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Presence of estrogen receptor (ER)-like proteins and endogenous ligands for ER in solanaceae

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Abstract

The synthesis of steroid hormones in different plant species and the possibility that these molecules could regulate cell growth, tissue differentiation and germination has been reported. However, the mechanism of action of these endogenous steroids in plant cells is still poorly understood. In the present work, binding experiments with [^{3}H]17β-estradiol in the presence and absence of an excess of unlabeled 17β-estradiol showed that *Solanum glaucophyllum* and *Lycopersicon esculentum* organs contain estrogen-binding sites. Scatchard analysis detected saturable [^{3}H]17β-estradiol-binding sites ($K_d \sim 6.6$ nM; $B_{max} \sim 1140$ fmol/mg protein) in *S. glaucophyllum callus* tissue. Estrogen-like compounds were detected in lipid extracts from *S. glaucophyllum* and *L. esculentum*. Moreover, the lipid fraction was able to compete with [^{3}H]17β-estradiol for binding to estrogen receptor (ER) from breast cancer MCF-7 cells as well as with estrogen-binding sites present in both plant species. Western blot analysis with monoclonal antibodies against different domains of the ER α, detected a ∼67 kDa band in various organs of both plant species. These proteins were able to bind estradiol in ligand blot assays using 17β-estradiol macromolecular derivatives as ligands. Western and ligand blot experiments of subcellular fractions from callus tissues of *S. glaucophyllum* showed that the ER-like protein of ∼67 kDa was most concentrated in the nuclear fraction. Reactive bands of lower molecular weight were also detected. Altogether these results provide evidence about the existence of estrogen-binding proteins and endogenous ligands in Solanaceae.

Keywords: *Solanum glaucophyllum*; *Lycopersicon esculentum*; 17β-Estradiol; Estrogen-binding proteins

1. Introduction

Steroid hormone receptors from vertebrates have the ability to control gene expression at the transcription level by binding to specific DNA regulatory elements (hormone response elements or HREs). In contrast to other transcription factors, their activities are modulated through binding of the cognate ligands. The interaction of ligand with receptor converts the inactive protein into a form that specifically recognizes and binds to HREs, and these interactions, ultimately, alter the rate of gene transcription [1–4]. In the case of estrogens, their biological effects are known to be mediated by specific receptor proteins (ERs) from either the α or β isoform type [5,6]. These two isoforms belong to the nuclear receptor gene superfamily of transcription factors, whose members bear highly conserved features in both structure and function [1–3]. The existence of a protein responsible for specific binding of 17β-estradiol was first recognized in the uterus [5], since then, ERs were identified to be widely expressed among different tissues and animal species.

Various species of bacteria, fungi, algae and vascular plants contain steroid-like compounds [7–18] and respond to steroids in a variety of ways [7,9,13,19,20]. The presence of 17β-estradiol has been reported for different plant species [7,9] and we have recently described evidence concerning the occurrence not only of estrogens but also of a putative estrogen receptor (ER)-like protein in in vitro cultures of *Solanum glaucophyllum* Desf. (*Solanum malacoxylon* Send.) [21].

The aim of the present work was to investigate the existence of estrogen-like compounds and estrogen-binding proteins related to the ER α in different organs of two members of the Solanaceae family: *S. glaucophyllum* and *Lycopersicon esculentum* Mill (tomato). 17β-Estradiol-binding sites were also characterized in in vitro cultures of *S. glaucophyllum* by saturation analysis. We first measured the relative contents of 17β-estradiol in plant extracts by immunochemical techniques. Moreover, by competition experiments we

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could demonstrate that lipid extracts from both species contain compounds that bind to the mammalian ER. We carried out radioligand-binding assays to determine the presence of sites for the specific binding of 17β-estradiol and of the corresponding endogenous ligands. Then we verified the possible relationship between these sites and the known ER α isoform by immunoblot and ligand blot approaches. Finally, we investigated the subcellular distribution profile of the estrogen-binding proteins in in vitro cultures of *S. glaucophyllum*.

The evidence obtained in this work point to the existence of novel estrogen-binding proteins in vascular plants that share structural similarity to the well-characterized mammalian ER α subtype.

