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Ethylene is a modulator of gibberellic acid-induced antheridiogenesis in *Anemia phyllitidis* gametophytes

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Abstract

In fern (*Anemia phyllitidis*) gametophytes cellulose in the walls of the antheridial zone cells which was organized in clusters and spots was transformed *via* dispersed form to fibrillar arrangement (layered in oblique and perpendicular array in relation to the transverse direction of cell expansion) during antheridiogenesis induced by gibberellic acid (GA_3) and/or enhanced by 1-aminocyclopropane-1-carboxylic acid (ACC). In the ACC-treated gametophytes, where antheridia were not induced, the cellulose was arranged in the same manner. Aminooxyacetic acid (AOA), which inhibits antheridiogenesis and development of fern gametophytes, produced in the cell walls both random and longitudinal type of organization of cellulose microfibrils, however, in the GA_3/AOA-treated plants the oblique type was also observed. The total numbers of cells with perpendicular and/or oblique type of cellulose microfibrils in the GA_3-, GA_3/ACC- and GA_3/AOA-treated gametophytes corresponded to the average number of antheridia formed. Moreover, it was found that the extracts from the gametophytes treated with GA_3 or with the mixture of GA_3 and ACC contained significantly less soluble sugars but more α-amylase- and endoglucanase-released sugars than the extracts from the gametophytes of the other series. Thin layer chromatography of the samples from the cell wall extracts hydrolyzed by endoglucanase contained xylose and cellobiose which suggested that these sugars built the xyloglucans, hemicellulose polymers responsible for tethering of walls of fern gametophyte cells like in higher plants.

Additional key words: aminooxyacetic acid, cellulose microfibrils, fern, sugars, xyloglucans.

Introduction

Plant morphogenesis is governed by coordinated cell divisions and cell expansion. A large body of evidence suggests that plant hormones are involved in the regulation of these processes also in *A. phyllitidis* gametophytes (Kaźmierczak 1998, 2003a,b, 2004). 12-d-old gametophytes of this fern are composed of three groups of cells (Kaźmierczak 2003a) making it a useful model to study morphogenesis and cell differentiation (Kaźmierczak 2003b, 2004). The first consists of large, longitudinally growing cells which are able to form rhizoids (basal zone), the second (antheridial zone) consists of cells of similar size, which are the most sensitive to gibberellic acid (GA_3), and the third (apical zone) consists of rather small, actively dividing cells (Kaźmierczak 2003a,b).

It is well known that antheridiogens, gibberellin derivatives, are the main and specific inducers of antheridiogenesis in homosporous ferns (Yamane 1998). Antheridiogens can activate cascade expression of sex determining genes (Strain *et al.* 2001) which induce the synthesis of a low molecular mass lipocain-like ANI1 protein (Wen *et al.* 1999). The studies performed with GA_3, which imitates natural effects of antheridiogens, showed that this process was also controlled by ethylene (Kaźmierczak 2003b, 2004). The most important markers of precociously induced antheridiogenesis – transient increment of nuclear chromatin dispersion and an increased number of S-phase cells (60 %) – were established during the first 12 h of treatment of *A. phyllitidis* gametophytes (Kaźmierczak 2003a,b). Whereas important morphological features of antheridiogenesis – induction of transverse growth of antheridia mother cells expressed as width to length (W/L) ratios and the number of antheridia – resulting from modification of developmental program – were manifested from 24 to 96 h. After 96 h the numbers of antheridia, which first appeared after 24 h (Kaźmierczak 2004), were about 2.7 and 5.7 in the GA_3- and GA_3/1-aminocyclopropane-1-carboxylic acid (ACC)-treated gametophytes, respectively, while the W/L ratio of antheridial mother

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; AOA - aminooxyacetic acid; DMSO - dimethyl sulfoxide; EDTA - (ethylenediamine)tetra acetic acid; GA_3 - gibberellic acid; TBS - Tris/NaCl buffer; TLC - thin layer chromatography; TMD - Tris/$MgCl_2$/DMSO buffer.

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cells reached the maximum (1.6) after 48 h (Kaźmierczak 2003a,b). Aminooxyacetic acid (AOA), the inhibitor of ACC synthesis, inhibited both GA_3-induced antheridiogenesis and development of untreated gametophytes by the reduction of cell growth. The primary experiments performed with fluorescently stained cellulose in the walls of *A. phyllitidis* gametophyte cells revealed that perpendicular and oblique directions microfibrils arrangement were characteristic of the transverse expansion of cells in the antheridial zone of gametophytes after 24-h treatment with GA_3 (Kaźmierczak 2003a,b).

