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Fusicoccin-Like Ligands in Higher Plants

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Abstract. Fusicoccin-like ligands in higher plants were sought by combining high performance liquid chromatography with radioreceptor analysis and radioimmunoassay. Such substances were found in genetically transformed cultured roots of horseradish, alteus and lupine as well as in native horseradish, cucumber, horse chestnut, and maize plants. In root crops such as carrot and sugar beet or in potato tubers, only traces of fusicoccin-like ligands were detected. Fusicoccin A was detected in genetically transformed cultured roots of horseradish by gas chromatography/mass spectrometry.

Key Words. Fusicoccin-like ligands—Radioreceptor analysis—Radioimmunoassay

The study of endogenous fusicoccins (FC) and fusicoccin-like substances has been going on for quite some while, yet the progress is rather slow. The first data on the possible presence of physiologic (Gronewald et al. 1979) and chemical (Muromtsev et al. 1980) FC analogs in higher plants were reported about 15 years after Ballio et al. (1964) found FC A in the culture medium of phytopathogenic fungus *Fusicoccum amygdali* Del. The finding and characterization of receptors for FC prompted a search for endogenous ligands (Aducci et al. 1980, 1995).

Two distinct approaches can be used to detect endogenous bioactive substances present in minor amounts (Muromtsev 1996): (1) conventional physicochemical identification, e.g. gas chromatography/mass spectrometry (GC/MS); (2) application of highly sensitive bioaffinity tests such as radioreceptor analysis (RRA) and radioimmunoassay (RIA), to reveal specific ligands for already known receptors. In both cases the identification is carried out on samples resolved by HPLC. With RRA and RIA one can screen a broad spectrum of compounds, and their sensitivity exceeds that of GC/MS.

In a series of works (Aducci et al. 1980, 1990, Ballio and Aducci 1987) endogenous ligands to FC receptors were reported in maize (*Zea mays* L.) roots and coleoptyles and in spinach (*Spinacia oleracea*) leaves. However, the amount of the isolated substance was not enough for complete chemical and biological characterization.

The use of RIA for detection of hormones in higher plants was advanced by Weiler (1979), and soon after that Federico et al. (1981) applied it to FC A detection in a competitive test with [^{3}H]dihydro-FC.

Since the mid 1980s 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). In one of the recent works (Babakov et al. 1995) we have proved the horseradish transgenic root culture to contain fusicoccin-like ligands (FCLLs), using both RRA and RIA. Our preliminary data (GC/MS) also suggested the presence of FC A in one of HPLC fractions.

In this work we report the results of our further work along this line on a larger number of plants.

Materials and Methods

Horseradish (*Armoracia rusticana* P.) plants were grown in open ground, and leaves and roots were examined. Horseradish-transformed root cultures were obtained as reported previously (Babakov et al. 1995).

Seeds of cucumber (*Cucumis sativus* L.) and maize were germinated for 4 days in the dark at 26°C. Primary roots (for cucumber) and sprouts (for maize) were used in routine extraction.

Abbreviations: FC, fusicoccin(s); GC/MS, gas chromatography/mass spectrometry; RRA, radioreceptor analysis; RIA, radioimmunoassay; HPLC, high performance liquid chromatography; FCLLs, fusicoccin-like ligands; PBS, phosphate-buffered saline; SIM, selected ion monitoring; TMS, tetratrimethylsilyl.

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Axial organs of horse chestnut (*Aesculus hippocastanum* L.) were kindly provided by Dr. N. Obrucheva. After 3 months in wet sand at 4°C, germinated seeds were cut to isolate the axial organs, which were then stored in liquid nitrogen until extraction (Obrucheva 1992).

Cultured roots of alteus (*Althaea officinalis* L.) and lupine (*Lupinus polyphyllus* L.) were transformed with *Agrobacterium rhizogenes* strain 15834 (Kuzovkina 1993). Potato tubers (*Solanum tuberosum* L.), sugar beet (*Beta vulgaris* L.), and carrot (*Daucus carota* L.) were also examined.

Primary screening of FCLLs in chloroform extracts was performed using RIA and RRA (Babakov et al. 1994). The concentration of FCLLs was assessed by competition with authentic radiolabeled FC as a reference and expressed in conventional FC units (Babakov et al. 1995).

