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Localization of Cytokinin Responses in the Moss *Funaria hygrometrica*

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Abstract. In the protonema of *Funaria hygrometrica*, predetermined cells run through a period of sensitivity to cytokinins, as part of the morphogenetic system with respect to bud formation. The cytokinin sensitivity is highest immediately after the formation of cells in question. The cells can react if enough hormone is present during the sensitive phase. Kinetin is cleaved very rapidly and at an increasing rate. Most probably the concerned enzyme ("kinetin oxidase") is induced by kinetin. Therefore, cells formed after the beginning of the treatment do not respond by forming buds.

In contrast to higher plants, in which hormones affect all cells of a tissue in almost the same manner, in lower plants such as liverworts (Grotha 1983), mosses (Bopp 1981) or fern prothalli (Schraudolf 1983), hormone responses can be localized in single cells and they also vary in distinct regions of these cells.

The two aspects very pronounced in the hormonal control of development in a plant are the localization of single target cells within the morphogenetic system and the site of reaction within the cells. However, not much is known about the events which lead a particular cell to behave as a target cell. The purpose of our research is to study in more detail: 1) the distribution of target cells and 2) the morphogenetic processes of these cells. When the two criterions are analysed in terms of the availability of hormones in specific cells, one should expect to understand better the regulation of morphogenesis by such hormones.

In the present investigation we have used cytokinin-induced bud formation in the moss *Funaria hygrometrica* as the experimental system.

MATERIAL AND METHODS

Raising of Cultures and Hormone Treatment

The moss cultures were raised using 12—18 days old protonema as described earlier (Lehnert and Bopp 1983). Cytokinins were supplied either in the agar phase or in 150 μl Knop's solution (pH 5.5; 5×10^{-4}M).

Extraction and Purification of Cytokinins

The hormone-treated protonemata were transferred in ice cold Knop-solution, washed 6 times in ice cold distilled water (250 ml each), then frozen in dry ice and stored at −20 °C. One to two grams of frozen material were homogenized in three parts of extraction medium (MeOH : chlorophorm : formic acid; 80 : 1 : 3) using sea sand, and extracted for 120 min by adding two more parts of the extraction medium. After centrifugation at 20 000 × *g*, the pellet was extracted twice for 60 min, each. The collected extracts were vacuum evaporated at 35 °C and frozen again after adjusting the pH to 2.5. Then the probe was thawed and centrifuged a second time. The pH of the aqueous phase was adjusted to 3. These probes were separated on a cellulose-phosphate cation-exchange column.

HPLC-Analysis

The cytokinin (nucleoside) containing fractions were collected and analysed using HPLC (column RP-18 Li Chro Sorb, 5 μm Merck Art. 50333, Steal 250 × 4 mm) at a flow rate of 1 ml min^{-1} of 55 % MeOH. The concentration was determined in comparison to a calibration series.

RESULTS

Localization of the Response to Kinetin

Treatment of 12 to 16 days old protonemata with kinetin (10^{-5}M) induces bud formation along the caulonema filaments. The process begins in certain well defined cells with the cessation of growth, about three hours after kinetin application. This is followed by swelling of the cell tip, and finally a typical sequence of cell divisions begins after about 24 h (Fig. 1a).

Normally buds appear in two regions of the protonema: 1) The side branches of caulonema cells themselves form buds between the third and sixth caulonema cells counted from the apical cell; 2) in older parts of the caulonema, beginning at the original eighth cell, buds arise as a side branch of the second order in the basal cell of a first order branch. In this paper we shall consider only the buds of the first category.

Each cell runs throughout the state of highest sensitivity immediately after cell division. If a protonema is treated with low concentration of kinetin (10^{-7} M), only such a cell on the caulonema forms a bud. For all other cells, kinetin concentration is too low for bud induction. With higher concentrations of kinetin, all cells between the third and sixth cells are transformed into buds as long as they are less than 80 μm long. Longer filaments lose the ability to respond. Moreover, the cells which are formed after the beginning of kinetin treatment, also do not produce buds normally. This seems to be surprising because they run through the most sensitive phase in the presence of kinetin in the substrate.

These observations are supported by the following findings. As soon as kinetin is removed, all induced buds (with only one or several cells) grow out as filaments after about four hours (Fig. 1b). The growing filaments respond to a new application of kinetin leading to typical bud formation, when they are not longer than 80 μm (Fig. 1c). Longer filaments, after a longer time interval between both kinetin treatments, continue to grow as filaments (Fig. 1d). They have lost the capacity to respond. In each case

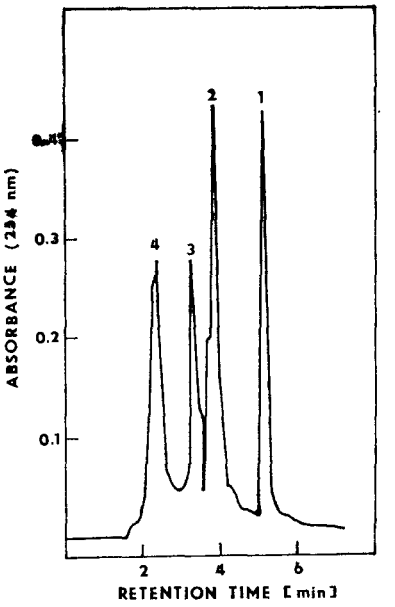

Fig. 2. HPLC of moss extract after kinetin treatment. The moss was treated for 1.5 h with 5×10^{-4} M kinetin. HPLC — flow rate 1 ml min^{-1}, injection volume 15 µl, elution with 55 % MeOH. 1 = kinetin; 2 = adenine; 3 = adenosine; 4 = nucleotides.

the first response concerns the very apical part of the cells, which is determined by the cell polarity. This polarity is not changed in induced or growing bud cells.

