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Specific binding sites for progesterone and 17β-estradiol in cells of *Triticum aestivum* L.

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The presence and location of specific binding sites for progesterone and 17β-estradiol in cells of wheat were estimated using radioligand binding assay. Membrane and cytosolic fractions of non-vernalized and vernalized plants were tested using tritium-labelled ligands. Specific binding of [^{3}H]progesterone and [^{3}H]17β-estradiol occurs in wheat cells. The binding sites are located in membranes and in the cytosol. Specific binding of [^{3}H]17β-estradiol is higher in the membranes than in the cytosol. Specific binding of both ligands in the cytosolic fraction is higher in vernalized plants than in non-vernalized ones. The possibility of the occurrence of steroid binding proteins specific for progesterone and 17β-estradiol, putative steroid receptors for these steroids in *Triticum aestivum* L., is discussed.

Keywords: *Triticum aestivum* L., progesterone, 17β-estradiol, radioligand binding assay, vernalization

INTRODUCTION

The growth and development of plants and animals is coordinated at the tissue and organ level by signalling molecules involved in intercellular communication. Hormone receptors recognise structural differences between ligands (for example steroids) in a highly specific way (Chow & McCourt, 2006). Two main modes of steroid action are known (Marcinkowska & Więdłocha, 2002; Norman *et al*., 2004). The first mode is gene regulation response mediated by nuclear receptors. A ligand-receptor complex is moved to the nucleus and promotes or represses gene transcription (Beato *et al*., 1995). An extensive nuclear receptor superfamily has been described in animals (Robinson-Rechavi *et al*., 2003). The existence of this kind of steroid receptor in plants was first reported by Janik and Adler in 1984 in *Gladiolus primulinus* Bak. Furthermore, specific binding of 17β-estradiol in the cytosolic and nuclear fraction of calli of *Solanum glaucophyllum* Desf. was detected by Milanesi and Boland (2004).

The second mechanism of steroid action is a rapid non-genomic response mediated by plasmalemma membrane receptors. The non-genomic steroid signalling alters secondary messenger levels, ion fluxes, and protein kinase activities *via* steroid carrier proteins which are yet to be precisely characterized, and through plasma membrane-associated receptors (Norman *et al*., 2004). An animal plasma membrane receptor has been cloned, namely a G-protein-associated membrane receptor for progesterone from fish oocytes (Zhu *et al*., 2003). In plants, steroid hormones known as brassinosteroids are recognised by the BRI1 cell surface receptor kinase (Wang *et al*., 2006; Li & Jin, 2007). A putative steroid-binding membrane protein for progesterone (MSBP1) has been identified and characterized as a regulator of cell elongation in *Arabidopsis thaliana* L. (Yang *et al*., 2005).

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Abbreviations: MSSH, mammalian steroid sex hormone; SBP, steroid binding protein.

Mammalian steroid sex hormones (MSSH), which include progesterone, 17β-estradiol and testosterone, are naturally present in plants, but their content depends on species, cultivar, plant organ and stage of development (Simons & Grinwich, 1989; Iino *et al*., 2007) (Fig. 1). MSSH applied exogenously stimulate plant cell division, pollen germination or plant growth and flowering (Janeczko & Skoczowski, 2005). Wheat is very sensitive to the application of MSSH. Our earlier experiments showed that growth of isolated immature embryos of wheat is moderated by MSSH in chemical structure-dependent manners (Janeczko *et al*., 2002). *In vitro* germination and growth of first leaf of isolated immature wheat embryos is strongly stimulated by androsterone and androstenedione. Callus induction is associated with these processes but its proliferation and growth are similar to the control (not treated with steroids). Estrone and progesterone inhibit the first leaf and a callus growth of immature embryos in *in vitro* culture (Janeczko *et al*., 2002). Progesterone and 17β-estradiol applied in *in vitro* culture of mature embryos of winter wheat stimulate the generative development of plants by increasing the percentage of heading plants and accelerating the heading (Janeczko & Filek, 2002). The high physiological activity of progesterone and estrogens in wheat is unquestionable, but the molecular mechanism of their action remains unclear. Based on the present knowledge about the involvement of steroid receptors in signal transduction and gene expression leading to physiological effects in living organisms, it can be assumed that in wheat cells specific steroid binding sites key to the mechanism of MSSH action may be present.