2. Materials and methods

2.1. Materials

[(2,4,6,7-^{3}H(*N*)]17β-estradiol with a specific activity of 80-115 Ci/mmol, chemiluminescence (ECL) blot detection kit and secondary antibodies were obtained from New England Nuclear (Boston, MA, USA). 17β-Estradiol, 17β-estradiol-6-(o-carboxy-methyl)oxime:BSA fluorescein isothiocyanate conjugate (E_2-BSA-FITC), 17β-estradiol-peroxidase (E_2-P), bovine serum albumin (BSA) and compounds for culture media were from Sigma–Aldrich (St. Louis, MO, USA). Anti-ER α mouse monoclonal antibody clones AER314, AER308, AER311 and TE111.5D11 were purchased from NeoMarkers (Fremont, CA, USA). Protein G plus/protein A–agarose was obtained from Calbiochem (San Diego, CA, USA). Molecular weight colored markers were bought from BioRad Laboratories (Richmond, CA, USA). All other reagents were of analytical grade.

2.2. Plants

S. glaucophyllum plant specimens were collected from their natural habitats in Buenos Aires Province, Argentina. *L. esculentum* plants were obtained from commercial seeds. All plants were grown under green-house conditions.

2.3. Cultures

Leaf-originated callus cultures from *S. glaucophyllum* were obtained as previously described [18]. Healthy aseptic leaf explants from plant renewals were inoculated into solidified (0.8% agar) Murashige-Skoog media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, 0.5 mg/l and 0.2 mg/l, respectively. In vitro tissue cultures were grown at 25 °C under darkness. Calli started to develop after approximately 15 days from inoculation. Subcultures were usually performed when growing tissue saturated the available surface inside culture flasks (ca. 2 months).

2.4. Isolation of cell fractions

Soluble fractions were obtained by grinding frozen tissues from *S. glaucophyllum* and *L. esculentum* plants (2 ml buffer/g tissue) or by homogenizing callus tissues (1 ml buffer/g tissue) with an Ultraturrax homogenizer (Janke and Kunkel, Germany) in TEKM buffer [10 mM Tris–HCl, pH 7.2; 1.5 mM EDTA; 0.3 M KCl; 2% polyvinylpyrrolidone; 2 mM β-mercaptoethanol] containing protease inhibitors [0.3 mM phenylmethylsulfonyl fluoride (PMSF); 20 μg/ml leupeptin; 20 μg/ml aprotinin]. The homogenates were filtered through two layers of nylon mesh and subsequently centrifuged for 1 h at 120,000 × *g* to obtain clear supernatants (soluble fractions).

Subcellular fractions from *S. glaucophyllum* callus were obtained by homogenizing callus tissues (1 ml buffer/g tissue) with an Ultraturrax homogenizer in TES buffer (10 mM Tris–HCl pH 7.2; 1.5 mM EDTA; 250 mM sucrose; 2% polyvinylpyrrolidone; 2 mM β-mercaptoethanol) containing the same mixture of protease inhibitors as above. The homogenate was filtered through two layers of nylon mesh and subsequently centrifuged at 800 × *g* for 15 min to remove the nuclear fraction. Further centrifugation of the resultant supernatant at 10,000 × *g* for 20 min allowed isolation of a mitochondrial-enriched pellet. The remaining supernatant was centrifuged for 1 h at 120,000 × *g* to obtain a microsomal pellet and a soluble supernatant (cytosol).

The protein concentration was measured by the method of Bradford [22] using bovine serum albumin as standard.

2.5. Preparation of lipid extracts and estradiol quantification

Lipid extracts from *L. esculentum* leafs were obtained following essentially the method described by Bligh and Dyer [23]. The starting plant material was first homogenized in chloroform–methanol (1:2, v/v; 3.6 ml/g f. weight) using an Ultraturrax homogenizer at maximum speed (2 min). The samples were further homogenized after the addition of chloroform (1.2 ml/g) and then after the addition of water (1.2 ml/g), for 30 s each time. The final homogenate was centrifuged at 4400 × *g* for 10 min. The lower lipid soluble phase was collected and evaporated by flushing nitrogen at 35 °C. The final residue was solubilized in ethanol (20 μl) and then phosphate buffer saline (PBS) solution was added (980 μl). A competitive test employing the lipid extracts, a biotin-conjugated polyclonal antibody specific for 17β-estradiol, 17β-estradiol labeled with ruthenium chelate and streptavidin-coated spheres was carried out. Estradiol-like compounds were then quantified by electrochemoluminescence (Elecsys Estradiol Immunoassay, Roche), according to the manufacturer's instructions.

