[^{3}H]DihydroFC was added to 2 ml of chloroform/water (1:1) to a final concentration of 10 nM; the mixture was left for 20 h at 4°C, then 100-μl aliquots were withdrawn from each phase, transferred to scintillation vials, and counted for radioactivity.

3. Results

The sensitivity of the RIA and RRA techniques is conditioned by two parameters: the affinity of receptors (antibodies) to the ligand under study, and the specific activity of the labeled ligand. Under optimal conditions of [^{3}H]dihydroFC synthesis [11], it had a sp.act. of 3 TBq/mmol (80 Ci/mmol), which permits FC assay in the 0.1–1.0 nM range. Our immunization protocol with booster injections [14] yielded polyclonal antibodies with 2.5-fold higher affinity as compared with that of monoclonal antibodies obtained by other authors [12]. Since receptor affinity for FC is only slightly dependent on the source of plasma membranes [4], we used maize root membranes which we have extensively studied in respect of the FC receptors [15]. To improve the assay, we first incubated the plasma membranes with the ethanolic extract and then introduced [^{3}H]dihydroFC; this doubled the RRA sensitivity.

Fig. 1 depicts the results of competitive binding assays for reference FC and the endogenous FC-like ligand. It also gives the respective dissociation constants calculated from the concentration dependencies of [^{3}H]dihydroFC binding with plasma membranes (Fig. 1a) and anti-FC antibodies (Fig. 1b). The higher affinity of antibodies vs. receptors pre-determines the higher sensitivity of RIA: thus the FC assay range is 0.05–0.5 ng (0.075–0.75 pmol) in RIA and 0.1–1.0 ng (0.15–1.5 pmol) in RRA. According to the RRA data, 50% inhibition of [^{3}H]dihydroFC binding is produced by 0.27 ng of reference FC or 0.12 μl of the ethanolic plant extract; the respective values in RIA are 0.18 ng and 0.10 μl. Similar determinations were conducted

Table 1
Quantitation of FC in ethanolic solutions obtained upon chloroform–water fractionation of plants extracts

Material and fraction	Yield (mg/g tissue)	Partition coefficient	FC-like ligand (pmol/g tissue)	
			RIA	RRA
Horseradish roots				
Chloroform[1]	3.5	15 (14)*	66	84
Water[2]	4.7		4	6
			70	90
Cabbage leaves				
Chloroform[1]	2.2	nd	9	11
Water[2]	66		nd	

[1]Residue after evaporation of the chloroform fraction.
[2]Residue after lyophilization of the aqueous fraction.
*Chloroform–water partition coefficient for endogenous ligand; parenthesized is the same for [^{3}H]dihydroFC.

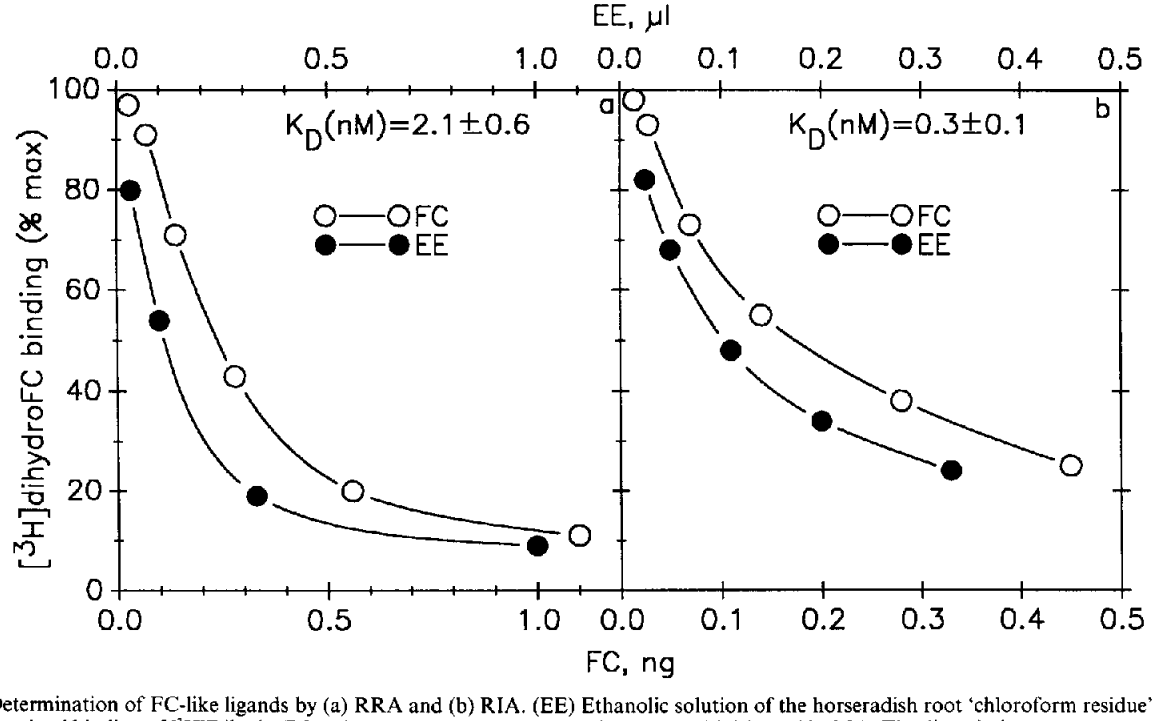

Fig. 1. Determination of FC-like ligands by (a) RRA and (b) RIA. (EE) Ethanolic solution of the horseradish root 'chloroform residue' (see section 2). The maximal binding of [^{3}H]DihydroFC (taken as 100%) was 0.02 pmol in RRA and 0.06 pmol in RIA. The dissociation constants were computed using an Enzfitter program from the concentration dependences of [^{3}H]dihydroFC binding measured in independent experiments.

with ethanolic extracts of the 'aqueous fraction' of horseradish roots and the 'chloroform fraction' of cabbage leaves (see section 2); the data are listed in Table 1. The table also presents the chloroform–water partition coefficients for [^{3}H]dihydroFC (determined experimentally) and FC-like ligand (calculated from the tabular data).