The aim of this study was to estimate the presence and location of specific binding sites for progesterone and 17β-estradiol in cells of winter wheat using the radioligand binding method. The radioligand-receptor binding assay is a useful method which provides information about specific binding of ligands tested in cells and the putative receptor-hormone interactions (Nowak, 1998). Membrane and cytosolic fractions of vernalized and non-vernalized plants of winter wheat were tested using tritium labelled ligands. Similarly to other winter plants, winter wheat requires vernalization, defined as the exposure of a plant to chilling (from a few days to a few weeks; temperature: +1°C to +10°C) which induces the plant's generative development. Generally, non-vernalized winter plants are not capable of generative development or their development is disturbed. As cold-treated plants are biochemically altered in comparison with plants growing at 20°C, these two different objects (non-vernalized and vernalized) were chosen for the ligand binding experiments.

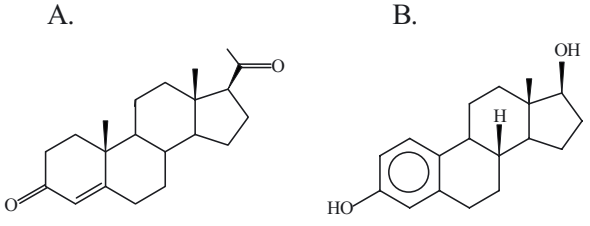

Figure 1. Chemical structure of mammalian sex hormones: progesterone (A) and 17β-estradiol (B).

MATERIALS AND METHODS

Plant material. Seeds of winter wheat cv. Kobiera were germinated for 4 days at 26°C/20°C (day/night), 8 h photoperiod and then transferred to the coldroom (5°C, 8 h photoperiod) where they grew for 8 weeks (vernalization). During this time wheat developed the first leaf while the second one was in a germ form. After vernalization, plants were allowed to grow in a greenhouse for 20 days (natural light conditions, March, latitude: 50°03' North, longitude: 19°55' East, about 12 h photoperiod; temperature: 16 h at 20°C, 8 h at 17°C) to reach the stage of tillering (main shoot and developing 1–2 tillers)). Non-vernalized plants after 4 days of germination (8 h photoperiod; temperature 26°C/20°C (day/night)) were then cultured for 6 days in a growth chamber (8 h photoperiod; temperature 20°C/17°C (day/night)). The seedlings with the first leaf developed (the second one was in a germ form) were moved to a greenhouse where they continued growth at natural light conditions (as above) to reach the tillering stage. All leaves were harvested for analysis.

Isolation of the microsomal and cytosolic fractions. Homogenisation and isolation of fractions was performed based on a modified protocol of Sommarin *et al*. (1985). Leaf samples (100 g about 200 plants) were homogenised using a Sorvall Omnimixer (USA; 2×30 s, maximum speed) in 400 ml of 10 mM Tris/HCl buffer (pH 7.8) containing 0.25 M sucrose, 1 mM EDTA, 2.5 mM DTT and 10 mM sodium molybdate (Sigma-Aldrich, Poland). The crude extract was filtered through 2 layers of Miracloth and centrifuged for 10 min at 10 000×*g* (Beckmann L3-50, rotor 15, USA) to remove the nuclear, chloroplast and mitochondrial pellet. The resulting supernatant 1 was then centrifuged for 30 min at 80 000×*g* (Beckmann L8-M, rotor SW 27, USA). The pellet obtained was the microsomal fraction (membrane fraction) while supernatant 2 was considered the cytosolic fraction. The pellet was resuspended in 6 ml of the buffer above and taken for analysis. Fifteen millilitres of supernatant 2 was concentrated using 2.5 g of dry Sephadex G 25 (Sigma-Aldrich, Poland) and taken for all further analyses.

