Induction of cell division and cell expansion at the beginning of gibberellin A₃-induced precocious antheridia formation in Anemia phyllitidis gametophytes

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Abstract

Few-celled haploid gametophytes of Anemia phyllitidis and gibberellic acid, which specifically imitates the activity of the natural antheridiogens were used to create the model of three-zonal structure of a fern gametophyte to study the cytomorphological background of male sex expression. Experiments showed that expression of male sex induced by gibberellin A₃ was preceded by (1) an increase in profile area of nuclei in gametophyte cells, (2) increase in the number of S phase cells, (3) transverse expansion of antheridial mother cells and was simultaneous with (4) proportional enlargement of cells from the apical zone of gametophyte.

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1. Introduction

Development of plants depends on different physiological and biochemical networks which are regulated and integrated by phytohormones. Gibberellins (GAs), a group of 126 naturally occurring diterpenoid compounds, belong to the classical plant hormones [1,2]. They control a wide range of processes in vascular plants, including growth and reproduction by modulating the expression of two main sets of genes: (1) the genes involved in cell-division cycles [3] and (2) the genes encoding proteins that act as cell-wall factors [4]. GAs and other plant hormones, auxin, cytokinins and ethylene, are also known as factors which control sexuality in many monococious and dioecious species [5,6]. However, GAs seem to be particularly important for male fertility in most dicotyledonous species. For example, GA-deficient mutants of Arabidopsis are male sterile whereas application of GAs to cucumber or hemp induces male sex [5]. GAs control sex expression also in lower plants [7]. In Chara vulgaris, exogenously applied gibberellic acid (GA₃) stimulates the development of antheridia and hastens the maturation of oogonia [8,9]. In homosporous ferns, GAs and structurally related compounds control the generative development of haploid gametophytes [10–13]. At least 12 compounds have been described, which are named antheridiogens [13]. Antheridiogens are the primary sex determinant in many species of fern gametophytes. On the basis of effectiveness of gibberellin inhibitors blocking the biological response to antheridiogens [13], of antagonistic responses of ABA to GAs [14] and of spectroscopic analysis, all known antheridiogens of homosporous ferns were identified as GAs including GA₉, GA₂₄, GA₂₅, GA₁₀₇, which were isolated from Anemia phyllitidis [1]. In the absence of an antheridiogen, a spore develops as a hermaphroditic gametophyte which produces and secretes the pheromone once it becomes insensitive to its male-inducing effects. When an antheridiogen is present, a spore develops as a male gametophyte [14]. Endogenous antheridiogens in young, sexually undetermined gametophytes promote the differentiation of antheridia, completely suppress the development of meristems and archegonia [13,15] and
inhibit the synthesis of an antheridiogen [14]. However, inhibitors of GAs biosynthesis do not significantly influence antheridiogen-induced antheridia formation [13]. The gametophytes of A. phyllitidis and other ferns respond to their native antheridiogens as well as to exogenous GAs [10,11,16]. The order of gibberellin activity in antheridia formation in A. phyllitidis was found to be: GA7 > GA4 ≥ GA1 > GA3 ≥ GA9 > GA5 ≥ GA8 [17]. Among these, GA3 was commonly used to induce antheridiogenesis in A. phyllitidis. The highest biological activity of GA3 has been observed at the concentration of 3 × 10⁻⁵ M [10–12].

The experiments reported in this paper were undertaken in order to get insight into some developmental aspects of GA3-induced male sex in fern gametophytes. The homosporous fern haploid gametophyte is a particularly useful system for studying hormonal regulation of sex determination and other aspects of plant development. To identify cytological and morphological features, the model of three-zonal organisation of the A. phyllitidis gametophyte, is proposed.