2.6. Detection of endogenous ligands for ER

To determine the presence of endogenous ligands for ER in both plant species, lipid extracts of *L. esculentum*

leaves and *S. glaucophyllum* leaves, seeds and calli were obtained as above and the final pellet was resuspended in 20 μl of ethanol for competition assays. The estrogen receptor preparation was obtained by homogenization of breast cancer MCF-7 cells (American Type Culture Collection, Rockville, MD, USA) in TES buffer followed by centrifugation at 120,000 × *g* for 1 h. The cytosolic fraction was collected and stored at −70 °C until use. Binding assays with lipid extracts were performed by incubating the samples (0.4 mg total protein) with 5 nM of [^{3}H]17β-estradiol alone or in combination with 5 μl of the resuspended pellet for 4 h at 4 °C. Free from bound radioactive steroid was separated by the hydroxylapatite procedure [24].

2.7. Saturation analysis of [^{3}H]17β-estradiol binding to *S. glaucophyllum* soluble fractions

Saturation analysis was typically measured by incubating 0.25–0.3 mg protein in 0.4 ml complete TEKM buffer with increasing concentrations of [^{3}H]17β-estradiol in the absence or presence of a 100-fold molar excess of unlabeled 17β-estradiol. The samples were incubated at 4 °C for at least 4 h. To separate bound from free sterol, 100 μl of a 0.5% dextran/5% charcoal suspension (pH 7.2) was added to each tube [25]. The samples were further incubated for 20 min at 4 °C by gently stirring and then centrifuged at 1200 × *g* (5 min) to pellet the charcoal. Aliquots (300 μl) from the obtained supernatant were taken for radioactivity measurements by liquid-scintillation spectrometry. Specific estradiol-binding sites were determined by subtracting the radioactivity bound in presence of [^{3}H]17β-estradiol alone and that retained by the non-specific tubes.

The steroid affinity constant (K_d) and the maximum number of binding sites (B_{max}), were estimated according to Scatchard, using the non-linear curve fitting LIGAND program for saturation analysis [26].

2.8. Radioligand-binding assays

[^{3}H]17β-estradiol-binding activity from soluble fractions of *L. esculentum* and *S. glaucophyllum* roots, stems, and leaves were typically measured by incubating 0.1 mg protein in 0.1 ml complete TEKM buffer in presence of 10 nM [^{3}H]17β-estradiol (total binding). A 50- and 200-fold molar excess of unlabeled 17β-estradiol was included in additional paired samples (non-specific binding). The samples were incubated at 4 °C for at least 4 h. Bound from free sterol was separated by the charcoal–dextran procedure described above. Aliquots (100 μl) from the obtained supernatant were taken for radioactivity measurements by liquid-scintillation spectrometry. Specific estradiol-binding sites were determined by subtracting the radioactivity bound in presence of [^{3}H]17β-estradiol alone and that retained by the non-specific tubes.

2.9. Detection of endogenous ligands for ER-like proteins

Lipid extracts of *S. glaucophyllum* and *L. esculentum* were used in competition assays to determine the binding capacity to the estradiol-binding sites present in both plants species. Lipid extracts of *L. esculentum* leaves and *S. glaucophyllum* leaves and calli were obtained as above and the final pellets were resuspended in 20 μl of ethanol. Binding capacity of these fractions was measured by incubating 0.4 mg protein from leaves and calli in 0.4 ml TEKM buffer in presence of 10 nM [^{3}H]17β-estradiol (total binding). For the non-specific binding, 0.5, 5, 10 and 20 μl of the respective lipid fraction from each organ were included in paired samples. The samples were incubated at 4 °C for at least 4 h. Bound from free sterol was separated by the charcoal–dextran procedure described above. Aliquots (300 μl) from the obtained supernatant were taken for radioactivity measurements by liquid-scintillation spectrometry. Specific estradiol-binding sites were determined by subtracting the radioactivity bound in presence of [^{3}H]17β-estradiol alone and that retained by the non-specific tubes.