Extraction

Fresh plant material (10–50 g) was homogenized in 100 mL of ethanol at room temperature for 18 h. Then 20 mL of water was added, and the mixture was centrifuged at 4°C for 30 min. Ethanol was evaporated, an equal volume of chloroform was added to the aqueous phase, and the mixture was shaken for 18 h at 4°C. The chloroform phase was collected, dehydrated with sodium sulfate, and evaporated. The dry material was stored at −20°C.

Sample Preparation for RIA and RRA

The dry residue of the chloroform extract (3–10 mg) was shaken with ethanol (1:10 w/v) at 4°C for 18 h. The mixture was centrifuged for 20 min at 9,000 ×*g* at 4°C. The supernatant was combined with 3–5 volumes of the assay medium (phosphate-buffered saline, pH 7.2 (PBS), or 5 mM $MgSO_4$ in 20 mM HEPES/NaOH, pH 7.2, for RIA and RRA, respectively). Any precipitate was removed by centrifugation. The supernatant was used for RIA and RRA.

RIA Procedure

Inhibition of [^{3}H]dihydro-FC (1 nM) binding to rabbit anti-FC antibodies in the presence of various amounts of the sample was measured in a final volume of 0.2 mL of PBS (Babakov et al. 1994). Series of threefold dilutions of each sample in PBS, starting with 1:50, were prepared, and 0.05 mL from each dilution was used for RIA. Each measurement was done in triplicate.

RRA Procedure

Inhibition of [^{3}H]dihydro-FC (1 nM) binding to plasma membrane receptors (0.015 mg) in the presence of various amounts of the sample was measured in a 0.2 mL final volume of medium containing 5 mM $MgSO_4$ in 20 mM HEPES/NaOH, pH 7.2. Series of threefold dilutions of each sample in the above buffer, starting with 1:3, were prepared, and 0.02 mL from each dilution was used for RRA. Each measurement was done in triplicate.

Sample Preparation and HPLC

The dry residue obtained after evaporation of chloroform was dissolved in methanol and stirred for 1 h at 4°C. After a 30-min centrifugation at 9,000 ×*g*, the supernatant was combined with 3 volumes of water and left for 30 min at 4°C. The precipitate was again removed by centrifugation, and the supernatant was adjusted to 50% in methanol. After a 30-min cooling at 4°C, the precipitate was removed by a 30-min centrifugation at 70,000 ×*g*, and the supernatant was fractionated by HPLC (Gilson, France) on a reversed phase Zorbax column (4.6 × 250 mm) thermostated at 45.5°C, with detection at 240 nm. Elution was with a 50–100% methanol gradient over 30 min followed by 45 min of pure methanol. Fractions were collected every 3 min at 1 ml/min.

GC/MS

MS identification of FC A was performed as described earlier (Muromtsev et al. 1987). GC/MS analysis was performed using a Hitachi M-80A mass spectrometer equipped with an M-003 computerized data processing system. The column was 2% OV-1 on GasChrom Q 100–120 mesh, column temperature of 300°C with preheating from 270 to 300°C at 10°C min^{-1}. The injector and separator temperature was 250°C. Ionization was at 70 eV. The HPLC fraction was silylated, and the mixture of trimethylsilyl derivatives was subjected to GC/MS analysis in the selected ion monitoring mode (SIM).

Results and Discussion

Primary screening for FCLLs in chloroform extracts from plant samples was performed using RIA and RRA, with a sensitivity threshold of about 0.1 ng (Babakov et al. 1994).

FCLLs were reliably detected by RIA and RRA (>10^{-9} mol/kg tissue) in the extracts from native horseradish roots (4 extracts) and leaves (1 extract), transgenic horseradish roots (13 extracts), as well as from cucumber roots (3 extracts), maize seed sprouts (10 extracts), horse chestnut axial organs (2 extracts), and transgenic root cultures of lupine (3 extracts) and alteus (4 extracts). In root crops (carrot, 2 extracts), sugar beet, (2 extracts), and potato tubers (3 extracts) only traces of FCLLs were found (about 5×10^{-10} mol/kg). The content of FCLLs in extracts obtained from different batches of transformed cultures was rather variable (see also Babakov et al. 1995). This is quite understandable because the root culture exhibits pronounced morphologic and, most likely, biochemical heterogeneity; thus the inoculi taken for individual flasks can hardly be identical.

