Physiologia Plantarum 128: 341–350. 2006

Presence of vitamin D_3 receptor (VDR)-like proteins in *Solanum glaucophyllum*

Lorena Milanesi and Ricardo Boland*

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur. San Juan 670, (8000) Bahía Blanca, Argentina

Correspondence
*Corresponding author,
e-mail: rboland@criba.edu.ar

Received 3 March 2006; revised 28 March 2006

doi: 10.1111/j.1399-3054.2006.00742.x

The detection of 1α,25(OH)$_2$-vitamin D_3 [1α,25(OH)$_2D_3$] in *Solanum glaucophyllum* and other plant species has been reported. Little is known about the mode of action of 1α,25(OH)$_2D_3$ in plants. The steroid acts in animal systems through binding to a specific receptor (VDR). In the present work, competition assays allowed the detection of binding sites for [^{3}H]1α,25(OH)$_2D_3$ in cultured *S. glaucophyllum* cells. The binding of [^{3}H]1α,25(OH)$_2D_3$ to total protein from calli was a saturable process with respect to the ligand concentration ($K_d = 3.49 \pm 0.75$ n*M*; $B_{max} = 255.7 \pm 18.6$ *f*mol/mg protein). Western blots employing antibodies directed against the avian VDR revealed three immunoreactive bands of approximately 72, 58 and 43 kDa in different plant organs, cells and callus cultures. The immunoreactive proteins were tyrosine phosphorylated, and the signal detected was increased using hypertonic buffers for protein extraction, suggesting that these proteins are associated with subcellular structures. When the callus cells were subjected to homogenization and differential centrifugation, a substantial proportion of the immunolabeling was localized in the nuclear and mitochondrial fractions. Ligand blot assays revealed the ability of the reactive bands to bind [^{3}H]1α,25(OH)$_2D_3$. Immunocytochemistry detection using Oregon Green-conjugated secondary antibodies showed immunofluorescent staining mainly in the nucleus, and in the nucleus, cytoplasmic corpuscles and outer cell membrane, for the monoclonal and polyclonal antibodies, respectively. [^{3}H]Thymidine incorporation assays and immunoblot analysis of mitogen-activated protein kinase (MAPK) indicated that 1α,25(OH)$_2D_3$ and cell lipid extracts stimulate DNA synthesis but do not activate MAPKs with TEY domains in *S. glaucophyllum* cells. These data demonstrate that *S. glaucophyllum* cells, which produce 1α,25(OH)$_2D_3$, also express a cognate binding protein(s) and are able to respond to 1α,25(OH)$_2D_3$. The exact functional role of the VDR-like protein (s) remains to be established.

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). The interaction of a specific ligand with the receptor converts the inactive protein into a form that recognizes and binds to HREs, thereby controlling the rate of gene transcription (Evans 1988, Laudet et al. 1992, Kuiper et al. 1997, Owen and Zelent 2000). The biological effects of 1α,25(OH)$_2$-vitamin D_3 [1α,25(OH)$_2D_3$] are mediated by a specific vitamin D receptor (VDR) protein, completely cloned several years ago (McDonell et al. 1987, Baker et al. 1988). Several types of bacteria, fungi, algae, and vascular plants in general, contain steroids (Wasserman et al. 1976, Geuns 1978, Buchala and Schmid 1979, Grunwald 1980, Mudd 1980, Mandava et al. 1982, Feldman et al. 1982, Boland 1986, Weissenberg et al. 1989, Agarwal 1993, Curino et al. 1998, Skliar et al. 2000) and have been

Abbreviations – 1α,25(OH)$_2D_3$, 1α,25(OH)$_2$-vitamin D_3; BSA, bovine serunm albumin; 2,4-D, 2,4-dichlorophenoxyacetic acid; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HRE, hormone response element; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.1% Tween-20; PCV, packed cell volume; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VDR, vitamin D receptor.

shown to undergo physiologic changes in response to these compounds, including vitamin D_3, 25(OH)-vitamin D_3 [25(OH)D_3] and 1α,25(OH)$_2D_3$ (Geuns 1978, Buchala and Schmid 1979, Vega et al. 1988, Talmon et al. 1989, Agarwal 1993). The presence of vitamin D_3 and its hydroxylated derivatives, in particular 1α,25(OH)$_2D_3$, has been detected in an appreciable number of flowering plants, including *Solanum glaucophyllum* Desf. (*Solanum malacoxylon* Send.) (Boland et al. 2003 and references therein).

We have recently described evidence concerning the occurrence not only of estrogens but also of a putative estrogen receptor-like protein in *S. glaucophyllum* and other Solanaceae species (Milanesi et al. 2001, Milanesi and Boland 2004).

The aim of the present work was to investigate the existence of 1α,25(OH)$_2D_3$-binding proteins related to the VDR in *S. glaucophyllum*.

We carried out radioligand-binding assays and ligand blots to establish the presence of specific binding sites for 1α,25(OH)$_2D_3$. We verified their possible structural relationship to the animal VDR by immunoblot analysis. In parallel, we investigated the subcellular distribution profile of the immunoreactive proteins in in vitro cultures of *S. glaucophyllum* by immunoblotting and immunocytochemistry. Finally, we evaluated the effects of 1α,25(OH)$_2D_3$ on cell proliferation by measuring [^{3}H]thymidine incorporation into DNA and mitogen activated protein kinase (MAPK) activity.

The evidence obtained in this work points to the existence, in a vascular plant, of novel 1α,25(OH)$_2D_3$-binding proteins that share structural similarity with the well-characterized VDR.

Materials and methods

Materials

[^{3}H]1α,25(OH)$_2D_3$ (specific activity: 171 Ci/mmol), [^{3}H]thymidine (specific activity: 50–60 Ci/mmol), chemiluminescence blot detection kit (enhanced chemiluminescence, ECL) and secondary antibodies were obtained from New England Nuclear (Boston, MA). Protein A agarose, bovine serum albumin (BSA), cytochrome *C* oxidase assay kit and compounds for culture media were from Sigma-Aldrich (St Louis, MO). Anti-VDR rat monoclonal antibody (MA1-710) against the VDR DNA-binding domain, and anti-VDR rabbit polyclonal antibody (PA1-711) against the VDR C-terminus, were purchased from Affinity BioReagents Inc. (ABR, CO). Anti-phosphotyrosine and anti-MAPK ERK1/2 polyclonal antibodies were obtained from Promega Corporation (Madison, WI). Anti-phospho-P44/42 MAPK antibody was from Cell Signaling Technology (Beverly, MA). Secondary fluorescent Oregon-Green-conjugated antibodies, propidium iodide (PI), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the mounting ProLong Antifade kit were from Molecular Probes (Eugene, OR). Molecular weight colored marker was bought from BioRad Laboratories (Richmond, CA). DNAzol® ES buffer for DNA extraction was from Molecular Research Center, Inc. (Cincinnati, OH). All other reagents were of analytical grade.