The above results show that antheridia formation in *A. phyllitidis* is a complex process, whose morphological aspects are based on the alteration of cell elongation to thickening (Kaźmierczak 1998, 2003a,b, 2004), and is connected with the specific kind of cellulose microfibrils arrangement in cell walls (Kaźmierczak 2004).

The present studies were undertaken to specify the role of synergistically acting ethylene and gibberellic acid on the antheridiogenesis and development of fern gametophytes by precisely timed detailed analysis of the arrangement of cellulose microfibrils in the cell walls as well as of soluble sugars released by α-amylase and endoglucanase; which were significant markers of differentiation of thalli and development of male sex organs in *Chara tomentosa* and *Chara vulgaris* (Kaźmierczak and Rosiak 2000, Kaźmierczak 2001).

Materials and methods

Plants and treatment: The spores and then gametophytes of *Anemia phyllitidis* were aseptically cultured in Petri dishes (5 cm in diameter) under continuous light (irradiance of 95 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in 5 cm^3 of Mohr's medium (for details see Kaźmierczak 2003a,b). 12-d-old thalli were treated with *a*) 30 μM GA_3, *b*) 30 μM GA_3 and 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC), *c*) 30 μM GA_3 and 100 μM AOA, *d*) 10 μM ACC and *e*) 100 μM AOA and cultured for 4 d. The material for analyses was collected after 0, 3, 6, 9, 12, 24, 48, 72 and 96 h.

Detection of types of cellulose arrangement in cell walls: Cellulose arrangement in the cell walls was studied in the gametophytes fixed in 4 % paraformaldehyde at pH 7.0 in TMD buffer (50 mM Tris, 5 mM $MgCl_2$, 5 % DMSO, pH 7.0) for 1 h at room temperature. To remove pectic polysaccharides, the gametophytes were treated with 100 mM EDTA (pH 7.0) at 85 °C for 4 h and washed three times in TMD buffer. Then the gametophytes were treated with 0.5 % Triton X-100 in TMD buffer, stained with 0.001 % (m/v) *Calcofluor White M2R* fluorochrome (*Sigma,* Poznań, Poland) for 15 min and washed with TBS buffer (10 mM Tris, 10 mM NaCl, pH 7.5). The thalli were mounted on slide glasses and observed using a *Optiphot-2* epi-fluorescence microscope (*Nikon*, Tokyo, Japan) equipped with excitation UV-2A (UV-light) filter. Photographs were taken using computerized *Act-1* system (*Precoptic*, Warsaw, Poland).

Extraction of free glucose; fractionation and analysis of cell wall enzymatic hydrolysates: To analyze soluble sugars, the gametophytes were homogenized with a mortar and pestle in 80 % (v/v) methanol. The homogenate was centrifuged for 20 min at 10 000 *g*, and re-extracted. Combined supernatant was used to determine soluble sugars and the pellet was extracted twice with chloroform:methanol (1:1, v/v) and twice with acetone and washed with distilled water. This material was treated with 0.1 M EDTA (pH 7.0) at 85 °C for a total of 3 h (the extraction solution was changed every 1 h), then washed 3 times with distilled water. The residue was then treated with 4 units cm^{-3} porcine pancreas α-amylase (type I, *Sigma*) in 25 mM sodium acetate buffer pH 6.5 for 4 h at 40 °C. The mixture was centrifuged for 15 min at 10 000 *g* and the α-amylase-released glucose was determined in the supernatants.

After being washed 3 times with distilled water, the residues of cell walls after α-amylase treatment were suspended and incubated with 1 cm of 100 milliunits cm^{-3} endoglucanase (Cellulase *Onozuka R-10* from *Trichoderma viride*; *Serva,* Heidelberg, Germany) in 2.5 mM sodium acetate buffer (pH 5.0) for 24 h (after this period the amount of the released glucose was constant) at 40 °C with the addition of a few drops of toluene. After being heated at 100 °C for 15 min to inactivate the enzyme, the incubation mixture was centrifuged and then the supernatant was collected; same of it was used for sugar determination and the rest was dried in a rotary evaporator after removing the non-neutral sugars through *Dowex 50Wx8*. The dried material was dissolved in 80 % methanol and after centrifugation thin layer chromatography was performed. The chromatogram of enzymatic hydrolysates was run on TLC plate (*Merck* silicagel 60), developed by 1-butanol/acetic acid/water (3:3:2, v/v/v) at room temperature, and visualized by spraying with 1 % (m/v) orcinol (*Sigma-Aldrich*, Poznań, Poland) containing 50 % (m/v) sulphuric acid and heating for 15 min in an oven at 100 °C.