2.10. Western blots

Protein samples from *L. esculentum* and *S. glaucophyllum* organs were analyzed for its ER α immunoreactivity content. Aliquots (20 μg of total protein) were combined with one-fourth of sample buffer (400 mM Tris–HCl, pH 6.8; 10% SDS; 50% glycerol; 500 mM DTT and 2 μg/ml bromophenol blue), boiled for 5 min and resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were incubated overnight at 4 °C with a 1:200 dilution of each primary antibody. After several washings with PBS-T, the membranes were incubated with anti-mouse conjugated to horseradish peroxidase as secondary antibody. Immunoreactive proteins were developed by means of enhanced chemiluminescence (ECL). The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

2.11. Ligand blots

Transferred proteins from SDS–PAGE were renatured by exhaustive washings with PBS-T (at least overnight at 4 °C). The membranes were blocked with 5% BSA–PBS and then incubated with E_2-BSA-FITC (10^{-6} M) or E_2-P (10–50 nM) for 1 h at room temperature [27]. Reactive bands were visualized under UV light and enhanced chemiluminescence, respectively. The final concentration of the macromolecular complexes, E_2-BSA-FITC (5–10 mol estradiol and 3–5 mol FITC/mol BSA) and E_2-P (1–2 mol estradiol/mol peroxidase

type VI), was calculated on the basis of their steroid content. Images from immunoblots and ligand blots were digitalized using a Hewlett-Packard 3200C scanner at a resolution of 300 dpi.

2.12. Immunoprecipitation

Subcellular fractions from *S. glaucophyllum* calli containing 500 μg of total protein were immunoprecipitated with 10 μl of a 50% suspension of protein G plus/protein A–agarose after incubating the extracts with 2 μg of a monoclonal antibody against ER α (clon AER 314). The immunoprecipitates were washed three times with wash buffer (50 mM Tris–HCl, pH 7.4; 1 mM EDTA; 1% Triton X-100; protease inhibitors: 2 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 10 μg/ml of soy bean trypsin inhibitor). The final pellets were obtained by centrifugation for 3 min at 10,000 × *g*, resuspended then in sample buffer (400 mM Tris–HCl pH 6.8; 10% SDS; 50% glycerol and 2 μg/ml bromophenol blue) without dithiothreitol, boiled for 5 min and resolved by SDS–PAGE. Fractionated proteins were electrotransferred to PVDF membranes and then blocked for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were incubated overnight at 4 °C with a 1:200 dilution of a primary monoclonal antibody against ER α (clon AER311). After several washings with PBS-T, the membranes were incubated with anti-mouse conjugated to horseradish peroxidase as secondary antibody. Immunoreactive proteins were developed by means of enhanced chemiluminescence. For the ligand blot experiments, transferred proteins from SDS–PAGE were renatured by exhaustive washings with PBS-T (at least overnight at 4 °C). The membranes were blocked with 5% BSA–PBS, incubated with E_2-BSA-FITC (10^{-6} M) for 1 h at room temperature and then visualized under UV light. The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

3. Results

We have recently shown [21] that *S. glaucophyllum* leaves contain 4 ng/kg FW of 17β-estradiol which was detected by means of an electrochemoluminescence immunoassay using a specific polyclonal antibody against the hormone. In the present study, applying the same assay technique we measured in lipid extracts of *L. esculentum* leaves 6 ng/kg FW of 17β-estradiol. To determine if *S. glaucophyllum* and *L. esculentum* contain endogenous compounds that exhibit binding affinity for the estrogen receptor, we tested the ability of plant lipid extracts to compete with [^{3}H]17β-estradiol for binding to the ER using cytosolic fractions from MCF-7 cells as a source of receptor. As shown in Table 1, varying quantities of endogenous ligand for ER were detected among tissues of *S. glaucophyllum*, lowest amounts being present in less differentiated calli and highest in seeds. In *L. esculentum*, only leaves were assayed which showed comparable levels of estrogen to that in *S. glaucophyllum* leaves.

