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Developmental Effects of Zeatin, Ribosyl-Zeatin, and *Agrobacterium tumefaciens* B_6 on Certain Mosses

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ABSTRACT

Eight species of mosses studied were divided into two groups on the basis of their developmental responses to ribosyl-*trans*-zeatin and *Agrobacterium tumefaciens* B_6. All eight produced either gametophores or callus on the protonema in response to 6-(γ,γ-dimethylallylamino)purine and *trans*-zeatin. Three which produced normal gametophores with *A. tumefaciens* yielded callus or abnormal gametophores with ribosyl-*trans*-zeatin. Ribosyl-*trans*-zeatin and *A. tumefaciens* were relatively ineffective on five other mosses. Characteristics of protonemal growth common to each of these two groups are described.

Various species of mosses respond to cytokinins with the production of buds that develop into callus or gametophores (2, 13). Whitaker and Kende (19) found that the same concentration of 6-(γ,γ-dimethylallylamino)purine, benzyladenine, or zeatin produce different numbers of buds on *Funaria hygrometrica* (L.) Sibth and that the ribosides of all three cytokinins are less active in comparison to their respective bases. Spiess (11) also showed that i^6Ade[1] and *trans*-zeatin are much more effective in inducing developmental changes on *Funaria hygrometrica* than are *cis*-zeatin or the *cis* or *trans* isomer of ribosyl-zeatin.

These cytokinins have been isolated from cultures of bacteria pathogenic on plants and from plant tumors induced by the bacteria. *Agrobacterium tumefaciens* produces i^6Ade (18), *Rhizobium japonicum* and *R. leguminosarum* produce a zeatin-like compound which is zeatin and/or ribosyl-zeatin (7), and *Corynebacterium fascians* produces *cis*-zeatin (8) and i^6Ade (3). Crown gall tumors resulting from an infection of *Agrobacterium* produce ribosyl-*trans*-zeatin (5, 17).

Spiess *et al.* (15) have shown that *Agrobacteria*, *Rhizobia*, and *Corynebacteria* induce developmental changes on the moss, *Pylaisiella selwynii*. Parallels between tumorigenesis in higher plants and gametophore induction on mosses were indicated by the need for physical contact of bacterium and moss (16), the moss responses to crown gall-related amino acids (13), and to virulent and avirulent bacterial strains (15). Because of the different responses of *Funaria* and *Pylaisiella* to cytokinins and the *Pylaisiella* response to *Agrobacterium*, it was of interest to examine other species of mosses as to their reaction to these agents.

MATERIALS AND METHODS

Moss plants and *Agrobacterium tumefaciens* (Smith and Town) Conn. strain B_6 were cultured as described by Spiess *et al.* (11, 13). The bacterial concentration used was approximately 10^8 cells/ml. Isomers of zeatin which had been synthesized by Nelson J. Leonard were the generous gift of Folke Skoog. The cytokinin i^6Ade was obtained from Calbiochem.

Funaria spores were a gift of Dr. Rolf Beiderbeck, University of Heidelberg, Germany, whereas other mosses were collected locally.

RESULTS

The eight species of mosses tested, each belonging to a different genus, may be divided into two groups on the basis of their response to cytokinins and to *A. tumefaciens* B_6. *Pylaisiella selwynii*, *Entodon seductrix*, and *Heterophyllum haldaneanum* (Table I) produce either callus or abnormal gametophores when supplied with i^6Ade or the isomers of zeatin and ribosyl-zeatin. The responses to i^6Ade and *trans*-zeatin are approximately equal. There was no significant difference in number of structures produced by ribosyl-*trans*-zeatin and *trans*-zeatin on *P. selwynii*, *E. seductrix*, or *H. haldaneanum*. The *cis* isomers of zeatin and ribosyl-zeatin produced fewer structures than the respective *trans* isomers. *A. tumefaciens* B_6 induced normal gametophores on all three mosses.