2. Materials and methods

2.1. Material, culture of prothallia

Spores of A. phyllitidis kindly provided by Schraudolf (Abt. Allgemeine Botanik, University of Ulm), were aseptically (sterilization 3 min with 0.875% Clorox) transferred into 5 ml of Mohr’s medium [12] and cultured for 2 days in the dark for synchronization of germination and then for 10 days in continuous cool white light on Petri dishes (5 cm in diameter) at 25 °C under two 40 W fluorescence tubes (95 μM m⁻² s⁻¹). The constant density of spores (1 g per 10 ml of Mohr’s medium), which eliminates the differences in time and frequency of appearance of antheridia resulting from endogenous antheridiogens was established on the basis of Warne and Lloyd’s [18] data. After 12 days, the prothalli were transferred to a fresh medium (control, C) or a medium supplemented with GA3 (30 μM) and cultured for the next 4 days.

2.2. Analysis of growth

Prothalli were fixed in cold Carnoy’s mixture of 96% ethanol and glacial acetic acid (3:1) for 1 h and washed with 96% and then 70% ethanol and, after hydration, were embedded in 86% glycerol. Fifty or more gametophytes per sample were used to estimate the number of cells and morphological parameters. Morphometric analysis was carried out under the computerized JenaMed-2 light microscope (ZEISS-Jena, Germany) and the prothalli were photographed under a Nikon microscope equipped with camera and ACT-1 software (Precoptic, Łódź, Poland). The mean number of antheridia per gametophyte was estimated on the basis of 300 prothalli for each series.

2.3. Fixation and staining for cytophotometry

For cytophotometrical estimation of nuclei of gametophyte cells, the prothalli (over 200) were fixed in cold Carnoy’s mixture. For Feulgen staining of gametophytes the method described earlier [12] was modified. The prothalli were hydrated, hydrolysed in 5 N HCl for 1 h at 20 °C and then stained in Schiff’s reagent (pH 2.2) prepared with 1% of pararosaniline (Sigma) and 2% of potassium pyrosulfite (Fluka) for 1.0 h at room temperature. Dissected prothalli placed in 45% acetic acid were squashed onto microscope slides. After freezing with dry ice, the cover slips were removed and the slides were dehydrated and mounted in Canada balsam (Serva, Germany). The absorbance of Feulgen-stained nuclei was measured at 565 nm and calibrated in arbitrary units (a.u.) under the computerized JenaMed-2 microscope (ZEISS-Jena) and IMAL-512 system for image analysis (Ultima, Łódź, Poland).

2.4. Fluorescence microscopy

For fluorescence microscopy, the prothalli fixed in Carnoy’s mixture were hydrated with TE buffer (100 mM Tris, 1 mM EDTA, pH 8.0) for 1 h and then washed for 24 h with TE buffer supplemented with 0.1% Triton X-100. Staining was carried out with DAPI (4,6-diamidino-2-phenylindole, 1 μg ml⁻¹) for 1 h. After two times rinsing with TE buffer, the prothalli were squashed on slides under the coverslip and observations were made using an Optiphot-2 epi-fluorescence microscope system (Nikon, Japan) equipped with excitation UV-2A (UV-light) filter illuminating at λ = 361–461 nm. Photographs were made using computerized ACT-1 system (Precoptic, Warsaw, Poland).

3. Results

3.1. Morphology of A. phyllitidis gametophyte

Filamentous, 12-days-old A. phyllitidis gametophytes comprised 15 cells on the average (series To; Fig. 1A). After application of GA3, the gametophytes reached a 20-celled stage (series GA; Fig. 1A) within 4 days, with the mean number of 2.5 antheridia per thallus. The number of cells in non-treated plants (antheridia were not observed) was about 25 per gametophyte (control series, C; Fig. 1A). The thalli grew from 240 μm (To, Fig. 1B) to 400 μm in the control (C, Fig. 1B) and to 370 μm after GA3 treatment (GA; Fig. 1B).
To establish morphological parameters characteristic of gametophytes that form the antheridia, the profile areas of all thallus cells were drawn for the control (Fig. 2A and B) and the GA 3-treated prothalli (Fig. 2 C). Morphological measurements revealed three categories of profile areas of cells, which allowed to divide the A. phyllitidis thalli into three regions. (I) ‘Basal’, formed by cells characterised by an intensive elongation (Fig. 2A–C), whose profile areas varied significantly (from 1300 to 2500 µm²) and by formation of rhizoids. (II) ‘Antheridial’, including cells which were susceptible to GA3 and which gave rise to male sex organs (antheridiogenesis, Fig. 2C), the sizes of these cells varied to a lesser degree reaching 1700 µm², on the average (To, Fig. 1C). Within 4 days of cultivation the cells at the ‘antheridial’ region grew to 3000 µm² in the control (C; Fig. 1C) and up to 2900 µm² in GA3-treated material (GA: Fig. 1C). (III) ‘Apical’ (Fig. 2A–C) containing small and actively dividing cells. In the control, an average profile area of cells at the apical region enlarged from 440 to 700 µm² (C; Fig. 1D) while the cells of the GA3 series grew to 900 µm² (GA; Fig. 1D). This morphologically varied picture of A. phyllitidis gametophyte was adopted as a model for cytological studies of GA3-induced antheridiogenesis.