Plants

S. glaucophyllum plant specimens were collected from their natural habitats in Buenos Aires Province, Argentina, and were grown under greenhouse conditions.

Cultures

Leaf-originated callus cultures from *S. glaucophyllum* were obtained as previously described (Curino et al. 1998). Healthy aseptic leaf explants from plant renewals were inoculated into solidified (0.8% agar) Murashige–Skoog medium 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 (approximately 2 months). Cell suspensions were initiated by mechanical dispersion of calli and then cultured in 125 ml Erlenmeyer flasks containing 50 ml of liquid Murashige–Skoog medium supplemented with hormones as above, without agar. The cells were grown in culture chambers with constant agitation (100 *g*) at 25°C in darkness. The growth of cultures was evaluated by counting cell numbers, protein content, cellular weight and packed cell volume (PCV). Typical growth curves were obtained. Cell viability was determined employing the fluorescein diacetate technique (Widholm 1972).

Protein extraction and isolation of cell fractions

Protein extracts were obtained by grinding frozen tissues from *S. glaucophyllum* plants (2 ml buffer/g tissue) or by homogenizing callus tissues or cells (1 ml buffer/g tissue or cells), at 4°C, with an Ultraturrax homogenizer (Janke and Kunkel, Germany) in TES buffer (10 m*M* Tris-HCl pH 7.2; 1.5 m*M* EDTA; 250 m*M* sucrose; 2% polyvinylpyrrolidone; 2 m*M* β-mercaptoethanol) or TEKM buffer (10 m*M* Tris-HCl, pH 7.2; 1.5 m*M* EDTA; 0.4 *M* KCl; 2% polyvinylpyrrolidone; 2 m*M* β-mercaptoethanol) containing protease inhibitors [0.3 m*M* phenylmethylsulfonyl

fluoride (PMSF); 20 μg/ml leupeptin; 20 μg/ml aprotinin; 10 μg/ml of soybean trypsin inhibitor]. The homogenates were filtered through two layers of nylon mesh and subsequently centrifuged for 1 h at 120,000 *g* to obtain clear supernatants.

Subcellular fractions from *S. glaucophyllum* callus were isolated by homogenizing callus tissues (1 ml buffer/g tissue) with an Ultraturrax homogenizer in TES buffer 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 sediment 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).

Measurements of protein concentration were performed by the method of Bradford (Bradford 1976), using BSA as standard.

Determination of mitochondria outer membrane integrity and detection of mitochondria in subcellular fractions were performed using a cytochrome *C* oxidase assay kit (Sigma-Aldrich) according to the manufacturer's instructions.

Radioligand-binding assays

[^{3}H]1α,25(OH)$_2$D$_3$-binding activity of whole cells from *S. glaucophyllum* calli was typically measured by incubating 1.5 ml of cultured cells in the presence of 2 n*M* [^{3}H]1α,25(OH)$_2$D$_3$ (total binding). A 500-fold molar excess of unlabeled 1α,25(OH)$_2$D$_3$ or 25(OH)D$_3$ was included in additional paired samples (non-specific binding). The samples were incubated at 25°C in dark and with constant shaking for 3 h. To separate bound from free steroid, cells were washed at least four times with fresh Murashige–Skoog medium. The radioactivity retained in the cells was extracted with 1% SDS/1 N NaOH (Monje and Boland 2002) followed by centrifugation at 10,000 *g*. Aliquots (100 μl) from the supernatant were taken for protein and radioactivity measurements by the method of Bradford (Bradford 1976) and liquid scintillation spectrometry, respectively. Specific binding sites were determined by subtracting the radioactivity bound in the presence of [^{3}H]1α,25(OH)$_2$D$_3$ alone and that retained by the non-specific tubes.

Saturation analysis was typically measured by incubating 0.1–0.2 mg of protein in 0.5 ml of complete TES buffer with increasing concentrations of [^{3}H]1α,25(OH)$_2$D$_3$ in the absence or presence of a 500-fold molar excess of unlabeled hormone. The samples were incubated at 4°C for at least 4 h. To separate bound from free sterol, 150 μl of a 0.5% dextran/5% charcoal suspension (pH 7.2) was added to each tube (Vega and Boland 1988). The samples were further incubated for 20 min at 4°C with gentle 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 binding sites were determined by subtracting the radioactivity bound in the presence of [^{3}H]1α,25(OH)$_2$D$_3$ 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 (Munson and Rodbard 1980).

Immunoblots

Protein samples from *S. glaucophyllum* organs, cells and calli were analyzed for their VDR-like immunoreactivity content. Aliquots (20 μg of total protein) were combined with one-fourth of electrophoresis sample buffer (400 m*M* Tris-HCl, pH 6.8; 10% SDS; 50% glycerol; 400 m*M* dithiothreitol (DTT); 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 (PVDF) membranes (Immobilon-P) and then blocked for 1 h with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). The blots were incubated overnight at 4°C with the corresponding dilution of each primary antibody according to the manufacturer's instructions. After several washings with PBS-T, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were developed by means of ECL. The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers. Total soluble proteins from chicken intestine extracted with TEKM buffer were used as positive controls.

Immunoprecipitation

Subcellular fractions from *S. glaucophyllum* calli containing 500 μg of protein were immunoprecipitated with 10 μl of a 50% suspension of protein A–agarose after incubating the extracts with anti-phosphotyrosine polyclonal antibody. The immunoprecipitates were washed three times with wash buffer (50 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA; 1% Triton X-100; protease inhibitors: 2 m*M* PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor). The final pellets were obtained by centrifugation for 3 min at 10,000 *g*, and then resuspended in electrophoresis sample buffer without

DTT, 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-T. The blots were incubated overnight at 4°C with primary monoclonal antibody against the DNA-binding domain of the avian VDR (MA1-710). After several washings with PBS-T, the membranes were incubated with anti-rat conjugated to horseradish peroxidase as secondary antibody. Immunoreactive proteins were developed by means of ECL. The apparent molecular weights of reactive bands were estimated by reference to a wide size range of protein markers.