Detection of glucose: Sugars were quantitatively determined by anthron (Antikainen and Pihakaski 1994) and expressed as equivalents of glucose per 1 g of fresh mass (f.m.). Water samples of sugars were evaporated and dissolved in methanol. 0.05 cm^3 of cold (4 °C) methanolic sample were mixed with 0.3 cm^3 methanol and 1.25 cm^3 of cold (4 °C) anthron reagent (200 mg in 100 cm^3 72 % sulphuric acid). The mixture was heated in boiling water bath for 11 min, then cooled on ice and absorption was measured at 630 nm (*Specol 10*, *Carl-Zeiss*, Jena, Germany).

Results

Type of cellulose arrangement in cell walls: Detailed fluorescence analyses of the walls of *A. phyllitidis* antheridial zone gametophyte cells stained with *Calcofluor White M2R* carried out 0 - 12 h and 24 - 96 h after treatments with GA_3, GA_3/ACC, ACC, GA_3/AOA or AOA showed different patterns of its deposition. At the beginning of the experiment (0 h) the cellulose was deposited as large or small clusters (Fig. 1*A*,*G*,*H*) in the

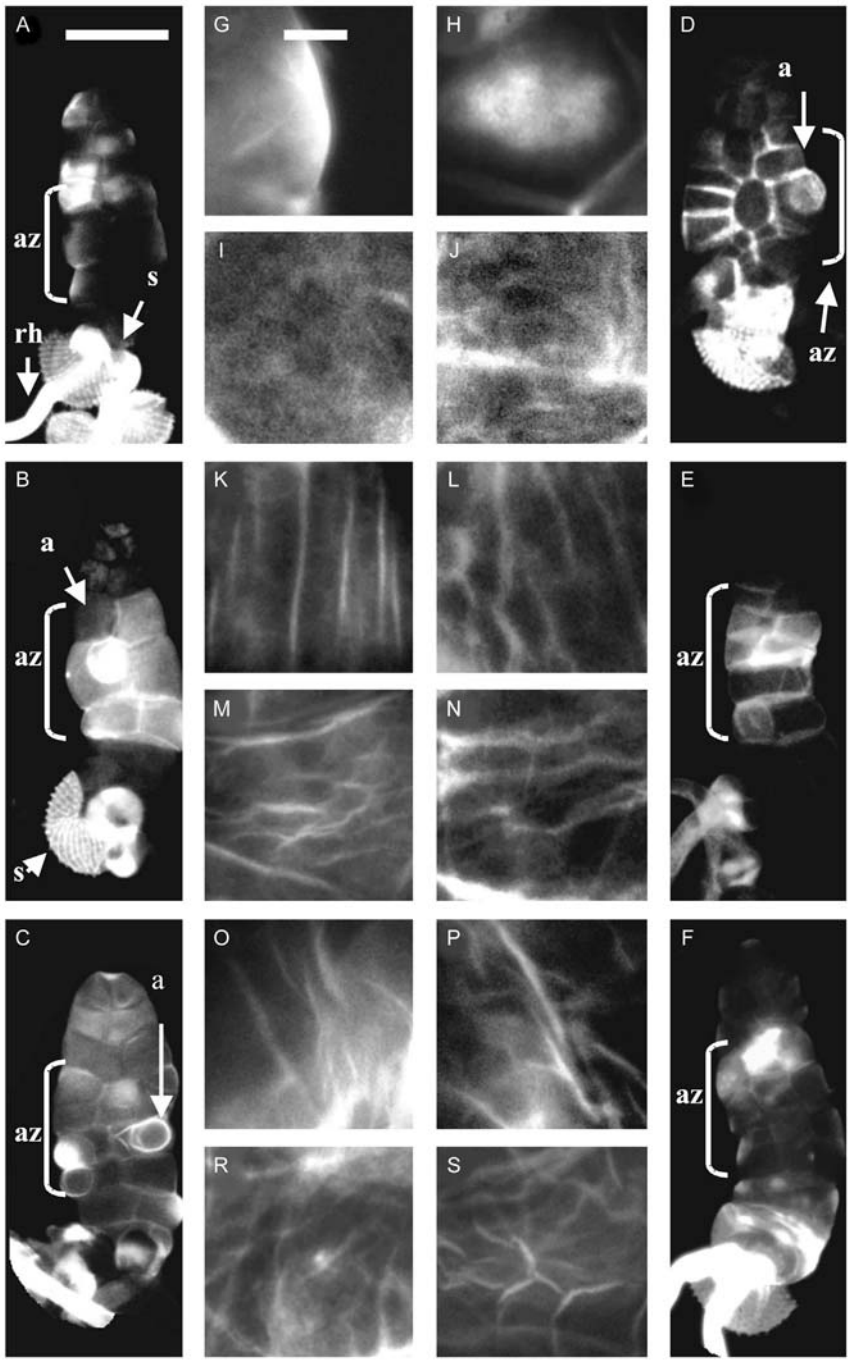

Fig. 1. *Calcofluor White M2R* visualization of the arrangement of cellulose in cell walls of the antheridial zone of control gametophytes (*A*) and those treated with 30 μM GA_3 (*B*), GA_3 with 10 μM ACC (*C*), GA_3 with 100 μM AOA (*D*), 100 μM AOA (*E*) or 10 μM ACC (*F*). Dispersed (*G*,*H*), clusters (*I*,*J*), transverse (*K*,*L*), longitudinal (*M*,*N*), oblique (*O*,*P*) and random (*R*,*S*) arrangement of cellulose microfibrils. Their direction is described in relation to the transverse expansion of the antheridial cells (a - antheridium, az - antheridial zone, rh - rhizoid, s - spore). *Bar* = 100 μm (*A-F*) and 10 μm (*G-S*).

4.0 ± 1.3 cells of the 15 ± 2.1-celled gametophytes. In the 0 - 6 h period these clusters disappeared (Fig. 1*I*,*J*) except for the ACC series where they constituted 8.8 % till 96 h. During the 6 - 12 h period in the GA_3- and in the GA_3/ACC-treated plants (Fig. 1*B*,*C*) oblique (Fig. 1*O*,*P*) and/or perpendicular (Fig. 1*K*,*L*) directions of cellulose microfibrils were detected in the cell walls, in relation to the transverse expansion of cells. During the 24 - 96 h period perpendicular (Fig. 1*K*,*L*) arrangement of cellulose microfibrils in the GA_3-treated (Fig. 1*B*) and in the ACC-treated (Fig. 1*F*) gametophytes was also induced while an oblique type (Fig. 1*O*,*P*) was induced in the GA_3/AOA-treated (Fig. 1*D*) and in the ACC-treated (Fig. 1*F*) ones. In the walls of GA_3/AOA-treated (Fig. 1*D*) and AOA-treated (Fig. 1*E*) gametophyte cells random (Fig. 1*R*,*S*) and longitudinal (Fig. 1*M*,*N*) arrangements of cellulose microfibrils were also induced.