Table 1
Quantification of endogenous ligand for ER in *Solanum glaucophyllum* and *Lycopersicon esculentum*

Plant species	Organ	Endogenous ligand for ER (mg/kg f. weight)
Solanum glaucophyllum	Calli	36 ± 8
	Seeds	800 ± 60
	Leaves	108 ± 20
Lycopersicon esculentum	Leaves	100 ± 22

Lipid extracts from *Lycopersicon esculentum* leaves and *Solanum glaucophyllum* leaves, seeds and calli were measured for their endogenous ligand content using a competitive binding plot made with the cytosolic fraction from MCF-7 cells as source of ER, 5 nM [^{3}H]17β-estradiol and different concentrations of non-radioactive 17β-estradiol. The values in pmoles obtained from the plot were converted to mg/kg f. weight. Values are the average of three independent determinations ± S.D.

Fig. 1 shows the results of a representative experiment demonstrating that the binding of 17β-estradiol to soluble

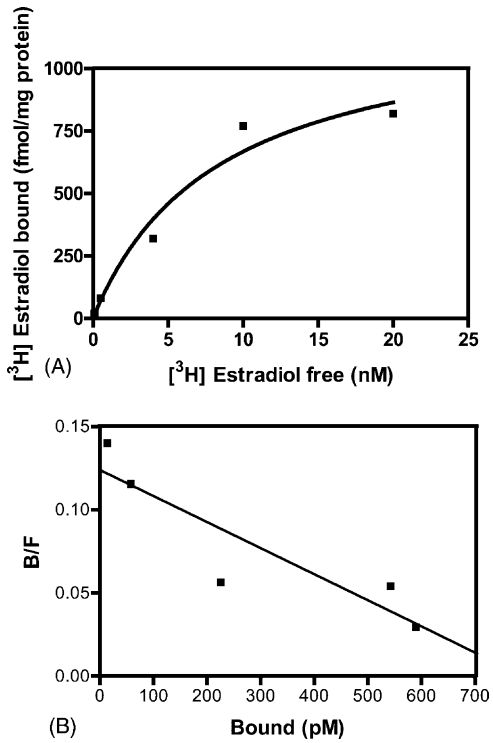

Fig. 1. Saturation analysis of [^{3}H]17β-estradiol binding to *Solanum glaucophyllum* soluble fraction. (A) 0.25–0.3 mg protein of soluble fraction obtained by centrifugation at 120,000 × *g* of *S. glaucophyllum* callus hormogenates were incubated with increasing concentrations of [^{3}H]17β-estradiol in the absence or presence of a 100-fold molar excess of unlabeled 17β-estradiol. Bound and unbound steroid were separated by the charcoal–dextran procedure as indicated in Section 2. Measured bound radioligand (B) was plotted against the concentration of free tritiated hormone in the incubation medium (F). A typical profile is shown and each point represents the mean of two independent determinations made in triplicate. (B) Plot derived from Scatchard analysis of the same data. Binding parameters at the equilibrium were estimated using the LIGAND program.

Table 2
[^{3}H]17β-estradiol-binding capacity of soluble fractions from roots, stems and leaves of *Solanum glaucophyllum* and *Lycopersicon esculentum*

Plant species	Organ	Specific binding site concentration (fmol/mg protein)	
		A	B
Solanum glaucophyllum	Roots	3676 ± 109	3340 ± 78
	Stems	1256 ± 74	1450 ± 110
	Leaves	198 ± 18	222 ± 11
Lycopersicon esculentum	Roots	1676 ± 107	1875 ± 43
	Stems	1100 ± 45	1202 ± 104
	Leaves	138 ± 21	106 ± 14

Samples of tissue soluble fractions were incubated with 10 nM [^{3}H]17β-estradiol alone or in combination with a 50-fold (A) and 200-fold molar excess (B) of unlabeled 17β-estradiol, for 4 h at 4 °C. Specific binding was determined as described in Section 2. Data represent the mean of two independent determinations made in duplicate ± S.D.

fractions of calli was a saturable process with respect to the ligand concentration. The saturation was nearly complete at ∼10 nM [^{3}H]17β-estradiol. Scatchard linearization of the saturation-binding data was consistent with a single set of binding sites (K_d = 6.59 ± 0.475 nM), with a maximum binding capacity of 1148 ± 87 fmol/mg protein.

