Relationship between formation of gametophore buds in the protonema of mosses and increase in ribonuclease activity

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Abstract

Changes in RNase activity similar to those accompanying cytokinin-induced formation of gametophore buds in mosses (a decrease in the early phase of bud formation and later an increase in enzyme activity) have also been found during spontaneous formation of gametophores in moss ontogenesis. Using various factors affecting the cytokinin-induced process of bud formation a correlation has been found between this process and the increase in RNase activity.

INTRODUCTION

The induction of gametophore buds in the protonema of mosses is one of the most striking morphogenetic effects of cytokinins (Bopp, 1962; Szweykowska, 1962). Schneider and Szweykowska (1974) studying activities of some enzymes during the kinetin-induced process of gametophore initiation have found considerable changes in RNase activity. In the preparatory stage for cell divisions (24 hours of kinetin treatment), RNase activity was inhibited, and after 48 and 120 hours a gradual increase in RNase activity was observed accompanying a gradual development of buds. Other data (from experiments with inhibitors) also indicated that the increase in RNase activity might be correlated with the bud-inducing effect of kinetin. The purpose of this study was a more detailed investigation of this correlation, both during the kinetin-induced and during the spontaneous process of the formation of gametophore buds.

MATERIAL AND METHODS

The experiments were carried out using sterile cultures of two species of mosses: *Ceratodon purpureus* (Hedw.) Brid., and *Funaria hygrometrica* (L.) Sibth. The species differ in their ability to form spontaneous buds in protonema cultures and in their response to the addition of cytokinins to the medium. *Ceratodon purpureus* forms single buds only after 50 days of protonema culture on agar media and requires 5 μM concentration of kinetin for the induction of gametophore buds in a younger protonema. *Funaria hygrometrica* forms spontaneous buds as early as after 20 days of culture and requires 0.5 μM conc. of kinetin for bud induction in 10-day-old protonema.

The spore capsules of *Ceratodon purpureus* were collected near Poznań in 1968 and those of *Funaria hygrometrica* in Bieszczady mountains in 1970.

The protonema cultures were raised either from spores or by vegetative propagation of a protonemal sterile clone culture. The method described by Szweykowska and Maćkowiak (1962) was used to obtain cultures from spores. The spore capsules were surface sterilized with 96% ethanol and 0.2% HgCl_2 . After washing with water the spores were released and inoculated on culture medium.

Composition of the medium and culture conditions

The basal medium was a modified mineral solution according to Kofler with microelements according to Heller (Szweykowska and al., 1971). The organic medium contained additionally glucose in concentration of 0.25%.

In experimental variants, kinetin in concentration from 0.5 to 1000 μM was added to the medium. In experiments with inhibitors of protein and RNA synthesis, an amino acid analogue, N-acetyl-DL-leucine, and two base analogues, 8-azaadenine and 6-azauracil, were used.

Depending on the kind of experiment, protonemas were cultured on liquid medium or on medium solidified with agar at 0.8%, in 100 ml Erlenmayer flasks. The surface of agar media was covered with sterile, cellophane discs on which small fragments of protonema were planted. Cultures were kept in a culture room under white fluorescent light of ca. 1000 lux, at a temp. of ca. 25° C and humidity of ca. 65%.

Preparation of enzyme extract

After several-time rinsing with water, the material was blotted with filter paper and weighed. For the extraction of soluble proteins the material was ground in a mortar with acid-washed sand and in the presence

of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.01 M NaCl. The homogenate was centrifuged at 1000 g for 10 min., the supernatant made up with the buffer to a volume corresponding to 100 mg fresh weight per 1 ml solution, and used for RNase estimation. The enzyme activity was determined by a modified method of Tuve and Anfinsen (Schneider and Szwedkowska, 1974), based on the hydrolysis of RNA by the enzyme extract at pH 5.0 and the spectrophotometrical (at 260 nm) measurement of the reaction product. Results were expressed in $E_{260\text{nm}}/50$ mg fresh weight or as per cent of control. Every experiment was three times repeated.