4. Discussion

The choice of the object for this work (cabbage leaves and transformed horseradish root culture) was not random. On the one hand, we wanted to compare the results of the RIA and RRA for FC-like ligands with our earlier data for cabbage [8,9]. On the other hand, the use of a sterile culture obviates the routine question of possible infection, e.g. with root microflora. Furthermore, we have initially given preference to the root as a source of endogenous FC [7] in view of the probable physiological role of this substance. The concentration of FC-like ligands in cultured horseradish roots (70–90 nmol/kg) proved eight-fold higher than in cabbage (see Table 1) and approached the range of physiologically active FC concentrations (0.1–10 μM) [11].

The data obtained allow preliminary conclusions concerning the chemical nature of the ligands revealed. First of all it should be noted that the FC-like ligands have a relatively low molecular weight and are non-polar, as they are much more soluble in chloroform than in water (15:1). The same partition coefficients together with the nice correspondence of the RIA and RRA determinations indicate that the endogenous ligands are chemically close to FC. At the same time, it is noteworthy that the content of endogenous FC-like ligands in cabbage leaves according to RIA and RRA is two orders of magnitude higher than the concentration of FC A determined earlier with GC/MS [8,9]. One of the causes of this discrepancy may be that in cabbage the FC-like ligand is represented not only by FC A but also by related compounds such as FCs B, C, D, and J (see FC classification in [15]) and probably by others not yet identified, which have the same affinity for the FC receptor [11]. Here again we can see a fair analogy with gibberellins of fungal and plant origin.

Pursuing this analogy, one may think that, much as the GA_3 is common in practical use is not at all a major endogenous gibberellin (although its close relative GA_1 is [17]), FC A is not the prevailing ligand in plants. Nevertheless, our quantitative data on the content of FC-like ligands in plants together with the evidence for the wide occurrence of FC receptors allow for the suggestion that plants harbour an operative hormonal system based on a novel group of phytohormones - fusicoccins.

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References

[1] Marré, E. (1979) Annu. Rev. Plant Physiol. 30, 273–288.
[2] Muromtsev, G.S., Sultonov, Yu.S. and Kazakova, V.N. (1989) Vestnik Selkhos Nauki 1, 141–144.
[3] Blatt, M.R. and Clint, G.M. (1989) Planta 178, 509–523.
[4] Aducci, P., Ballio, A., Fogliano, V., Fullone, M.R., Marra, M. and Verzili, D. (1992) in: Transport and Receptor Proteins of Plant Membranes (Cooke, D.T. and Clarkson, D.T. Eds.) Plenum Press, New York, p. 179–184.
[5] Ballio, A., Chain, E.B., De Leo, P., Erlanger, B.F., Mauri, M. and Tonolo, A. (1964) Nature 203, 297.
[6] Muromtsev, G.S., Voblikova, V.D., Kobrina, N.S., Koreneva, V.M., Krasnopolskaya, L.M. and Sadovskaya, V.L. (1994) J. Plant Growth Reg. 13, 39–40.
[7] Muromtsev, G.S., Kobrina, N.S., Voblikova, V.D. and Koreneva, V.M. (1980) Izv. AN SSSR, Ser. Biol. 6, 897–902.
[8] Muromtsev, G.S., Voblikova, V.D., Kobrina, N.S., Koreneva, V.M., Sadovskaya, V.L. and Stolpakova, V.V. (1987) Fiziologiya Rastenii 34, 980–987.
[9] Muromtsev, G.S., Voblikova, V.D., Kobrina, N.S., Koreneva, V.M., Sadovskaya, V.L. and Stolpakova, V.V. (1989) Biochem. Physiol. Pflanzen. 185, 261–268.
[10] Marra, M., Aducci, P., Fogliano, V., Fullone, M.R. and Verzili, D. (1990) Physiol. Plant. 79, 184.
[11] Abramycheva, N.Yu., Babakov, A.V., Bilushi, S.V., Danilina, E.E. and Shevchenko, V.P. (1991) Planta 183, 315–320.
[12] Maisuryan, A.N., Khadeeva, N.V. and Bobkova, A.F. (1993) Fiziologiya Rastenii 40, 273–277.
[13] Feyerabend, M. and Wieler, E.W. (1987) Plant Physiol. 85, 835–840.
[14] Eisen, H.N. and Siskind, G.W. (1969) Biochemistry 3, 996–1000.
[15] Abramycheva, N.Yu., Nesterenko, M.V. and Babakov, A.V. (1993) Planta 189, 301–305.
[16] Ballio, A. (1977) in: Regulation of Cell Membrane Activity in Plants (Marré, E and Ciferri, O. Eds.) Elsevier, Amsterdam, p. 217–223.
[17] Pharis, R.P. (1985) Annu. Rev. Plant. Physiol. 36, 517–568.