Protein content. Preparation of Bradford reagent: 100 mg Coomassie Brilliant Blue (Fluka, Poland) was dissolved in 50 ml of 95% ethanol; subsequently, 100 ml of 85% (w/v) phosphoric acid was added. The resulting stock solution was diluted to 1 litre and filtered through filter paper immediately before use.

Protein content was estimated based on the protocol of Sedmak and Grossberg (1977). Two microlitres of 10% water solution of Triton X-100 (Sigma-Aldrich, Poland) was mixed with 10 μl of the microsomal fraction and kept for 10 min in ice. Subsequently, 2 ml of water and 2 ml of Bradford reagent were added. After 10 min absorbance was recorded (595 nm) using a Biochrom Ultrospec II spectrophotometer (LKB, Sweden). Measurements were carried out with 3 repetitions. Bovine serum albumin (Sigma-Aldrich, Poland) was used as the calibration standard. Albumin was diluted in the buffer for the isolation of cell fractions and 2 μl of 10% water solution of Triton X-100 was added.

17β-estradiol and progesterone binding sites

Ligands. [(1,2,6,7-^{3}H[N])-Progesterone (specific activity 82 Ci/mmol)] and 2,4-[^{3}H]17β-estradiol (specific activity 32 Ci/mmol) were purchased from Lencomm Trade International S.J. (Poland). Unlabelled progesterone and 17β-estradiol were obtained from Sigma-Aldrich (Poland).

Elimination of free steroids. In a preliminary experiment microsomal and cytosolic fractions were treated with dextran-coated charcoal to eliminate free steroids. The fractions were incubated with the charcoal (0.5%)/dextran (0.05%) suspension for 10 min at 4°C (shaking). Then the samples were centrifuged (800×*g*, 10 min, 2°C) to remove the charcoal and supernatants were used for progesterone- and 17β-estradiol-binding site determination. Because the preincubation with charcoal/dextran did not change progesterone and 17β-estradiol binding in the microsomal and cytosolic fraction, it was ommited in the final assays.

Binding assay of 17β-estradiol. Aliquots of the microsomal or cytosolic fraction containing about 150 μg of protein were incubated in triplicate with 5 nM [^{3}H]17β-estradiol in a buffer (5 mM Tris/HCl, 1 mM EDTA, 1 mM DTT, 10 mM sodium molybdate, 10% glycerol, pH 7.4) at 4°C for 16 h. Identical samples were incubated in triplicate with a 500-fold excess of unlabelled 17β-estradiol added to determine nonspecific binding. The final volume of each sample was 200 μl. After incubation, 200 μl of charcoal (1%)/dextran (0.1%) suspension was added to each sample. The samples were incubated for 10 min at 2°C, centrifuged at 800×*g* for 10 min at 4°C and 200 μl of the supernatant was removed and the radioactivity of [^{3}H]17β-estradiol bound was measured in a scintilation counter (Beckmann LS 335 liquid scintillation counter, USA). Specific binding was determined by subtracting nonspecific binding from total binding, and results were expressed as femtomoles (fmol) of specifically bound 17β-estradiol per mg of protein.

Binding assay of progesterone. For the determination of microsomal and cytosolic progesterone binding sites 10 nM [^{3}H]progesterone was used. In order to measure nonspecific binding, a 500-fold excess of unlabelled progesterone was applied. In a preliminary experiment microsomal and cytosolic fractions were pretreated with 1 μM cortisol to exclude the possibility of progesterone binding with the corticosteroid-binding globulin and glucocorticoid receptor. Because cortisol did not influence progesterone binding in the fractions investigated, it was not added in the final assay. Conditions of the incubation and separation of the bound from the free steroid were the same as described above for 17β-estradiol.