RESULTS

CHANGES IN RNase ACTIVITY IN MOSS ONTOGENY

Because changes in RNase activity accompany the bud-inducing effect of kinetin and because buds form also spontaneously after some time of the protonema culture in control medium, an interesting question was whether changes in RNase activity occur also during an unaffected moss

Fig. 1. RNase activity during unaffected moss ontogeny in cultures of *Funaria hygrometrica* and *Ceratodon purpureus*

The cultures were grown from spores in liquid mineral medium. RNase was determined in crude enzyme extract by a modified Tuve and Anfinsen method

ontogeny. After 10, 20, 30 and 50 days of culture of the protonema of *Funaria hygrometrica* from spores on liquid medium, samples of the gametophyte (protonema + gametophores, if present) were collected, and RNase activity was determined. At the stage of formation of young gametophore buds (20 days of culture) a small decrease of the enzyme activity was found. As the appearance of new gametophores and their maturation proceeded, the RNase activity increased and later maintained at a high level (Fig. 1).

Since the ontogenetic process in *Ceratodon purpureus* is slower than in *Funaria hygrometrica*, samples of gametophyte in this species were collected after 20, 40 and 50 days of culture of the protonema from spores on liquid medium. At the period of formation of young gametophore primordia (40 days of culture), a distinct decrease in RNase activity, and after 50 days a strong increase in the enzyme activity were also found.

RELATIONSHIP BETWEEN BUD-INDUCING EFFECT OF KINETIN AND INCREASE IN RNase ACTIVITY

Schneider and Szweykowska (1974) suggested that a correlation exists between the increase in RNase activity and the bud induction under the influence of kinetin. The correlation between bud formation and changes in RNase activity was confirmed by our results concerning the enzyme activity during the normal ontogeny of mosses. To obtain further evidences for this relationship, various factors affecting bud-inducing activity of kinetin were used (culture in the dark, various concentrations of kinetin, inhibitors of protein and RNA syntheses), and their effect on RNase activity was determined.

1. Effect of light and dark on bud induction and on RNase activity

In the protonema of *Ceratodon purpureus* kinetin has been found to induce gametophore buds also in the dark (Szweykowska 1963), whereas the protonema of *Funaria hygrometrica* is deprived of this property. It was therefore decided to examine the effect of kinetin treatment on the RNase activity in protonemas of these two species in conditions of light and dark.

With the appearance of the bud-induction response in *Ceratodon purpureus*, a distinct increase in RNase activity has been found in the dark as well as in the light. On the contrary, in *Funaria hygrometrica*, the bud-induction response to kinetin, as well as the increase in RNase acti-

vity were absent in the dark (Fig. 2). This would indicate a dependence of the increase in RNase activity under the influence of kinetin on the bud-inducing effect of this growth regulator.

Fig. 2. Effect of kinetin on the RNase activity and bud induction in light and dark cultures of the protonema of *Ceratodon purpureus* and *Funaria hygrometrica*

Prior to the kinetin treatment, the protonemas were cultured from spores on media containing glucose at 0.25% (*Funaria* in liquid medium for 10 days, and *Ceratodon purpureus* on agar medium for 14 days and then on liquid medium for 7 days, in light conditions). Activity of enzyme was determined after 5 days of kinetin treatment in light or dark conditions, by a modified method of Tuve and Anfinson

C-control, K-kinetin, — no buds present, ++ distinct bud-inducing effect, +++ strong bud-inducing effect

2. Effect of the concentration of kinetin on the formation of gametophores and on RNase activity

An experiment was carried out using kinetin concentrations higher than those usually applied for the bud-inducing effect. A concentration of 25 μM appeared to be optimal for this effect. At this concentration, also a highest increase in RNase activity was found. With higher kinetin concentrations (50, 100 and 500 μM , resp.), the bud-inducing effect, as well as RNase activity gradually decreased (Fig. 3). The concentration of 1000 μM appeared to be toxic for the protonema. This experiment showed the existence of a correlation between the intensity of the bud-inducing effect and RNase activity.

Fig. 3. Effect of kinetin concentration on the activity of RNase and on the bud induction in *Ceratodon purpureus*

A 30-day-old protonema was pre-cultured on mineral agar medium and transferred for 5 days to liquid medium with various concentrations of kinetin. RNase activity was determined in crude extracts by a modified Tuve and Anfinsen method.

— no buds present, + single buds present, ++ distinct bud-inducing effect, +++ strong bud-inducing effect, ++++ very strong bud-inducing effect

3. Effect of inhibitors of protein and RNA syntheses on bud induction and on RNase activity

Investigations of Szweykowska and Schneider (1967) showed that some inhibitors of protein synthesis had an abolishing effect on the kinetin-induced bud formation, with a reversibility of this effect and no signs of toxicity. On the other hand, 8-azaguanine, used as inhibitor of RNA synthesis, only slightly inhibited the bud-inducing effect of kinetin. In a later paper, these authors (Schneider and Szweykowska, 1974) examined the effect of m-fluorophenylalanine, 5-methyltryptophane and 8-azaguanine on kinetin induced increase in RNase activity and showed that fluorophenylalanine and methyltryptophane in concentrations abolishing bud-induction decreased the RNase activity to the level of control. The azaguanine, which only slightly inhibited the bud-inducing effect, also decreased the RNase to a small degree only.