We investigated the presence of specific binding sites for tritiated 17β-estradiol in soluble extracts from roots, stems and leaves of *S. glaucophyllum* and *L. esculentum*. As shown in Table 2, a reproducible binding activity could be measured in the different organs using a 50- and 200-fold molar excess of cold 17β-estradiol. The specific binding detected was higher in roots than in stems and leaves for both species.

Table 3 shows results obtained when different amounts of lipid extracts of *L. esculentum* leaves and of *S. glaucophyllum* leaves and callus were employed in competition assays using soluble fractions from the respective organs. Reproducible specific binding activity could be measured in all the samples analyzed indicating that lipid extracts contain compounds that could bind to ER-like proteins present in both plant species and are able to compete for the endogenous-binding sites in a dose-dependent manner.

We carried out Western blot assays to investigate estrogen receptor immunoreactivity in the two Solanaceae, using monoclonal antibodies against distinct domains of the ER α (Fig. 2A). An immunoreactive band of ∼67 kDa, similar to the molecular weight of the mammalian ER α and the estrogen-binding protein of *S. glaucophyllum* calli [21] was detected with the different antibodies. A higher MW band picked up in roots by the monoclonal antibody AER314 could not be reproduced in various experiments and was not detected with the other antibodies employed. To corroborate if these immunoreactive proteins were able to bind 17β-estradiol, we used fluorescent 17β-estradiol covalently linked to BSA (E_2-BSA-FITC) and 17β-estradiol-peroxidase (E_2-P) as estrogen ligands for ligand blot assays. In this technique, we determine the presence of functional estrogen-binding domains after their separation in SDS–PAGE, transference to PVDF membranes and renaturation. Fig. 2B shows the results obtained in both plant species. It is clear that there is labeling by the derivatives of a ∼67 kDa protein band in all the samples analyzed. The complete profile of the immunoprecipitation assay in Fig. 3A describes the distribution of the immunoreactive proteins, using monoclonal antibodies against ER α, in total homogenates and subcellular fractions from *S. glaucophyllum* callus cultures. The ∼67 kDa immunoreactive band was resolved as close multiple bands, usually a triplet, and appeared to be more abundant in the nuclear fraction. The 35–28 kDa bands were detected in the microsomal fraction. Two novel immunoreactive bands of ∼45 and ∼50 kDa were detected and appeared to be more abundant in the microsomal and cytosolic fraction, respectively. All these bands were able to bind the 17β-estradiol hydrophilic derivative in ligand blot assays (Fig. 3B).

4. Discussion

These studies have been focused on the characterization of the estrogen-binding sites detected in soluble fractions of *S. glaucophyllum* callus [21] and the presence of estrogen-like compounds and estrogen-binding proteins not only in callus but also in differentiated organs of *S. glaucophyllum* and *L. esculentum*. Thus, here we report that these plants species contain endogenous compounds that are functional and structural similar to 17β-estradiol. It has been previously shown that these plants could synthesize

Table 3
Binding capacity of soluble fractions from *Lycopersicon esculentum* leaves and *Solanum glaucophyllum* leaves and callus for endogenous ligands of the respective organs

Plant species	Organ	Specific binding site concentration (fmol/mg protein)			
		A	B	C	D
Solanum glaucophyllum	Callus	74 ± 5	102 ± 9	465 ± 15	412 ± 10
	Leaves	10 ± 2	120 ± 12	318 ± 5	374 ± 7
Lycopersicon esculentum	Leaves	60 ± 4	235 ± 12	540 ± 12	790 ± 14

Protein samples were incubated with 10 nM [^{3}H]17β-estradiol alone or in combination with 0.5 μl (A), 5 μl (B), 10μl (C) and 20 μl (D) of organ lipid extract, for 4 h at 4 °C. Specific binding was determined as described in Section 2. Data represent the mean of two independent determinations made in duplicate ± S.D.