Immunocytochemistry

Cells derived from calli were grown for 7 days in Murashige–Skoog medium at 25°C under darkness. Aliquots (1.5 ml) were washed with fresh medium and fixed for 15 min with freshly prepared 2% paraformaldehyde in PBS. To permeabilize cells and allow nuclear antigen labeling, 0.05% Triton X-100 was included in the paraformaldehyde solution. After fixation, cells were rinsed three times with PBS and treated with 50 μg/ml ribonuclease A to avoid cytoplasmic staining with nucleic acid dyes. Non-specific sites were blocked for 30 min in PBS containing 2% BSA. Cells were then incubated for 60 min in the presence or absence (negative controls) of primary antibodies (1 : 40 dilution) or the nuclear stains (PI and DAPI) as a reference for nuclear localization. After PBS washing, cells were incubated for another 60 min with Oregon Green-conjugated secondary antibodies. The antibodies were prepared in PBS containing 2% BSA, and all steps were carried out at room temperature. After three PBS washes, cells were mounted with the commercial ProLong Antifade reagent according to the manufacturer's protocol. A Zeiss Axiolab fluorescence microscope (Carl Zeiss, Thornwood, NY) was employed for conventional microscope visualization, equipped with standard filter sets to capture fluorescent signals.

Images from immunoblots and immunocytochemistry were digitalized using a Hewlett Packard 3200C scanner at a resolution of 300 dpi.

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 2 n*M* [^{3}H]1α,25(OH)$_2$D$_3$ (at least overnight at 8°C in darkness) (Monje and Boland 2001), alone or in combination with a 500-fold molar excess of unlabeled 1α,25(OH)$_2$D$_3$. After several washes with PBS-T, the membranes were air-dried and the immunoreactive bands, obtained by western blots, were cut using a wide size range of protein markers as reference. Membrane samples were measured for their radioactive contents by liquid scintillation spectrometry. Positive (total soluble proteins from chicken intestine) and negative (non-immunoreactive proteins and PVDF membrane without protein) controls were also included.

MAPK determination

For MAPK determination, 5 ml of cultured cells were treated for 20 min, under darkness and with constant shaking, with 50 n*M* 1α,25(OH)$_2$D$_3$, 500 μl of lipid extract obtained from cell cultures by the Bligh and Dyer method (Bligh and Dyer 1959), or vehicle (isopropanol), followed by protein extraction with lysis buffer (1% Triton X-100; 50 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA; 2% polyvinylpyrrolidone; 20 μg/ml leupeptin; 20 μg/ml aprotinin; 2 μg/ml soybean trypsin inhibitor; 1 m*M* PMSF; 0.1 m*M* sodium orthovanadate). The samples were then centrifuged at 120,000 *g* for 20 min at 4°C. Aliquots were taken from the supernatant for protein content measurements and assays of MAPK immunoreactivity by western blot analysis employing anti-MAPK ERK 1/2 and anti-active phospho-MAPK (anti-phospho-P44/42 MAPK) antibodies.

[^{3}H]Thymidine incorporation

Aliquots (1 ml) of cultured cells were treated for 20 min with 1α,25(OH)$_2$D$_3$ (1 n*M*, 10 n*M* and 50 n*M*), lipid extract (1 μl, 50 μl, 100 μl) or vehicle (isopropanol) and then incubated for 3 h in medium containing 0.5 μCi/ml [^{3}H]thymidine. Cells were then washed three times with fresh medium, and the DNA was extracted employing DNAzol® ES buffer. Chloroform was added (1 : 1 v/v, buffer/solvent) and samples were subjected to centrifugation for 5 min at 10,000 *g*. From the aqueous fractions, 200 μl aliquots were taken for protein and radioactivity measurements by the method of Bradford (Bradford 1976) and liquid scintillation spectrometry, respectively.

Statistical evaluation

The statistical significance of data was evaluated using Student *t*-test (Snedecor and Cochran 1967). Quantitative data were expressed as the mean ± SD from the indicated set of experiments, and probability values below 0.05 ($P < 0.05$) were considered significant.

Results

As a first step in the identification of 1α,25(OH)$_2$D$_3$-binding proteins in *S. glaucophyllum*, the presence of specific binding sites for [^{3}H]1α,25(OH)$_2$D$_3$ was assayed