Kinetin Metabolism

Our earlier findings have shown (by TLC-separation) that cytokinins, especially kinetin, are not stable in the moss protonema (Bopp and Erichsen 1981, Bopp 1984). The side chain of kinetin cleaves and dominantly adenine and adenine derivatives are formed. After HPLC-studies moss protonema treated with kinetin (5×10^{-4}M) accumulates it in the cells during the first 1.5 h (Fig. 2). Whereas after 1.5 h under continuous application the internal kinetin content decreases so that after 6 h only 40 %, and after 20 h less than 10 % of the amount found after 1.5 h are left (Fig. 3). After prolonged kinetin treatment an enhanced degradation seemed to be induced by the hormone.

To prove this assumption we pretreated the protonemata on agar medium with 10^{-4}M kinetin for 48 h. Thereafter the protonema was transferred to a new treatment with kinetin (5×10^{-4}M) in solution. In this case we also observed an increase in kinetin concentration within the cells during the first 1.5 h of treatment. But it was significantly less than in the former case. The following cleavage was much faster so that after 6 h only 5 % of the initial value (recorded after 1.5 h) was found and this low level of kinetin in the cells disappeared completely after further 6 h. Therefore it can be assumed that the treatment with kinetin induces a kinetin cleaving "oxidase" (compare Scott *et al.* 1982).

A comparison of the cleavage of kinetin with that of BAP (Fig. 3) has shown that the slope for BAP is quite similar in the first part. But two important differences are to be considered. 1) Equimolar concentration of BAP leads

to more than 50 % higher initial accumulation as compared to kinetin. 2) Even after 42 h the BAP values did not reach zero. Always a remarkable and more or less constant amount of BAP was found.

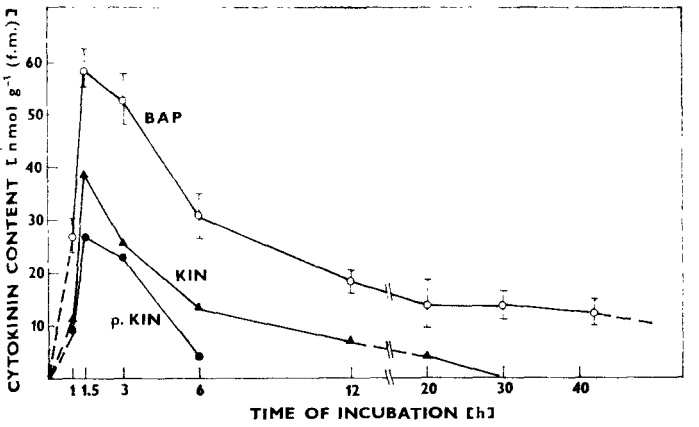

Fig. 3. Cytokinin content of moss extracts after the application of external cytokinins. Slope of cytokinin content in the extract beginning after the application of hormonal solution (5×10^{-4}M). KIN = kinetin; BAP = 6-benzylaminopurine; p.Kin = preatreatment for 48 h on kinetin agar (10^{-4}M).

DISCUSSION

Our results have shown that in the protonema of *Funaria hygrometrica*, no free kinetin can be detected with the HPLC method, after a continuous application of kinetin for more than 20 h. This depends on the very quick cleavage of the N^6 side chain of kinetin, perhaps by a kinetin-oxidase system (PALNI *et al.* 1984), which results in the formation of adenine and adenine derivatives. It is apparent from the presented results with kinetin pretreatment that this system is induced by kinetin itself.

As long as kinetin is present in the substrate at concentrations higher than 10^{-6}M, its uptake is sufficient enough to saturate cytokinin-dependent sites, responsible for induction and maintenance of buds for a certain time. At lower concentrations, however, only one bud per caulonema filament is induced immediately after the start of the treatment, and this concerns only that cell which has just arisen from the caulonema. The gradually decreasing internal kinetin content is sufficient only to keep the induced bud in continuance, more buds can not be formed. The low internal kinetin level after more than 3 h explains also why side branches produced after the beginning of kinetin treatment are not able to be transformed to buds.

Although other cytokinins (BAP, IPA) are metabolized too, a certain amount of them always remains present as a consequence of the equilibrium between influx in the cells and metabolism, which is reached after about 20 h. The amount is high enough to induce buds further in the growing caulonema. This may be the reason why other cytokinins are more effective than kinetin in inducing buds at lower concentrations (HAHN and BOPP 1968, SZWEYKOWSKA *et al.* 1970, BOPP 1983).