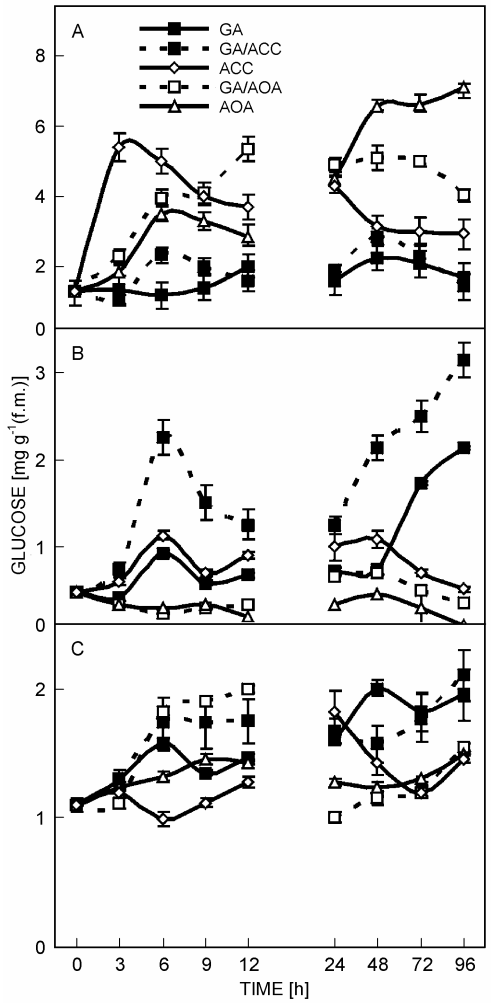

Fig. 2. Amount of free glucose (*A*), α-amylase (*B*) and endoglucanase (*C*) released glucose in *A. phyllitidis* gametophytes after treatment with GA_3, GA_3/ACC, ACC, GA_3/AOA and AOA during the 0 - 12-h and the 24 - 96-h periods. *Error bars* represent ± SE of means of three repetitions of two independent experiments.

The number of cells with perpendicular and oblique type of cellulose arrangement in the 6 - 12 and 24 - 96-h periods, expressed per one gametophyte, was similar to the average number of antheridia induced in the GA_3-, GA_3/ACC- and in the GA_3/AOA-treated plants (Kaźmierczak 2003a,b, 2004).

Sugar contents: The contents of sugars in all fractions were expressed as glucose equivalents. The average soluble sugar content was highest [about 4.4 and 4.5mg g^{-1}(f.m.)] in the extracts from the GA_3/AOA- and AOA-treated gametophytes with the maximum at 12 h [5.4 mg g^{-1}(f.m.)] and 96 h [7.7 mg g^{-1}(f.m.)], respectively, while the extracts from the ACC-treated gametophytes contained free sugars at about 3.9 mg g^{-1}(f.m.) on average, with the maximum [5.4 mg g^{-1}(f.m.)] after 3-h treatment.

Statistically insignificant ($P < 0.05$) fluctuations of glucose contents throughout the experiments in both the GA_3- and the GA_3/ACC-treated gametophytes were detected. The average soluble sugar contents in these series were about 1.7 and 1.9 mg g^{-1}(f.m.), respectively (Fig. 2*A*).

α-Amylase-released glucose:. The average glucose content released by α-amylase from the cell wall fraction (Fig. 2*B*) was highest in the GA_3/ACC-treated gametophytes and was about 1.7 mg g^{-1}(f.m.) with the maxima after 6 h [2.2 mg g^{-1}(f.m.)] and after 96 h [3.1 mg g^{-1}(f.m.)] of treatment. In the GA_3-treated gametophytes the average amount of glucose was about 0.9 mg g^{-1}(f.m.) in the 0 - 96 h period with the maxima after 6 h [0.9 mg g^{-1}(f.m.)] and after 96 h [2.1 mg g^{-1}(f.m.)] of treatment. In the GA_3/AOA- and AOA-treated gametophytes the fluctuation of glucose

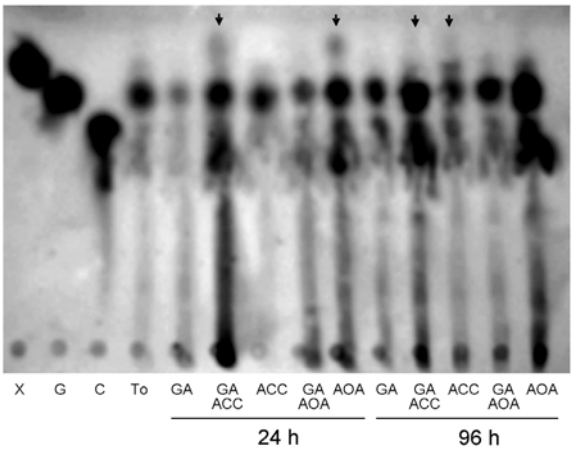

Fig. 3. TLC of standard: *lane X* - xylose, *lane G* - glucose, *lane C* - cellobiose and endoglucanase hydrolysates from the cell wall fractions obtained from *A. phyllitidis* gametophytes after 24 and 96-h treatment with GA_3, GA_3/ACC, ACC, GA_3/AOA and AOA. TLC was developed with the solvent of butanol, acetic acid and water. The upper spots correspond to xylose (marked also by *arrows*), glucose and cellobiose.

contents released by α-amylase was statistically insignificant ($P < 0.05$); the average being about 0.4 and 0.3 mg g^{-1}(f.m.), respectively (Fig. 2*B*). In the extracts from the ACC-treated gametophytes the average amount of α-amylase-released glucose was about 0.8 mg g^{-1}(f.m.) with the maxima after 6 and 48 h [1.1 mg g^{-1}(f.m.)].