When the FCLL content in a dry residue of extract exceeded 0.2 ng/mg (in conventional FC units), this extract was subjected to HPLC, and the fractions were again tested by RIA and RRA. Figs. 1 and 2 depict the results for HPLC-fractionated extracts from transformed and native horseradish roots (Fig. 1, *A* and *B*), transformed roots of lupine and alteus (Fig. 1, *C* and *D*), and cucumber roots, horse chestnut seed axial organs, and maize seed sprouts (Fig. 2, *A–C*).

In most cases the substances revealed by RIA were less polar (*right* side of each figure), whereas those detected by RRA were more polar (*left* side).

In a number of cases, instead of the expected inhibition of labeled FC A binding, the latter was enhanced, usually in RRA. This fact, which at first sight looks paradoxical, can be explained as follows. HPLC fractions obviously contain a complex mixture of different substances, some of which might act as enhancers, e.g. by altering allosterically the conformation of the recep-

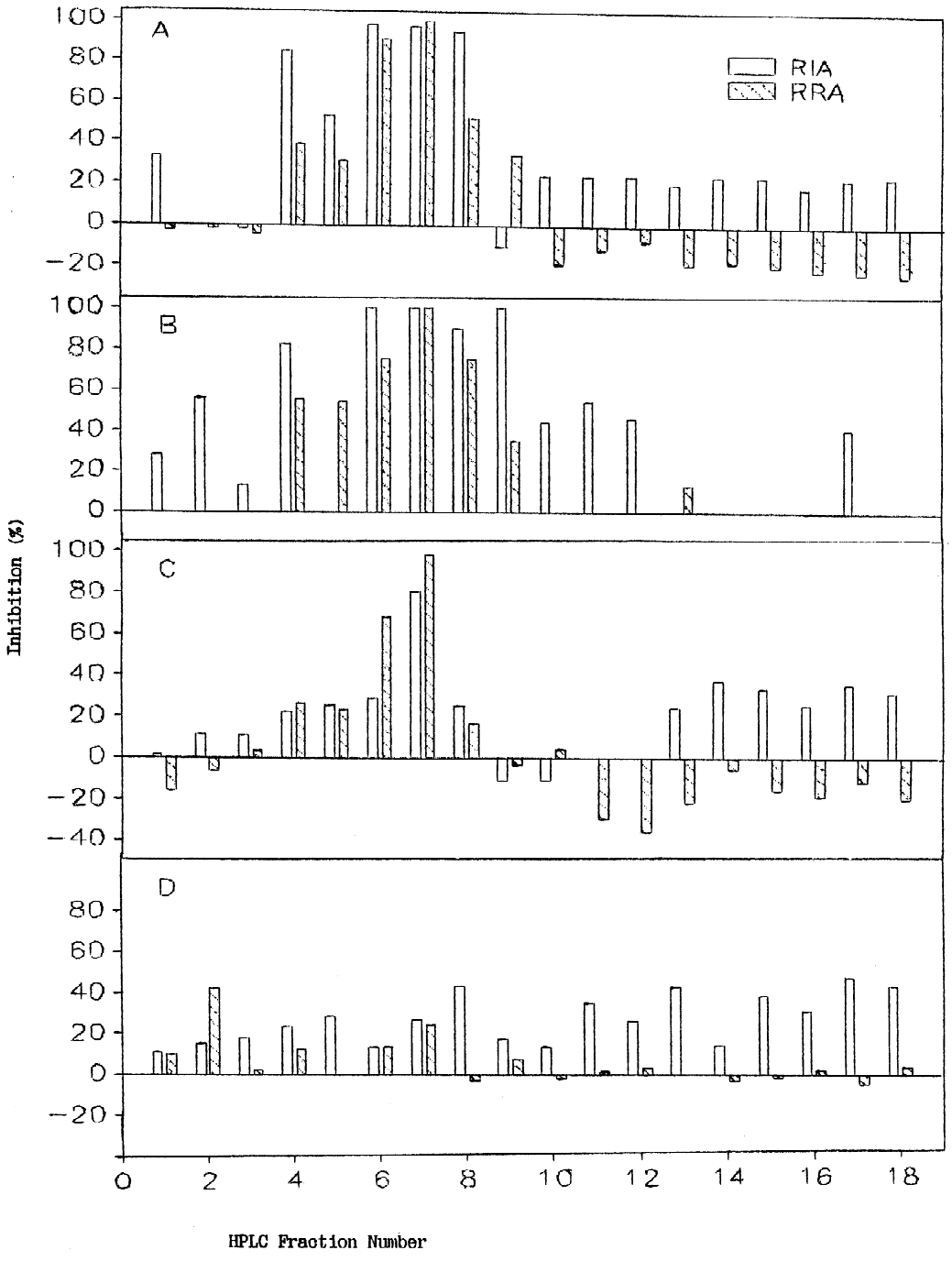

Fig. 1. HPLC of FC-like ligands in the higher plants. Inhibition of [^{3}H]dihydro-FC binding in RIA and RRA tests. *A,* horseradish native roots; *B,* horseradish-transformed root culture; *C,* lupine-transformed root culture; *D,* alteus-transformed root culture.