3.2. GA3-stimulated expansion of cells

Although the sizes of antheridial zones expressed as the profile surface areas of cells were nearly identical in Control and GA3 series (Fig. 1C), the direction of expansion of GA3-treated cells changed. The antheridial mother cells (cells susceptible to GA3-induced antheridia formation) grew mostly transversely (series GA; Fig. 3A). For that reason the width to length ratio (W:L), used as a parameter to describe the cell morphology, was found to be about 1.6 (Fig. 3A). The cells in the apical zone grew both transversely and longitudinally (Fig. 3B) and the W:L ratio was 1.28 (series C) and 1.26 (series GA; Fig. 3B). The mean profile area of cells bearing antheridia, formed by divisions of the antheridial mother cell, was about 2000 µm², and the W:L ratio was about 1.2.

3.3. GA3 changes the morphology of nuclei of A. phyllitidis cells

GA3 increases the mean nuclear profile area (NPA) of cells in the antheridial and apical zones. The NPA of cells in the antheridial zone with maxima after 3 and in 18 h was about 50% bigger as compared to the apical part (maximum after 3 h; Fig. 4A). The increase in the NPA resulted in a decrease in the mean optical density (MOD) of Feulgen-stained nuclear DNA in cells of both zones, especially in cells from the antheridial zone of GA3-treated gametophytes. A gradual increase in nuclear DNA content, induced by GA3, in antheridial and apical parts caused an enhancement of optical density, which diminished to the control level after 48 h (Fig. 4B).

3.4. GA3 increases DNA content and regulates the number of cells of the cell cycle phases

Induction of cell-division activity was analyzed in the antheridial and apical zones of A. phyllitidis thalli. At various times (0, 3, 6, 12, 18, 24 and 48 h) after GA3 treatment, Feulgen-cytophotometric analysis was performed to measure the distribution of cells in the G1, S and G2 phases (Fig. 5). In both antheridial and apical
parts of thalli, an increase in the number of S phase cells was observed, from 30% (0 h) to 60% (in 9 h) and from 17% (0 h) to 63% (12 h), respectively (Figs. 5 and 6). Accumulation of cells in the S phase suggests that a subpopulation of cells in the G1 phase was induced to enter a new cell cycle in a synchronous manner. Moreover, the increase in number of cells in S phase resulted in an increase in mean nuclear DNA contents from 1.30 (0 h) to 1.70 \times 10^3 a.u. (48 h). At the beginning of cultivation, the mean nuclear DNA contents, were higher in the antheridial than in the apical zone of gametophyte (1.61 \times 10^3 and 1.41 \times 10^3 a.u.; respectively) and were higher also during the GA_3 treatment period.
Fig. 5. Histograms displaying the distribution frequency (%) of cells in G1 (peak around $10 \times 10^2$ a.u.), G2 (peak around $20 \times 10^2$ a.u.) and S (presented as gaps between G1 and G2 peaks) phases in the control (0 h) and after GA$_3$ treatment of A. phyllitis thalli in antheridal (column A) and apical (column B) zones.
4. Discussion