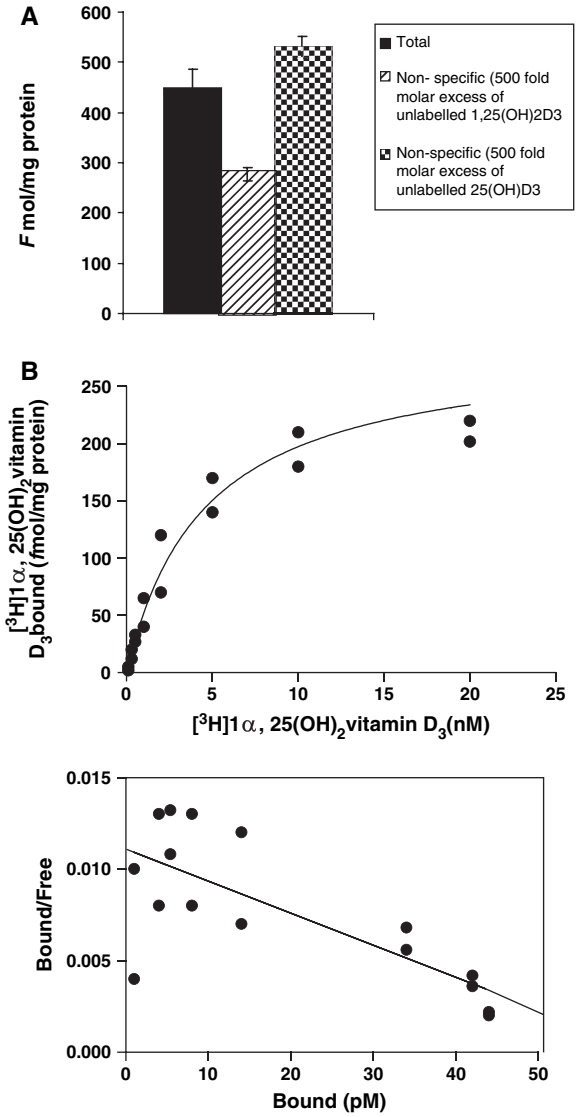

Fig. 1. (A) [^{3}H]1α,25(OH)$_2$-vitamin D$_3$ binding capacity of *Solanum glaucophyllum* cell cultures. 1α,25(OH)$_2$-vitamin D$_3$ specific binding was measured by radioligand-binding assays with whole cells in culture, as described in Materials and methods. *S. glaucophyllum* cells were incubated with 2 n*M* [^{3}H]1α,25(OH)$_2$D$_3$ alone (total binding) or in combination with a 500-fold molar excess of non-radioactive steroids (non-specific binding). Results are expressed as *f*mol/mg cellular protein and represent the mean of three independent experiments ± SD. (B) Saturation analysis of [^{3}H]1α,25(OH)$_2$-vitamin D$_3$ binding to *S. glaucophyllum* callus proteins. Callus tissue total protein (0.1–0.2 mg) obtained by centrifugation of homogenates at 120,000 *g* were incubated with increasing concentrations of [^{3}H]1α,25(OH)$_2$-vitamin D$_3$ in the absence or presence of a 500-fold molar excess of unlabeled 1α,25(OH)$_2$-vitamin D$_3$. Bound and unbound steroid were separated by the charcoal/dextran procedure as indicated in Materials and methods. Measured bound radioligand (upper panel) was plotted against the concentration of free tritiated hormone in the incubation medium. A typical profile is shown, and each point represents the mean of duplicate determinations. The lower panel shows the plot derived from Scatchard analysis of the same data. Binding parameters at equilibrium were estimated using the ligand program.

in whole cells. As shown in Fig. 1A, reproducible specific binding activity could be measured. The specific binding (total – non-specific binding) was found to average approximately 190 *f*mol/mg protein. 25(OH)D$_3$ was tested for its ability to interact with [^{3}H]1α,25(OH)$_2$D$_3$-binding sites. Competition studies showed that a 500-fold molar excess of 25(OH)D$_3$ was ineffective in displacing the radioactive ligand (Fig. 1A). Figure 1B shows the results of a representative experiment demonstrating that the binding of [^{3}H]1α,25(OH)$_2$D$_3$ to total proteins from calli was a saturable process with respect to the ligand concentration. The saturation was nearly complete at approximately 10 n*M* [^{3}H]1α,25(OH)$_2$D$_3$. Scatchard linearization of the saturation binding data was consistent with a single set of binding sites ($K_d = 3.49 \pm 0.75$ n*M*), with a maximum binding capacity of 255.7 ± 18.6 *f*mol/mg protein.

For immunochemical experiments, monoclonal MAI-710 and polyclonal PAI-711 antibodies directed to the avian VDR DNA-binding domain and C-terminus (contains the ligand-binding domain), respectively, were selected to search for VDR-like immunoreactivity in protein extracts from different organs of the plant body and in in vitro cultures of *S. glaucophyllum* by western blot analysis (Fig. 2). An immunoreactive band with

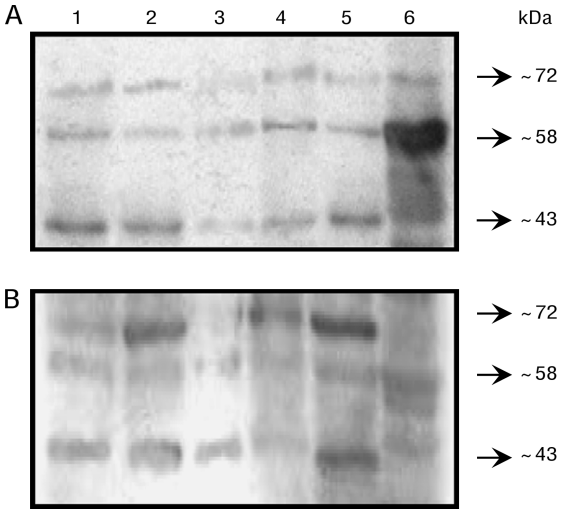

Fig. 2. Western blot profiles of protein extracts from different organs and cell and callus cultures of *Solanum glaucophyllum*, using two different anti-VDR antibodies. Equal protein samples (20 μg) were fractionated by SDS-PAGE, electrotransferred to Immobilon membranes and incubated with the anti-VDR monoclonal MAI-710 antibody (A) or the anti-VDR polyclonal PAI-711 antibody (B). A similar pattern of immunoreactivity was obtained for the two antibodies. Three major protein bands were consistently observed. A representative immunoblot from six independent experiments is shown. Lane 1: cells. Lane 2: calli. Lane 3: leaves. Lane 4: stems. Lane 5: roots. Lane 6: total soluble protein extract from chicken intestine (positive control).

a molecular mass matching that of the avian VDR (approximately 58 kDa) was revealed on the blots with either MAI-710 or PAI-711 antibodies. Interestingly, both antibodies labeled two other intense reactive bands with molecular masses of approximately 72 and 43 kDa. The signal detected was increased using a hypertonic buffer for protein extraction, suggesting that these proteins are associated with subcellular structures (Fig. 3A,B).

Immunoblots of cell fractions isolated from calli were carried out to establish the subcellular distribution of the immunoreactive bands described above. To that end, equivalent protein amounts of subcellular fractions were subjected to SDS-PAGE and western blot analysis using anti-VDR monoclonal (Fig. 4A) and polyclonal (Fig. 4B) antibodies. High expression levels of the reactive proteins were detected in nuclear and mitochondrial fractions with both antibodies. The monoclonal antibody also detected a substantial proportion of immunoreactivity in the cytosol. The polyclonal antibody was more immunoreactive with the ~58 kDa band than with the ~72 kDa and ~43 kDa bands, opposite to what is shown in Fig. 2B, an observation that may be explained by the concentration of the ~58 kDa protein with respect to total proteins in the different fractions.