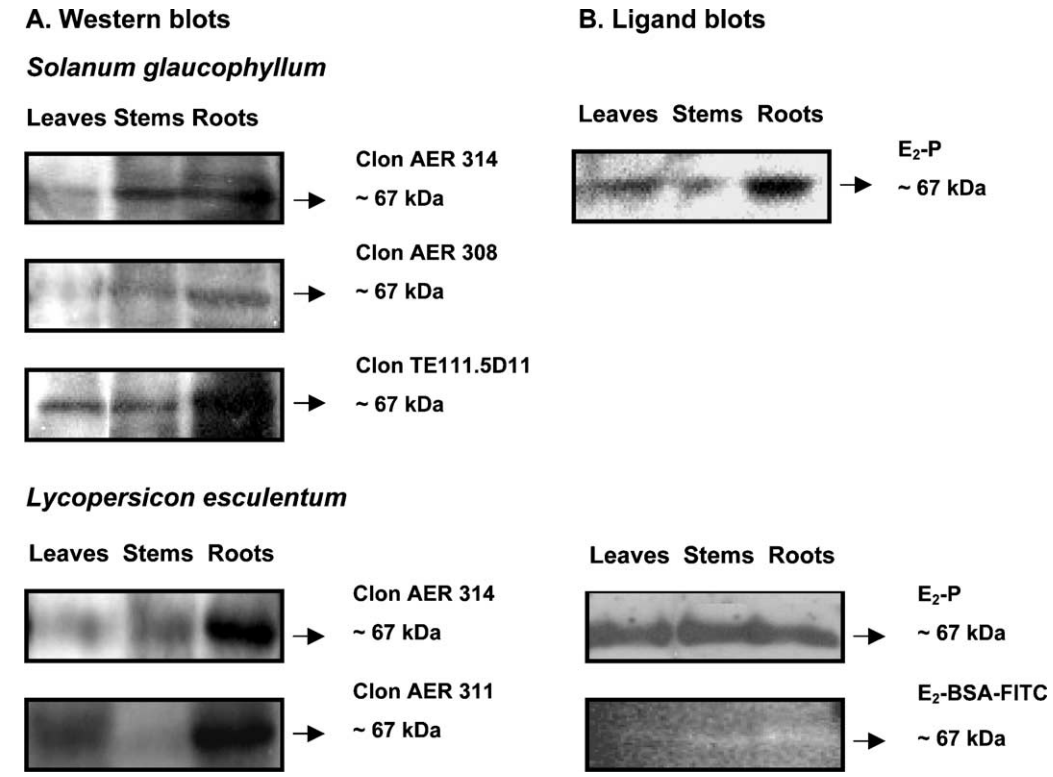

Fig. 2. ER α-like estrogen-binding proteins from *Solanum glaucophyllum* and *Lycopersicon esculentum*. The ER α immunochemical reactivity using the indicated antibodies (A) has been compared to the expression of estrogen-binding proteins (B). Total protein (20 μg) from *S. glaucophyllum* and *L. esculentum* were fractionated by SDS–PAGE, transferred to PVDF membranes and incubated with (A) anti-ER α monoclonal IgGs directed to linear aminoacid sequences localized primarily in the transactivation (AER 314), heat shock protein (AER 308) and steroid hormone-binding domains (AER 311 and TE111.5D11). Either E_2-BSA-FITC or E_2-P were used for the ligand blotting assays (B). A strong ~67 kDa band is noted. Representative blots from five independent experiments are shown.

appreciable quantities of other steroid hormones and related metabolites [15–18], and, as here, it has been demonstrated that these compounds are present in seeds at considerably higher concentrations than in other parts of the plant body [9,21]. The quantities of estrogen-like compounds detected by competition assays were higher than those detected by electrochemoluminescence immunoassays, which is in keeping with the fact that the lipid extracts contain molecules, known as phytoestrogens, that are able to compete for ER-binding sites but are not recognized by the antibody employed. Thus, some phytoestrogens such as coumestrol, genistein, apigenin, naringenin and kaemperol