Endoglucanase-released sugars. Glucose amount in endoglucanase hydrolysates from the cell wall fractions, previously digested by α-amylase, were determined by spectrophotometry (Fig. 2*C*) while TLC was used to detect xylose, glucose and cellobiose (Fig. 3).

In the gametophytes treated with GA_3 or GA_3/ACC the amount of released glucose was highest [about 1.7 and 1.8 mg g^{-1}(f.m.) on average] with the maxima after 48 h [2.0 mg g^{-1}(f.m.)] and after 96 h [2.1 mg g^{-1}(f.m.); Fig. 2C] of treatment, respectively.

The pattern of TLC chromatograms (Fig. 3) showed that endoglucanase released xylose from the cell wall fractions, obtained from gametophytes after 24-h treatments with GA_3/ACC and AOA as well as after 96-h treatments with GA_3/ACC, ACC. Glucose and cellobiose were released by endoglucanase from gametophytes after 24-h and 96-h treatments with GA_3, GA_3/ACC, GA_3/AOA, AOA and ACC. The presence of xylose and cellobiose suggested that the endoglucanase in cell walls cleavaged xyloglucans.

Discussion

One of the cellular processes controlled by hormones is the stimulation of transverse growth of the antheridial zone cells in *A. phyllitidis* gametophytes during GA_3-induced antheridiogenesis (Kaźmierczak 2003a,b). In most higher plants growth of cells is mediated by the organization of plasma membrane-associated cortical microtubules (Nick 1999) which together with actin are supposed to be one of the cellular, peripheral structures of plant cells (Baluška *et al.* 2004), determining the orientation of cellulose microfibrils in cell walls. Gibberellins and ethylene regulate both processes (Huttly and Phillips 1995, Martin *et al.* 2001) by independent (Collett *et al.* 2000) or cooperative way (Rijnders *et al.* 1997, Tadeo *et al.* 1997, Voesenek *et al.* 2003, Calvo *et al.* 2004). Gibberellins promote elongation of *Lemna minor* roots (Inada *et al.* 2000), hypocotyls of *Arabidopsis thalliana* (Cowling and Harberd 1999) as well as shoots of *Oryza sativa* (Vriezen *et al.* 2003) and *Rumex palustris* (Voesenek *et al.* 2003) inducing and/or maintaining transverse array of microtubules or relaxation of cell wall structure (Cosgrove 2000) before altering cellulose microfibrils organisation (Huttly and Phillips 1995, Fujino *et al.* 2000, Vriezen *et al.* 2003); whereas, ethylene stimulates growth of cells which leads to thickening of *A. thallina* roots (Pierik *et al.* 1999) and *Cucumis sativus* hypocotyls (Collett *et al.* 2000) and stems of others plants (Pospíšilová 2003). Oblique orientation of cellulose microfibrils indicates that cells reach the end of elongation phase while modification from oblique to perpendicular orientation reflects a shift of cell expansion into transverse direction; while randomly organized cellulose shows inhibition of cell growth (Vriezen *et al.* 2003). In fact ACC in *A. phyllitidis* gametophytes induced transverse expansion of cells in the GA_3/ACC- and ACC-treated gametophytes (Kaźmierczak 2003a,b) by perpendicular and oblique types of organization of cellulose microfibrils in the walls of the antheridial zone gametophyte cells. Inhibition of the cell growth in the GA_3/AOA- and AOA-treated gametophytes caused by AOA, an ethylene synthesis inhibitor (Kaźmierczak 2004), was connected with random arrangement of the cellulose microfibrils evoking rigidification of cell walls. However, longitudinal arrangement of the cellulose microfibrils in the walls of cells in the GA_3/AOA- and in the AOA-treated gametophytes was controlled in that fern by gibberellins *e.g.* GA_3 and/or endogenous antheridiogens.