Tyrosine phosphorylation of the *S. glaucophyllum* VDR-like proteins was investigated. Figure 5 shows the

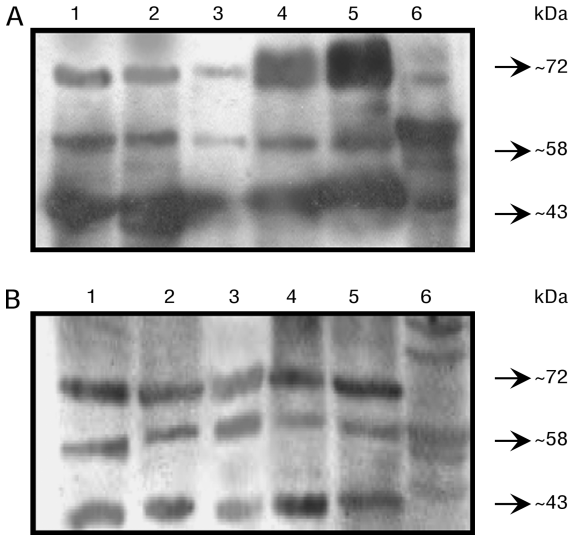

Fig. 3. VDR-like immunoreactivity profiles of protein extracts from different organs and cell and callus cultures of *Solanum glaucophyllum* obtained with high ionic strength buffer (TEKM). Equal protein samples (20 μg) were resolved by SDS-PAGE, followed by western blot analysis using anti-VDR monoclonal MAI-710 antibody (A) or anti-VDR polyclonal PAI-711 antibody (B). Representative immunoblots from five independent experiments (A) and two independent experiments (B) are shown. Lane 1: cells. Lane 2: calli. Lane 3: leaves. Lane 4: stems. Lane 5: roots. Lane 6: total soluble protein extract from chicken intestine (positive control).

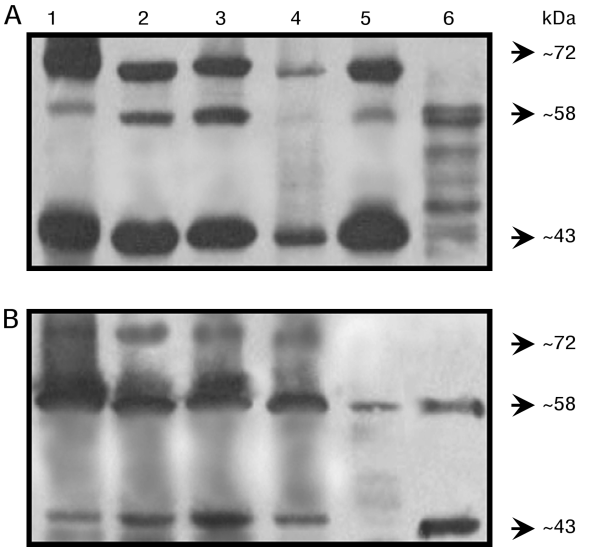

Fig. 4. Subcellular distribution of VDR-like immunoreactivity in *Solanum glaucophyllum* callus. Subcellular fractionation was performed by homogenization of calli in TES buffer followed by differential centrifugation as described in Materials and methods. Equivalent protein samples of subcellular fractions (20 μg) were resolved by SDS-PAGE and then subjected to western blot analysis using anti-VDR monoclonal (MAI-710, A) or VDR polyclonal (PAI-711, B) antibodies. Immobilon membranes were developed simultaneously in order to obtain comparable relative intensities of immunolabeling. A representative immunoblot from five independent experiments is shown. Lane 1: total homogenate. Lane 2: nuclear fraction. Lane 3: mitochondrial fraction. Lane 4: microsomal fraction. Lane 5: cytosolic fraction. Lane 6: total soluble protein extract from chicken intestine (positive control).

immunoreactive profile when subcellular fractions derived from callus were immunoprecipitated with an anti-phosphotyrosine specific antibody and then blotted with

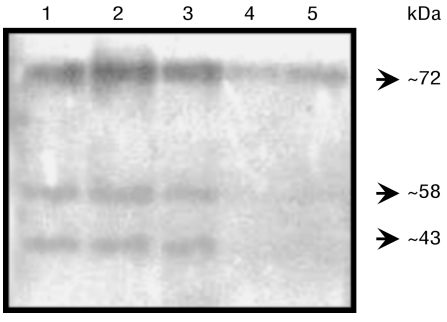

Fig. 5. Tyrosine phosphorylation of the VDR-like immunoreactive bands from *Solanum glaucophyllum* calli. Subcellular fractions from *S. glaucophyllum* calli containing equivalent amounts of protein were immunoprecipitated with anti-phosphotyrosine polyclonal antibody, followed by SDS-PAGE and western blot analysis. The immunoreactive bands were revealed using anti-VDR monoclonal antibody MAI-710. A representative immunoblot from three independent experiments is shown. Lane 1: total homogenate. Lane 2: nuclear fraction. Lane 3: mitochondrial fraction. Lane 4: microsomal fraction. Lane 5: cytosolic fraction.

Table 1. Cytochrome *C* oxidase activity of subcellular fractions from *Solanum glaucophyllum* callus. Different fractions were isolated from callus tissue homogenates and assayed for cytochrome *C* oxidase activity as described in Materials and methods. Data represent the mean of two different experiments ± sd. 1, nucleus; 2, mitochondria; 3: microsomes; 4: cytosol.

	1	2	3	4
Cytochrome c Oxidase activity μg/min/mg	n.d.	4.12 ± 0.77	n.d.	n.d.

the anti-VDR monoclonal antibody. The major reactivity was concentrated in the nuclear and mitochondrial fractions, thus showing that the associated VDR-like proteins also contain tyrosine phosphorylated residues, as was previously described for the avian VDR (Buitrago et al. 2000).

Assays of cytochrome *C* oxidase activity revealed no mitochondrial contamination of nucleus, microsomes or cytosol (Table 1), and a mitochondrial outer membrane integrity of 79%. These results suggest that the technique of isolating cell fractions with low ionic strength TES buffer, described in Materials and methods, allows a high percentage of mitochondria without any membrane damage to be recovered, which might also be the case for the other fractions (nucleus and microsomes), although unoccupied sterol-binding proteins could leave their native localization during subcellular fractionation (King and Greene 1984).