Determination of 17β-estradiol and progesterone receptors in rat uterus. In order to verify the method used, simultaneously with the determination of progesterone and 17β-estradiol binding sites in plant fractions, progesterone and 17β-estradiol receptors were assayed in rat uteri. Uteri of two immature (20-day old) female rats were homogenised in Tris/HCl buffer, centrifuged at 800×*g* for 10 min at 4°C and subsequently the supernatant was subjected to high speed centrifugation (105000×*g*, 1 h, 2°C) and the supernatant obtained was used as the cytosolic fraction. The concentration of progesterone and 17β-estradiol receptors in the cytosol from rat uteri was determined as described above for plant fractions. Specific 17β-estradiol binding was 444 fmol×mg^{-1} protein and specific progesterone binding was 376 fmol/mg protein in the rat uterus. These values are similar to those described by other authors (Garófalo & Raymondo, 1995).

RESULTS AND DISCUSSION

Specific binding of [^{3}H]17β-estradiol and [^{3}H]progesterone in membrane and cytosolic fractions of non-vernalized and vernalized plants of winter wheat was investigated. Data were obtained using the method applied universally for the determination of estrogen and progesterone receptors in mammalian tissues (Hammond *et al*., 1979; Holinka *et al*., 1980). Additionally, cytosolic fraction from immature rat uterus was used as a positive control for the studies.

Since the presence of endogenous MSSH should reduce radioligand binding, in preliminary

experiments we used fractions after removing putative free steroids. We did not observe any change in progesterone and 17β-estradiol binding after the fractions were preincubated with charcoal/dextran suspension, which confirms the absence of substantial amounts of endogenous free progesterone or estrogens in the fractions of winter wheat investigated (not shown). However, it cannot be excluded that they are present as glucoside or glucosyl esters, which is common for plant hormones (Sembdner *et al.*, 1994). Conjugates might act as storage forms, important in regulation of physiologically active hormone levels (Sembdner *et al.*, 1994). There is rather little knowledge about the presence of progesterone and estrogens in green parts of plants of the *Graminae* family. Progesterone was found in the shoots and ears of rice (Iino *et al*., 2007) and in the inflorescence of *Zea mays* L. (Simons & Grinwich, 1989). Progesterone and estrogens are present in the leaves of *Agropyron intermedium* (Host) Beauv. and *Hordeum vulgare* L. (Simons & Grinwich, 1989).

To exclude [^{3}H]progesterone binding to the corticosteroid-binding globulin and glucocorticoid receptor, in preliminary experiments, plant fractions were pretreated with cortisol, but this treatment did not change radioligand binding either (not shown). These results, and the fact that the presence of EDTA and dithiothreitol in the buffer destroys the binding activity of the sex-steroid binding proteins, show that the radioactive ligand is bound specifically.

We found specific binding of [^{3}H]progesterone in the membrane fraction of wheat (Table 1). In non-vernalized plants the specific binding of [^{3}H]progesterone was 31.0 fmol/mg protein. In plants vernalized for 8 weeks, the specific binding of progesterone was 18.1 fmol/mg protein. This suggests the presence of steroid-binding factors — possibly steroid binding proteins (SBPs) — in wheat. SBPs are well known in animals, where they function as steroid receptors or steroid carrier proteins. SBPs are described as plasma or membrane proteins (MSBP), according to their subcellular localisation (Bordin & Petra, 1980; Stanczyk *et al*., 1989; Cenedella *et al*., 1999). Most likely, then, a membrane steroid binding protein is present in wheat cells. A putative membrane steroid binding protein (MSBP1) was identified in *Arabidopsis thaliana* L. (Yang *et al*., 2005). The *MSBP1* gene encodes a 220-amino-acid protein which can bind progesterone (high affinity) and 5-dihydrotestosterone, 24-epibrassinolide (one of brassinosteroids), and stigmasterol (low affinity) (Yang *et al*., 2005). Genes encoding putative progesterone-binding proteins are present in *Arabidopsis* (*MSBP1, MSBP2)* and rice (*OsMSBP1,2*) (Iino *et al*. 2007). Based on the available plant Expressed Sequence Tags data Iino *et al.* (2007) hypothesise that progesterone binding membrane proteins may be present in various plant species, including wheat.