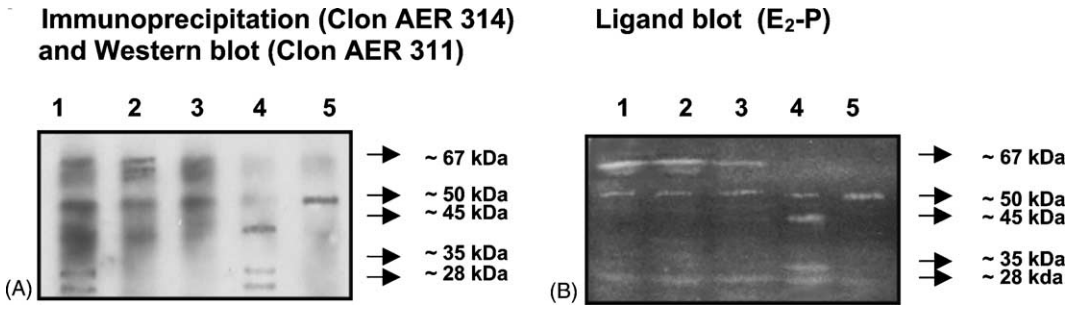

Fig. 3. Western and ligand blot analysis of ER α-like subcellular distribution in *Solanum glaucophyllum* calli. Subcellular fractions from *S. glaucophyllum* calli containing an equivalent protein amount were immunoprecipitated with a monoclonal antibody (AER 314), resolved by SDS–PAGE and processed for Western (A) or ligand blot (B) analysis. (A) ER α-immunolike partitioning. The immunoreactive bands were revealed using another monoclonal antibody (AER 311). (B) Ligand blot analysis to determinate the distribution of the estrogen-binding proteins. The blot was revealed using E_2-BSA-FITC conjugate. The estrogen-binding proteins identified coincided with the bands detected using the antibody. Lane 1: total homogenate; lane 2: nuclear fraction; lane 3: mitochondrial fraction; lane 4: microsomes; and lane 5: cytosol.

effectively compete with 17β-estradiol for binding to ER; furthermore, some of these plant phytoestrogens stimulate the transcriptional activity of ER [28].

We previously detected estrogen-binding sites in in vitro cultures of *S. glaucophyllum* [21]. Now, we have characterized by Scatchard analysis the binding of the soluble fraction sites present in these cultures. As classical receptors, these sites exhibited ligand affinity in the low nanomolar range and were saturable with respect to the ligand concentration. We also describe that these estrogen-binding sites are present in differentiated organs of both the Solanaceae members *S. glaucophyllum* and *L. esculentum*. The estrogen-binding site concentration recovered in soluble fractions of roots and stems was higher to that usually detected in ER-enriched mammalian tissues and in soluble fractions from callus tissues [21,27]. We have also shown that lipid extracts of leaves and calli are able to compete with [^{3}H]17β-estradiol for binding not only to the mammalian ER but also to the estrogen-binding sites present in both plants species.

Using highly specific monoclonal antibodies against the ER α, we have detected a plant antigen of ∼67 kDa that is coincident with the molecular weight of the ER α. The same molecular weight band was also detected using 17β-estradiol hydrophilic derivatives prior renaturation of the proteins resolved in SDS–PAGE, suggesting that the ER α-like protein would correspond to a real estrogen binder. In addition to the classical ER α-related molecule, in subcellular fractions of *S. glaucophyllum* calli, we have detected the presence of another antigens localized, principally, in cytosol (∼50 kDa) and microsomal (∼45, 35, and 28 kDa) fractions. These low molecular weight bands could also bind 17β-estradiol hydrophilic derivatives, thus possibly being structurally and functionally related to the ER. We speculate that these estrogen-binding proteins could represent primitive estrogen receptors and that the endogenous ligands for ER, estrogen-like compounds that could mediate physiological events through binding to the estrogen-binding proteins. However, the possibility that the sterol-binding sites may also function as sterol carrier or storage molecule cannot be excluded.

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