In order to correlate immunoblotting results with radioligand assays, we carried out ligand blot experiments. Protein samples were subjected to SDS-PAGE, electrotransferred to membranes, re-natured, and then incubated with [^{3}H]1α,25(OH)$_2$D$_3$ alone or in combination with a 500-fold molar excess of unlabeled 1α,25(OH)$_2$D$_3$. Table 2 shows the distribution of radioactivity retained in the membranes. Positive (chick intestine soluble protein extract) and negative (non-immunoreactive proteins and Immobilon membrane without protein sample) controls were also tested in order to ensure specifity of the binding. Table 2A shows the radioactivity retained when the membranes were incubated with [^{3}H]1α,25(OH)$_2$D$_3$ alone. Combination with a 500-fold molar excess of unlabeled steroid displaced approximately 50–60% of the radioactivity, revealing specific labeling by the steroid hormone selectively associated with the ~72 kDa, ~58 kDa and ~43 kDa immunoreactive bands (Table 2B), suggesting that these reactive entities could also bear 1α,25(OH)$_2$D$_3$-binding sites.

Immunocytochemistry detection of fixed cells derived from callus with secondary antibodies conjugated to Oregon Green is shown in Fig. 6. Immunofluorescent staining appeared in the nucleus when the monoclonal antibody was employed as primary antibody, and in the nucleus, unidentified cytoplasmic corpuscles and outer cell membrane with the polyclonal antibody.

Figure 7 shows western blot assays for the active phosphorylated MAPKs with TEY domains after treatment of *S. glaucophyllum* cells with 1α,25(OH)$_2$D$_3$ or the plant lipid extract. Neither the hormone nor the extract induced phophorylation of MAPKs; weak immunoreactive bands

Table 2. Ligand blot analysis of the immunoreactive proteins from *Solanum glaucophyllum* callus employing [^{3}H]1α,25(OH)$_2$-vitamin D$_3$. Immobilon membranes containing 20 μg of protein from each subcellular fraction were re-natured as described in Materials and methods and then incubated overnight at 4°C with 2 n*M* [^{3}H]1α,25(OH)$_2$-vitamin D$_3$ alone (A) or in combination with a 500-fold molar excess of cold hormone (B). Radioactivity bound was determined as described in Materials and methods. Results are expressed as c.p.m. and represent the mean of two independent experiments ± SD. Lane 1: total homogenate. Lane 2: nuclear fraction. Lane 3: mitochondrial fraction. Lane 4: microsomal fraction. Lane 5: cytosolic fraction. Lane 6: total soluble protein extract (0.4 *M* KCl) from chicken intestine (positive control). Lane 7: non-immunoreactive protein. Lane 8: PVDF membrane without protein sample.

A

kDa (approx.)	1	2	3	4	5	6	7	8
72	1680 ± 130	1870 ± 200	1650 ± 120	1540 ± 90	1530 ± 120	310 ± 90		
58	1790 ± 180	1480 ± 130	2040 ± 200	1910 ± 180	2410 ± 200	2520 ± 360		
43	1230 ± 90	1760 ± 150	1830 ± 110	1390 ± 80	1100 ± 80	810 ± 100		
							500 ± 40	
								300 ± 20

B

kDa (approx.)	1	2	3	4	5	6	7	8
72	750 ± 100	690 ± 50	580 ± 80	400 ± 30	800 ± 120	540 ± 80		
58	410 ± 70	710 ± 90	800 ± 140	490 ± 60	890 ± 100	1320 ± 140		
43	500 ± 80	900 ± 100	630 ± 110	590 ± 70	510 ± 70	700 ± 110		
							490 ± 40	
								390 ± 30

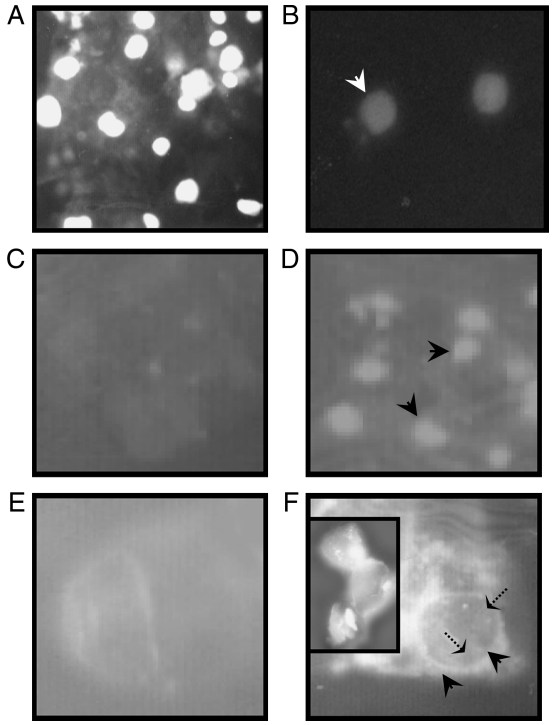

Fig. 6. Localization of VDR-like proteins in *Solanum glaucophyllum* cells from callus tissue by indirect immunofluorescence microscopy. VDR-like immunoreactivity was detected in paraformaldehyde-fixed and permeabilized cells using the monoclonal (D) and polyclonal (F) antibodies (see Materials and methods). The fluorescence signal from Oregon Green represents VDR-like indirect visualization obtained by incubation with fluorescent secondary antibodies. Immunostaining of the nucleus (D; black arrows), unidentified cytoplasmic corpuscles (F; dashed arrows) and outer cell membrane (F; black arrows) is denoted. Nuclear staining with PI and DAPI resulted in a red (A) and blue (A; white arrow) signals, respectively. Negative controls using secondary antibodies alone rendered negligible cellular fluorescence (C, E). Original magnification = 400×.

were detected (Fig. 7A), which could correspond to MAPK activation by the vehicle (isopropanol). However, 1α,25 $(OH)_2D_3$ and the lipid extract were able to stimulate DNA synthesis in a dose-dependent manner, as shown by a significant increase in [^{3}H]thymidine incorporation into DNA at three different concentrations (Table 3).