These results showed that the effects of ethylene and gibberellins on the arrangement of cellulose microfibrils in *A. phyllitidis* gametophytes were similar to those described in higher plants (Vriezen *et al.* 2003). Moreover, the number of cells in the antheridial zone with perpendicular and oblique array of cellulose microfibrils in the walls was almost the same as the number of antheridia induced (Kaźmierczak 2003a,b), indicating that the pattern of arrangement of cellulose microfibrils expressed the current program of cell differentiation. For that reason, the number of cells with the perpendicular and oblique types of cellulose microfibrils organization can be a useful index for determining the important and specific features characterizing the ability of the antheridial zone cells to expand transversely and divide asymmetrically, which leads to formation of initial antheridial cells and then antheridia. These cells, known as antheridial mother cells are characterized by: *1*) the oblique or perpendicular array of cellulose microfibrils in the cell walls in relation to the transverse cell expansion; *2*) transient increment of nuclear profile area (30 - 100 %; Kaźmierczak 1998, 2003a,b); *3*) induction of S phase (DNA synthesis) and cell divisions (Kaźmierczak 1998, 2003a,b), as well as *4*) enlargement of the cell profile area to 2900 - 3000 μm^2 with width to length ratio at about 1.6 (Kaźmierczak 2003a,b).

Plants have many physiological and metabolical sinks of sugars. Sugars are the basis of energetical support in a simple or storage form (Huttly and Phillips 1995) as well as structural and matrix equipment of cell walls (Martin *et al.* 2001, Cosgrove 2000). Gibberellic acid regulates the sugar content (Cao and Shannon 1997, Kaźmierczak 1998). The average contents of different fractions of glucose *i.e.* low of free glucose and high of that released

by α-amylase- and endoglucanase from cell walls of the gametophytes treated with the GA_3 and GA_3/ACC can suggest: *1*) a high ratio of energetical turnover of simple sugars, *2*) synthesis of starch in a great number of chloroplasts during GA_3-induced antheridiogenesis (Kaźmierczak 1998), and/or *3*) relaxation of hemicelluloses, xyloglucan-like or xylan-like ones (Martin *et al.* 2001, Cosgrove 2000), which tether the newly synthesized cellulose chains in the walls of gametophyte cells. The low level of α-amylase- and endoglucanase-released glucose as well as the high content of free glucose in the GA_3/AOA- and AOA-treated gametophytes indicated reduction of the rate of glucose built-in into polysaccharides of the cell walls when cell expansion was reduced or inhibited (Kaźmierczak 2004). These results as well as the average amount of free, α-amylase-released and cell wall-bound glucose in gametophytes of ACC series in comparison to all the other series mean that ethylene seems to be a modulator of sugar metabolism during the development of fern gametophytes and antheridiogenesis.

TLC plate analyses of endoglucanase hydrolysates obtained from the gametophytes showing small amount of xylose or cellobiose indicated that xyloglucans could play a tethering role for cell wall components which are important for dynamic changes during transverse growth of the antheridial zone cells.

This paper presenting the effects of GA_3, ACC and AOA on the arrangement of cellulose microfibrils in cell walls as well as sugar contents in *A. phyllitidis* gametophytes shows the links between gibberellins and ethylene in regulation of antheridiogenesis and extends the knowledge about important steps of fern development in the early (0 - 12 h) as well as later (24 - 96 h) period of responses. Taking together the effects of GA_3, ACC and AOA on the re-arrangement of cellulose microfibrils, fluctuations of the levels and the average amount of soluble and bound sugars as well as their quality it seems that ethylene but not gibberellin is the plant hormone which induces antheridiogenesis in *A. phyllitidis* fern gametophytes. However, without GA_3 precocious antheridia formation in this fern was not induced (Kaźmierczak 2003b). These results seem to suggests that gibberellin upregulates ethylene responses. In the first period (0 - 12 h) GA_3 induced dispersion of chromatin (Kaźmierczak 1998, 2003a,b), which was one of the aspects of initiation of transcription which preceded the cell division and cell expansion in *A. phyllitidis* fern gametophytes (Kaźmierczak 2003a,b). This period may be the time in which GA_3 de-repressed signals of ethylene responses can be generated by DELLA-like protein (Achard *et al.* 2003). In the next steps, 24 - 96 h, by DELLAcate balance (Fleet and Sun 2005) the delicate changes took place which resulted from correlation between gibberellin (gibberellic acid and/or endogenous antheridiogens) and ethylene. These changes triggered modulation of cell wall structural reorganization and of sugar metabolism, which activated appropriate pathways controlling cell growth by specific mechanisms which by asymmetrical cell division led to antheridia formation. Participation of glucose as a signal molecule in the regulation of antheridiogenesis and development of cell walls of fern gametophytes cannot be excluded (Roitsch and González 2004).

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