The basis of sex determination in homosporous ferns is different from that of organisms with sex chromosomes [13–15]. The development of sex in ferns is determined by antheridiogens and depends on: (1) the type and concentration of an inducer, (2) the species, (3) the age of the gametophyte and (4) the interaction between specific set of genes controlling sex development. Genetic analysis based on a model of sex-determining pathway identified five classes of mutants of Ceratopteris ferns: her (‘hermaphroditic’), tra (‘transformer’), man (‘many antheridia’) [14] fem (‘feminization’), [19] and abr type (‘ABA resistant’) [20]. The genes of these mutants are involved in epistatic interactions during the sex determining process. In the presence of ACE (antheridogen of Ceratopteris) the genes HER and FEM1 in wild-type gametophyte are active and promote the expression of male sex and simultaneously repress the female one inhibiting the expression of TRAI and MANI genes. When ACE is absent TRAI and MANI genes are active and repress HER and FEM1 genes promoting female sex [13,15,19]. Moreover, ACE induces a transient expression of ANII gene coding the proteinous substance (ANII) within 3 h after treatment, which may be the carrier necessary for initiation of male sex. In the absence of ACE, the expression of ANII is directly or indirectly prevented by the sex determining gene, TRAS5. In contrast, ACE inactivates this gene leading to the upregulation of ANII [21].

Antheridiogens are natural pheromones which specifically control development of fern gametophytes. However, GA3 can imitate their effect and thus initiate and maintain the expression of male sex. Cytomorphometrical analysis showed that GA3 increased NPA of cells in an antheridial and as well as in an apical region. The increase in NPA of apical cells was observed between 0 and 3 h of treatment. Parallel to the increment of NPA the decrease in MOD was observed. Moreover, the number of S phase cells was increased and reached the maximum in antheridial zones earlier (9 h) than in apical (12 h) parts of gametophytes. The activation of cell division resulting in an increased number of S phase cells in the antheridial part of gametophyte was detected as a GA3-stimulated effect of antheridial formation. Whereas in the apical part, dividing cells increased the size of thallus. The differences in time in which the number of S phase cells reached the maximum in apical and antheridial zones may reflect the directions of gametophyte cell differentiation. Whereas the increment of NPA, may be a general effect of GA3 action evoking chromatin remodeling during induction of cell division. The GA3-stimulated increase in NPA of cells of A. phyllitidis gametophytes was presented elsewhere [12] and was described as an example of higher transcriptional activity. Moreover, the cytophotometrical measurements described in that paper indicated that GA3 rather arrested cells in the G1 phase than inducing cell-division cycle. An induction of cell-division cycle mediated by GA3 activating G1/S phase transition of cell cycle was observed in rice plants. In the intercalary meristems of internodes of Oryza sativa stems, the induction of cell-division cycle mediated by GA3 was preceded by the increase in expression of histone H3 (marker for the S phase), growth replication factor (Os-GRF1) [22] and replication protein A1 (RPA1) [23]. GA3 also activates the expression of specific cell-division factors: cdc2Os-3 kinase and kinase dependent cyclins (cyclA1;1) [24]. The presence of these cell cycle factors proves that GA3 plays a crucial role in regulation of cell cycle progression [3]. Growth of young few-celled gametophytes results from co-ordinated production and expansion of new cells [10,14]. GA3 stimulates expansion of gametophyte cells of ferns [10–12]. It was observed that GA3 stimulated expansion of cells both in antheridial and apical zones. However, the former cells grew mostly transversely whereas the latter longitudinally and transversely. This was due to the fact that antheridial mother cells divided asymmetrically to form male sex organs.

The final form of a plant depends on cell division and cell elongation. It is well documented that the entry into cell cycle depends on the cell size [25]. The development of Arabidopsis hypocotyls [26], Pisum sativum [27] and Oryza sativa [24] internodes is controlled by regulating growth of individual cells. The cell growth is controlled by GA3, which determines the reorientation of cortical microtubules and redeposition of cellulose microfibrils from oblique/longitudinal to transverse directions [2,28].
These modifications are induced by changes in composition of primary cell wall components (xyloglucans), which results from modulation of endotransglycosylase expression [4,29]. Interrelations between GA3-regulated morphogenesis in fern gametophytes is a process involving specific mechanisms regulating cell divisions closely related to cell size and could be compared to that described for higher plants.

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References