Discussion

The natural occurrence of sex steroid hormones in several vascular plants has been extensively documented (Geuns 1978, Buchala and Schmid 1979, Grunwald 1980, Mudd 1980, Mandava et al. 1982, Boland 1986, Weissenberg et al. 1989, Agarwal 1993, Curino et al. 1998, Skliar et al. 2000). *S. glaucophyllum* has been previously shown to contain appreciable quantities of the secosteroid hormone 1α,25$(OH)_2D_3$ and related metabolites (Boland et al. 2003 and references therein). In order to investigate the existence of 1α,25$(OH)_2D_3$-binding proteins in *S. glaucophyllum* tissue cultures, we first designed radioligand-binding assays to detect binding sites for the steroid hormone. Surprisingly, we found that cell cultures derived from callus contained binding sites for 1α,25$(OH)_2D_3$. The binding exhibited specificity, as 25$(OH)D_3$ could not compete with the tritiated steroid hormone for the binding sites. We characterized, by Scatchard analysis, the binding sites present in callus cultures. As classic receptors, these sites exhibited ligand affinity in the nanomolar range and were saturable with respect to the ligand concentration. Specific antibodies against the VDR recognized a plant antigen (approximately 58 kDa) that exactly co-migrates with the avian VDR after SDS-PAGE separation. This plant antigen was detected in protein extracts derived from different organs of the plant body and in vitro tissue cultures. Two additional bands of approximately 72 and 43 kDa were also detected. Interestingly, the same immunoreactive pattern was obtained with protein extracts derived from two other Solanaceae species, *Lycopersicon esculentum* and *Nicotiana glauca* (data not shown). The immunoreactive entities were best recovered using hypertonic

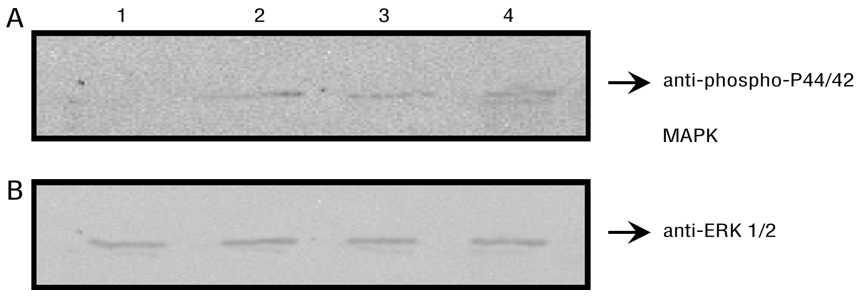

Fig. 7. Lack of effect of 1α,25$(OH)_2$-vitamin D_3 and lipid extract on MAPK activity in *Solanum glaucophyllum* cells. *S. glaucophyllum* cell cultures were treated for 20 min with 50 n*M* 1α,25$(OH)_2D_3$, 0.5 ml of plant lipid extract or vehicle (isopropanol), followed by homogenization. Equivalent protein samples (20 μg) were resolved by SDS-PAGE and processed for western blot analysis using (A) anti-active phospho-MAPK (anti-phospho-P44/42 MAPK) antibody or (B) anti-MAPK ERK1/2 antibody. A representative immunoblot from three independent experiments is shown. Lane 1: control. Lane 2: vehicle. Lane 3: 1α,25$(OH)_2$-vitamin D_3. Lane 4: *S. glaucophyllum* lipid extract.

Table 3. Effect of 1α,25(OH)$_2$-vitamin D$_3$ and lipid extract on DNA synthesis in *Solanum glaucophyllum* cell cultures. Aliquots (1 ml) of cultured cells were incubated for 3 h in a medium containing 0.5 μCi/ml [^{3}H]thymidine prior to treatment with 1 n*M* (A), 10 n*M* (B) or 50 n*M* (C) 1α,25(OH)$_2$-vitamin D$_3$, or 1 μl (A), 50 μl (B) or 100 μl (C) of *S. glaucophyllum* lipid extract or vehicle (isopropanol). [^{3}H]Thymidine incorporation was determined as described in Materials and methods. Results are expressed as c.p.m./mg DNA and represent the mean of two independent experiments ± SD. *$P < 0.05$, with respect to the control.

Treatments	A	B	C
Control	11754 ± 823	11754 ± 823	11754 ± 823
Vehicle	11840 ± 750	11024 ± 602	11513 ± 637
1α,25(OH)$_2$D$_3$	16659 ± 1022*	21851 ± 1748*	22774 ± 1594*
Lipid extract	13905 ± 873	15763 ± 1099*	17688 ± 1208*

buffers for protein extraction, suggesting that these proteins are associated with subcellular structures and are tyrosine phosphorylated, as shown by immunoprecipitation assays with a specific phosphotyrosine antibody. The subcellular distribution pattern revealed a nuclear–mitochondrial localization of the immunoreactivity. Also, the monoclonal antibody detected substantial cytosolic localization of the reactive bands. However, by immunocytochemistry assays, the monoclonal antibody did not recognize a cytosolic reactive entity. We propose that the cytosolic immunoreactivity could be attributed to a partition into the cytosol of the nuclear protein when the cell is disrupted, and thus the cytosolic reactivity may represent an extraction artifact, as shown for estrogen receptors (King and Greene 1984). Immunocytochemical detection of cytoplamic corpuscles with the polyclonal antibody (Fig. 6F) may be tentatively ascribed to the presence of VDR-like proteins in mitochondria, in agreement with the immunochemical data.

By means of ligand blot assays using [^{3}H]1α,25(OH)$_2$D$_3$, we established that these immunoreactive proteins are real 1α,25(OH)$_2$D$_3$ binders. The relative contribution of each reactive band to hormone binding is uncertain, as it depends basically on the degree of re-naturalization of the receptor-like entities.

To search for a role of 1α,25(OH)$_2$D$_3$ in *S. glaucophyllum* cells, we investigated the effects of 1α,25(OH)$_2$D$_3$ on cell proliferation by means of MAPK activation and [^{3}H]thymidine incorporation into DNA. Stimulation of MAPK in cell proliferation responses has been demonstrated in plant cells (Hirt 1997, Huang et al. 2000, Ligternik and Hirt 2001, Taylor et al. 2001). We showed that both the hormone and the lipid extract (which contains appreciable quantities of 1α,25(OH)$_2$D$_3$) (Boland et al. 2003) induce DNA synthesis in a dose-dependent manner, and probably cell proliferation, as was previously described for vitamin D$_3$ in *Phaseolus vulgaris* roots (Vega et al. 1988), by a mechanism that is, however, independent of the activation of MAPKs with TEY domains. The results obtained in this study indicate that both the steroid hormone and the lipid extracts affect DNA synthesis similarly to plant growth factors of known mitogenic activity. Stimulation of [^{3}H]thymidine incorporation into DNA probably indicates entry of cells into the S-phase of the cycle. The data also indicate that *S. glaucophyllum* cells, which produce 1α,25(OH)$_2$D$_3$, are able to respond to the steroid. Whether the VDR-like protein(s) play a functional role in this response remains to be established.