tor. Hence the RRA data in some cases may result from a superposition of two opposite processes: competition and enhancement.

Under the conditions used, the retention time for FC A was 18–21 min, which corresponds to fraction 7 (see Figs. 1 and 2). It was just in this fraction that FCLLs were detected in almost all cases by both RIA and RRA, often with good correlation between them.

We examined this fraction by GC/MS, using SIM with tetratrimethylsilyl FC A (TMS-FC A): *m/z* 253, 289, 349, 385, 409, 410, 715, and 716. The method has been thoroughly described earlier (Muromtsev et al. 1987, 1989).

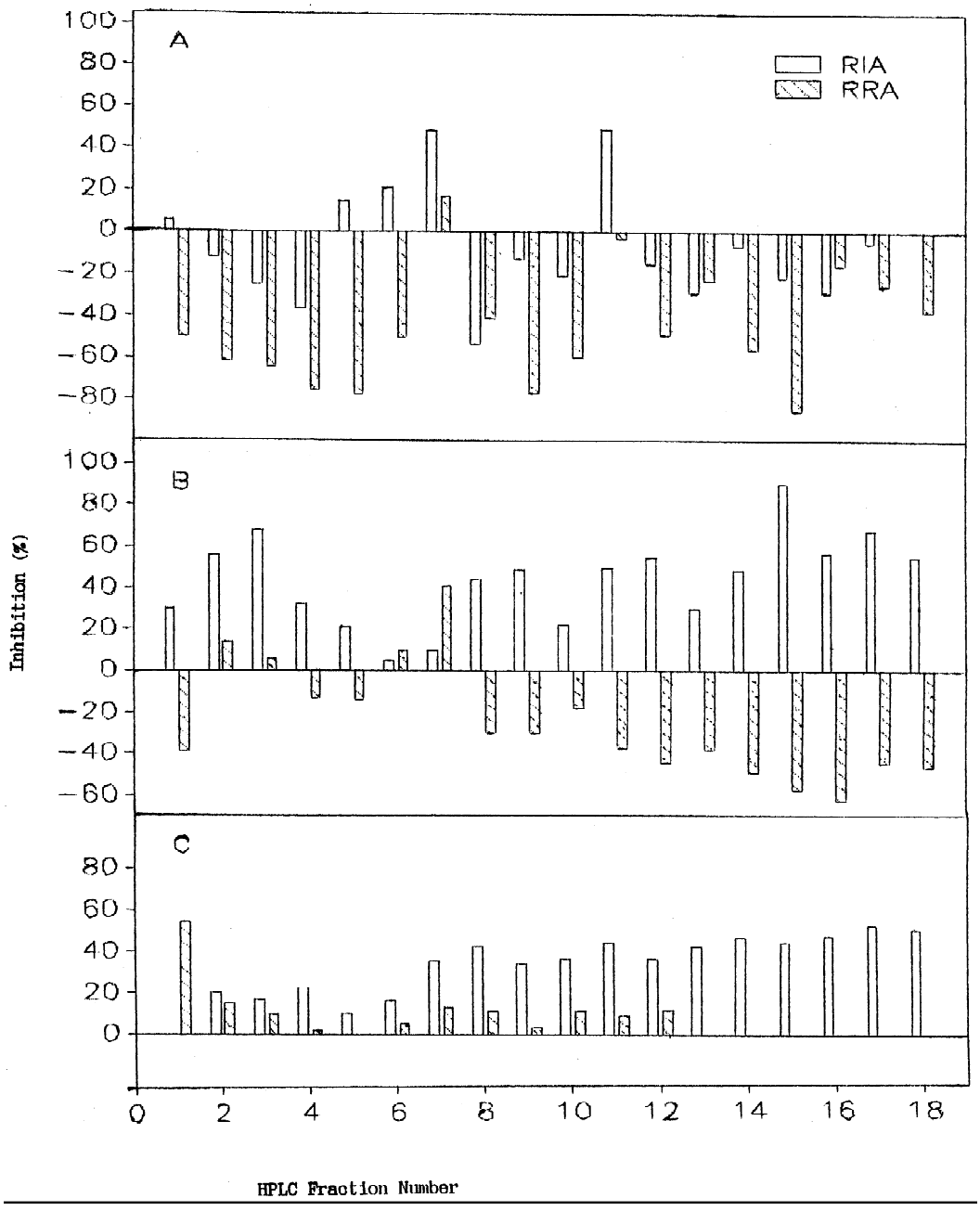

Fig. 2. HPLC of FC-like ligands in higher plants. Inhibition of [^{3}H]dihydro-FC binding in RIA and RRA tests. *A*, cucumber seeds; *B*, horse chestnut axial organs; *C*, maize seeds.

As shown in Fig. 3, the SIM patterns of fraction 7 from the horseradish transgenic root extract match with TMS-FC A in retention time (5.7 min, *left vertical line*) and show a similar set of ions, suggesting the presence of FC A in horseradish transgenic root extract.

Another set of additional ions can be seen in Fig. 3, which appears somewhat later (6.3 min). Considering our experience in GC/MS analysis of FC metabolites in *F. amygdali* culture media, these probably represent an FC A isomer yielding a mass spectrum almost similar to that of FC A although differing slightly in ion intensities (Sadovskaya et al. 1986).

Fraction 7 of the extract from transgenic alteus was also studied. However, neither FC A nor closely related substances were found. Probably their concentration in the sample was lower than the GC/MS sensitivity threshold, which for our device was 30 ng/sample. It should be noted that in much more numerous experiments with