As a consequence of the fact that no detectable amount of free kinetin is present in the protonema, the removal of an external cytokinin source causes a reversion of the buds to the filamentous growth, retaining the capacity to respond to cytokinins with new bud formation. That this is only possible before the outgrowth is longer than 80 μm is in agreement with the behaviour of primary side branches of the caulonema. To understand that the special reaction, the swelling of the tip leading to bud formation, which appears in hours must be compared with the rapid swelling in minutes, which occurs as the first step of phototropic reaction (HARTMANN 1984). The latter is evidently not dependent on the cell length.

It is still open to question why, with respect to bud formation, the longer cells lose their "responsiveness" to kinetin. It may be a consequence of the geometry of such cells or a change in the stability of the cell tip structure.

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Fig. 1 is at the end of the issue.

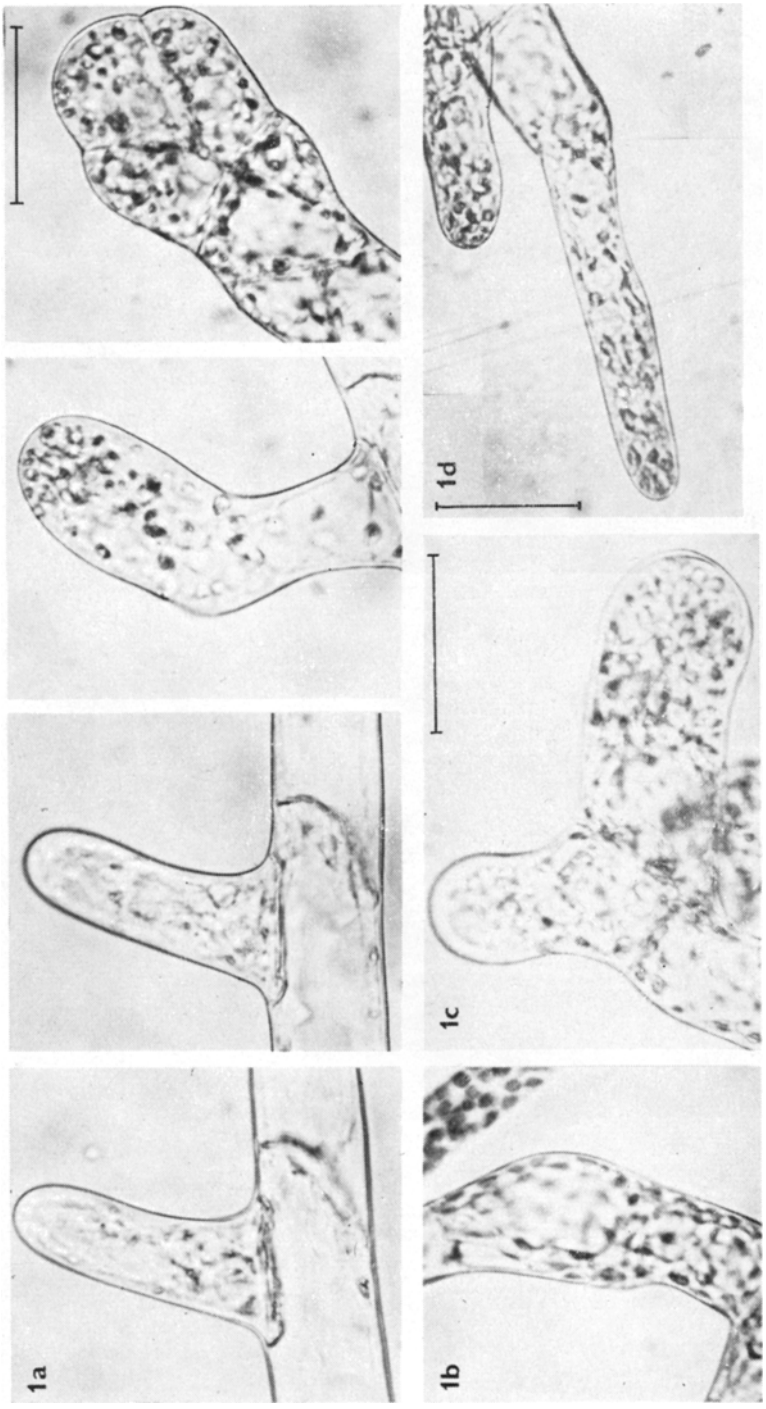

Fig. 1a. Bud formation after kinetin (10^{-5}M) treatment. Side branch of the fourth caulonema cell. Development at 3, 6, 24 and 90 h after kinetin treatment; (bar = 40 μm).
Fig. 1b. Reversion of a bud to filamentous growth. The protonema was treated for 16 h with kinetin (10^{-5}M). Photographs are taken 12 h after removal of the kinetin.
Fig. 1c,d. Bud induction by a second kinetin treatment on filaments arising from buds after removal of kinetin. The first kinetin treatment 16 h, the second treatment 8 h, resp. 24 h later. The second treatment lasted 18 h.