In this study, some other inhibitors of protein and RNA syntheses were also tried, N-acetyloleucine, 8-azaadenine and 6-azauracil, resp. Acetyloleucine and azaadenine in concentrations of 500 μM , and azauracil in a concentration of 100 μM completely abolished the bud-inducing effect of kinetin. At the same time the level of RNase activity decreased to the level of control, in the case of azaadenine even below the control (Fig. 4).

This provided additional evidence for a relationship between the bud-inducing effect of kinetin and the increase in RNase activity.

Fig. 4. Effect of inhibitors of protein and RNA syntheses on the RNase activity and the bud induction by kinetin in *Ceratodon purpureus*

A 30-day-old protonema was pre-cultured on mineral agar medium and transferred for 5 days to liquid medium with kinetin (5 μ M) and inhibitors

— no buds present, +++ strong bud-inducing effect; AL — N-acetyl-leucine, 500 μ M; AA — 8-azaadenine, 500 μ M; AU — 6-azauracil, 100 μ M

DISCUSSION

The effect of cytokinins on the activity of RNase was particularly investigated in connection with the prevention by cytokinins of leaf senescence. Most authors showed that kinetin abolished the increase in RNase activity accompanying the process of senescence of detached leaves of vascular plants, e.g. in wheat (Sodek and Wright 1961) and barley (Srivastawa and Ware 1965).

A difference in response of isolated tomato leaves to cytokinins, depending on the age of leaves, has been shown by Dove (1971). Kinetin increased the RNase activity in young leaves and inhibited it in old ones. Also Schneider and Szwedkowska (1974) showed two ways of kinetin action on RNase activity in the moss protonema. In the preparatory stage for cell divisions, kinetin depressed the enzyme activity in the protonema of *Ceratodon purpureus*. In the second phase of kinetin action, when a development and growth of gametophore buds occurred, a strong increase in RNase activity appeared.

In the present paper, it has been shown that a decrease in enzyme activity occurs also in the normal ontogeny at the time of the spontaneous formation of gametophores. On the other hand, in the stage of gametophore maturation, a high level of RNase activity was found. A similar relationship was shown by Pilet and Brown (1970) in tips of *Lens culi-*

naris roots, where intensive cell divisions and RNA syntheses were accompanied by a low RNase activity, which then increased towards the base of the root, in the maturation zone.

In experiments in which kinetin was used, a high level of RNase was found only accompanying the presence of a large number of over-grown gametophore buds, after 5 days of kinetin treatment. Abolishing the kinetin effect by a lack of light, by inhibitors of protein and RNA syntheses, or high concentrations of kinetin lowered the RNase activity to the level of control. Particularly indicative are data from experiments carried out with *Funaria hygrometrica* in the dark in which the bud-induction effect was absent, and RNase activity, in spite of the presence of kinetin, was also on the control level.

The increase in RNase activity seems thus to be correlated with the presence of maturing gametophores or over-grown gametophore buds. It seems also that this increase in enzyme activity is rather a consequence of the morphogenetic action of kinetin on the protonema and represents a non-specific and secondary response. Feierabend (1970) has found a similar non-specific and quantitative response to cytokinins in the case of enzymes associated with photosynthetic apparatus in rye seedlings. This author emphasizes that "it depends on the special circumstances (e.g. developmental stage, growth temperature, auxin level, light, competition between different synthetic processes, pattern of active genes) which enzymes will be influenced by kinetin and to what extent".

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*Zależność między tworzeniem się pąków gametoforowych w splątku
mchów a wzrostem aktywności rybonukleazy*

Streszczenie

W okresie spontanicznego tworzenia się gametoforów podczas ontogenezy mchów stwierdzono zmiany aktywności rybonukleazy podobne do tych, jakie występują podczas procesu pączkotwórczego indukowanego przez cytokininę (spadek aktywności we wczesnej fazie tworzenia pączków, a później wzrost aktywności enzymu). Stosując różne czynniki wpływające na indukowany kinetyną proces pączkotwórczy, wykazano korelację między wzrostem aktywności RNazy a tym procesem.