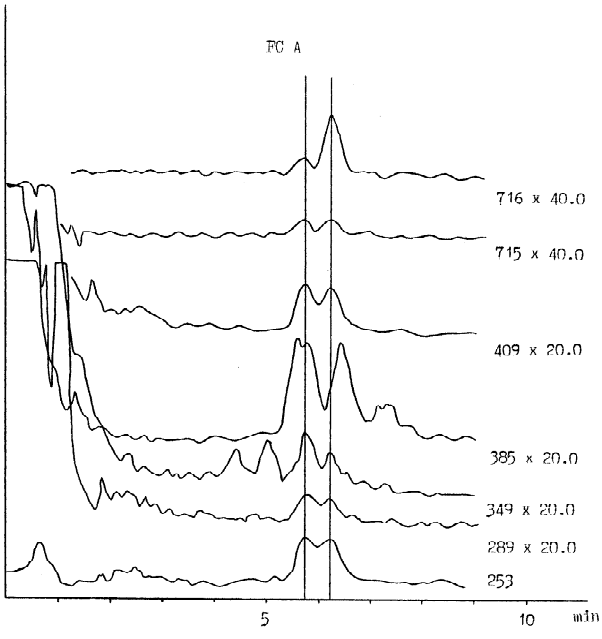

Fig. 3. GC/SIM profiles identifying FC A in HPLC fraction 7 from the horseradish-transformed root culture.

transgenic horseradish roots, only twice could FC A be detected with GC/MS.

All of these data suggest the existence of a multiple set of FCLLs in higher plants. These substances, as well as already known FCs, are highly soluble in chloroform and can be almost completely extracted with it from the water phase, whereas sugars or other highly polar compounds remain in the water phase. In cases when FCLLs are detected in fraction 7 (which coincided in retention time with FC A) by both RRA and RIA with a good correlation between them, the presence of FC A or quite closely related substances is highly probable. If so, the negative GC/MS results may merely reflect a low concentration of FC A in the sample, inasmuch as the sensitivity of RIA and RRA is 2–3 orders of magnitude higher than that of GC/MS. On the other hand, when the RIA and RRA data on fraction 7 differ markedly, this is likely to reflect interference of some other FC-like substances. The data presented hence give serious grounds for considering the endogenous FCCLs as terpenoid phytohormones, together with gibberellins, abscisic acid, and brassinosteroids.

Our experience suggests that detection and identification of endogenous FCLLs require a complex of analytical methods, including RRA, RIA, HPLC, and GC/MS. Unfortunately, we failed to find any reports on application of such combined approach to detect endogenous FC-like substances in the available literature. We could only wish that such works be expanded to prove or disprove that FC are indeed phytohormones.

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