We also examined specific binding of [^{3}H]17β-estradiol in wheat membranes (Table 1). The specific binding of [^{3}H]17β-estradiol in the cell microsomal fraction was 41.7 fmol/mg protein and 28.8 fmol/mg protein for non-vernalized and vernalized plants, respectively. The presence of estrogen binding sites in the microsomes of *Solanum glaucophyllum* calli has also been reported by Milanesi & Boland (2004).

In the cytosol of non-vernalized wheat, specific binding of [^{3}H]17β-estradiol was not detected. It appeared, however, in vernalized plants (10.9 fmol/mg protein) (Table 1). In the cytosolic fraction of wheat, [^{3}H]17β-estradiol was bound by certain steroid binding factors, supposedly by SBPs. This seems to be consistent with the findings of Milanesi and Boland (2004) who described specific binding of 17β-estradiol in *S. glaucophyllum* Desf. and *Lycopersicon esculentum* Mill. Using highly specific monoclonal antibodies against estrogen receptor α, they found a plant antigen of about 67 kDa (the molecular weight of the estrogen receptor) localised in the cell nuclear fraction and one of about 50 kDa in the cytosol.

The results of our work show that [^{3}H]progesterone is also specifically bound in the cytosolic fraction of plant cells, which suggests the presence of SBPs for progesterone in wheat cytoplasm as well. The specific binding of [^{3}H]progesterone was 4.9 fmol/mg protein and 21.3 fmol/mg protein for non-vernalized and vernalized plants, respectively (Table 1). According to Yang *et al*. (2005) and Iino *et al*. (2007), genes for soluble SBPs are present in *Arabidopsis* and rice.

Generally, for both the radioligands tested in our experiment, specific binding is higher in plant membranes than in the cytosolic fraction. Moreover, the level of specific binding seems to be dependent on the induction of plant generative development (vernalization). This phenomenon, however, requires more studies

Table 1. Specific binding of [^{3}H]progesterone and [^{3}H]17β-estradiol in microsomal and cytosolic fractions of winter wheat cells.

Results are expressed as mean values ±S.E.; n=3; ND, not detectable. Statistical significance of mean values for vernalized *versus* non-vernalized plants according to paired Student's *t*-test was: *, $P<0.04$; **, $P<0.0001$.

Winter wheat plants	[^{3}H]17β-estradiol	[^{3}H]progesterone
	(fmol/mg protein)	
	microsomal fraction	
Non-vernalized	41.7±10.8	31.0±5.5
Vernalized	28.8±3.3	18.1±4.2*
	cytosolic fraction	
Non-vernalized	ND	4.9±0.5
Vernalized	10.9±1.9	21.3±0.6**

using other wheat varieties. According to Milanesi and Boland (2004), specific binding of 17β-estradiol depends on the plant organ. As our earlier studies show, progesterone and 17β-estradiol stimulate flowering in winter wheat vernalized for a short period, insufficient for the normal development (Janeczko & Filek, 2002). Specific binding of these compounds in wheat cell suggests that the mechanism of their action may be related to the presence of their receptors. MSSH may be among the natural players in the pathway of development in wheat.

Despite the examples of steroid binding sites mentioned above and the presence of SBPs in plant cells, knowledge about this problem is quite insufficient in comparison with the data available for animals.

To conclude, our work has shown, for the first time, that specific binding of progesterone and 17β-estradiol occurs in wheat cells. The binding sites are located in membranes and in the cytoplasm. Most likely, SBPs with an affinity to progesterone and 17β-estradiol are present which may be putative steroid receptors for these ligands in the cells of *Triticum aestivum* L. The different number of binding sites for radioligands in vernalized and non-vernalized plants seems to be determined by the physicochemical state of the plant tissue, which depends on the growth temperature and stage of plant development. This phenomenon deserves more attention in further experiments as a possible element of the mechanism of vernalization.

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