Finally, to the best of our knowledge, these are the first results showing a potential plant VDR-like molecule. From an evolutionary point of view, the conservation of epitopes and molecular weight of these related binding proteins gives rise to the existence of steroid receptor molecules in plant species, similar to those known for animals, and opens the possibility of the operation of a regulatory system within the plant body.

Acknowledgements – This research was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Sur, Argentina. Lorena Milanesi is recipient of a graduate research fellowship from CONICET.

References

Agarwal MK (1993) Receptors for mammalian steroid hormones in microbes and plants. FEBS Lett 322: 207–210

Baker AR, McDonell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85: 3294–3298

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917

Boland R (1986) Plants as a source of vitamin D$_3$ metabolites. Nutr Rev 44: 1–8

Boland R, Skliar M, Curino A, Milanesi L (2003) Vitamin D compounds in plants. Plant Science 164: 357–369

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72: 248–254

Buchala AJ, Schmid A (1979) Vitamin D and its analogues as a new class of plant growth substances affecting rhizogenesis. Nature 280: 230–231

Buitrago C, Vazquez G, Boland AR de, Boland R (2000) Activation of src kinase in skeletal muscle cells by 1, 25(OH)$_2$-vitamin D$_3$ correlates with tyrosine phosphorylation of the vitamin D receptor (VDR) and VDR-Src interaction. J Cell Biochem 79: 274–281

Curino A, Skliar M, Boland R (1998) Identification of 7-dehydrocholesterol, vitamin D$_3$, 25(OH)-vitamin D$_3$ and 1, 25(OH)$_2$ vitamin D$_3$ in *Solanum glaucophyllum* cultures grown in the absence of light. Biochim Biophys Acta 1425: 485–492

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240: 889–895

Feldman D, Do Y, Burshell A, Stathis P, Loose DS (1982) An estrogen-binding protein and endogenous ligand in *Saccharomyces cerevisiae*: possible hormone-receptor system. Science 218: 297–298

Geuns JMC (1978) Steroid hormones and plant growth and development. Phytochem 17: 1–14

Grunwald D (1980) Steroids. In: Bell ED, Charlwood BV (eds) Encyclopedia of Plant Physiology, Secondary Plant Products, Vol. 8. Springer–Verlag, Berlin, pp 221–256

Hirt H (1997) Multiple roles of MAP kinases in plant signal transduction. Trends in Plant Science 2: 11–15

Huang H, Li H, Gupta R, Morris P, Luan S, Kieber JJ (2000) ATMPK4, an *Arabidopsis* homolog of mitogen-activated protein kinase, is activated in vitro by AtMEK1 through threonine phosphorylation. Plant Physiol 122: 1301–1334

King WJ, Greene GL (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307: 745–747

Kuiper G, Carlsson B, Grandien K, Enmark E, Hagglad J, Nilsson S, Gustafsson J (1997) Comparison of the ligand binding specificity and transcript distribution of estrogen receptors α and β. Endocrinology 138: 863–870

Laudet V, Hanni C, Coll J, Catzeflis F and Stehelin D (1992) Evolution of the nuclear receptor gene superfamily. EMBO J 11: 1003–1013

Ligternik W, Hirt H (2001) Mitogen-activated protein (MAP) kinase pathways in plants: versatile signaling tools. Int Rev Cytol 201: 209–275

Mandava NB, Sasse JM, Yop JH (1982) Brassinolide, a growth-promoting steroidal lactone II. Activity in selected gibberellin and cytokinin bioassays. Physiol Plant 53: 453–461

McDonell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW (1987) Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235: 1214–1217

Milanesi L, Boland R (2004) Presence of estrogen receptor (ER)-like proteins and endogenous ligands for ER in *Solanaceae*. Plant Science 166: 397–404

Milanesi L, Monje P, Boland R (2001) Presence of estrogens and estrogen receptor-like proteins in *Solanum glaucophyllum*. Biochem Biophys Res Commun 289: 1175–1179

Monje P, Boland R (2002) Expression and cellular localization of naturally ocurring beta estrogen receptors in uterine and mammary cell lines. J Cell Biochem 86: 136–144

Monje P, Boland R (2001) Subcellular distribution of native estrogen receptor α and β isoforms in rabbit uterus and ovary. J Cell Biochem 82: 467–479

Mudd JB (1980) Sterol interconversions. in: Stumpf K, Conn Ee (eds) The Biochemistry of Plants. Lipids: Structure and Function. Academic Press, New York, pp 509–534

Munson PJ, Rodbard D (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107: 220–239

Owen GJ, Zelent A (2000) Origins and evolutionary diversification of the nuclear receptor superfamily. Cell Mol Life Sci 57: 809–827

Skliar M, Curino A, Milanesi L, Benassati S, Boland R (2000) *Nicotiana glauca*: another plant species containing vitamin d$_3$ metabolites. Plant Sci 156: 193–199

Snedecor GW, Cochran WG (1967) Statistical Methods. Ames, IA. The Iowa State University Press, pp 120–134

Talmon V, Vega M, Boland R (1989) Cytohistological studies on the action of vitamin D$_3$ and stigmasterol on *Phaseolus vulgaris* roots growing in vitro. Plant Sci 59: 183–190

Taylor A, Kim J, Low P (2001) Involvement of mitogen-activated protein kinase activation in the signal-transduction pathways of the soya bean oxidative burst. Biochem J 355: 795–803

Vega M, Boland R (1988) Presence of sterol-binding sites in the cytosol of French-bean (*Phaseolus vulgaris*) roots. Biochem J 250: 565–569

Vega M, Fernandez L, Boland R (1988) Mediation of sterol induced calmodulin synthesis in *Phaseolus vulgaris* roots by Ca^{2+} and its possible relationship to plant growth regulators. Physiol Plant 75: 499–505

Wasserman RH, Henion JD, Haussler MR, McCain TA (1976) Calcinogenic factor in *Solanum malacoxylon*: evidence that it is 1,25-dihydroxy-vitamin D$_3$-glycoside. Science 194: 853–854

Weissenberg M, Levy A, Wasserman RH (1989) Distribution of calcitriol activity in *Solanum glaucophyllum* plants and cell cultures. Phytochem 28: 795–798

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47: 189–194

Edited by D. Van Der Straeten