The other five species used may be placed in a second group on the basis of their low response to ribosyl-zeatin and to *A. tumefaciens* B_6 (Table II). The few structures found on plants treated with these agents were normal gametophores. All five mosses responded to i^6Ade and to *trans*-zeatin although there was variation in this response from callus to normal gametophores. *Thuidium delicatum* and *Climacium americanum* were more prolific in response to i^6Ade and *trans*-zeatin than were *Atrichum undulatum*, *Polytrichum commune*, or *Funaria hygrometrica* and completely unresponsive to *cis* isomers of zeatin and ribosyl-zeatin. *Cis*-zeatin was much less effective in inducing developmental structures on *A. undulatum* and *F. hygrometrica* than was *trans*-zeatin, the few structures produced being normal gametophores. Ribosyl-*cis*-zeatin showed about the same activity on these two mosses as did ribosyl-*trans*-zeatin.

Statistical significance of differences between average number of structures observed on moss protonemata under each pair of treatments was tested by a nonparametric rank test (Kruskal-Wallis test as illustrated in ref. 9). For example, in *P. selwynii*, comparison of *trans*-zeatin (3.05 callus structures) with ribosyl-*trans*-zeatin (2.35 callus structures) gave a value for H (adjusted) equal to 1.24, which is distributed as χ^2 with 1 degree of freedom, and thus indicated no significant difference. In *F. hygrometrica*, comparison of the same treatments for structures produced (5.32 *versus* 0.37) gave a very large value for H which is highly significant (null hypothesis rejected).

A few morphological comparisons may be made also on these mosses. In the first group, the developmental structures were not localized at any one part of the protonema but were scattered at random on the filaments. No rhizoids appeared directly from the spore upon germination but only later from the gametophore structures. No clear difference could be noted between the chloronema and caulonema filaments. All grow in nature on

[1] Abbreviation: i^6Ade: 6-(γ,γ-dimethylallylamino)purine.

decaying wood and are slow growing, being three to four times smaller than mosses in the second group at a given time.

The developmental structures on *A. undulatum*, *P. commune*, and *F. hygrometrica* were localized to cells nearest the spore center of the protonema or at a constant distance from the spore. The callus on *T. delicatum* and *C. americanum* was more widely distributed although the first structures appeared near the spore center. All five mosses in this second group formed a rhizoid directly from the spore immediately after appearance of the first protonema cells. The natural habitat of these mosses is soil or rocks.

DISCUSSION

Mosses vary in their morphological development including the position on the protonema at which buds and subsequent gametophores or callus may appear (1). In many mosses cytokinins induce bud formation at the caulonema stage of protonemal development whereas in other mosses the caulonema stage may be bypassed, buds appearing at the chloronema stage (10). In this study of *P. selwynii*, *E. seductrix*, and *H. haldaneanum*, which did not show clearly defined chloronema and caulonema stages, buds appeared randomly scattered on the protonema. On the other five mosses which did show the two stages of protonema development, the buds appeared in more specific locations indicating a possible apical dominance effect as postulated by Larpent-Gourgaud (4). The appearance of the rhizoid from the spore, as in the second group of mosses or only later from the gametophore as in the first group, is an observation for which no physiological reason can be offered at this time.

Bud formation in response to kinetin has been extensively documented (2). Other cytokinins such as benzyladenine, i^6Ade, and zeatin have been shown to have variable activities on bud and gametophore or callus induction on different mosses (10, 13, 19).

Pylaisiella selwynii is sensitive to cytokinins, producing structures that vary from callus (masses of unorganized cells), to cabbage buds (some structural organization), to abnormal gametophores (leaf structures formed but no elongation of gametophore), and to a very few normal elongating gametophores depending upon concentration and cytokinin used (13). Various ratios of different concentrations of i^6Ade to indoleacetic acid failed to enhance bud initiation over that obtained with cytokinin alone or to permit normal gametophore development (14). Other factors are implicated in regulation of bud development.

The comparisons made by Whitaker and Kende (19) of benzyladenine, i^6Ade, and zeatin and their ribosides on *Funaria* show that the ribosides were almost ineffective in bud induction. The same result was reported by Spiess with respect to zeatin and ribosyl-zeatin on *Funaria* (11).

Whether there is a correlation between the ability of a moss to respond to a cytokinin riboside and to the bacterium *Agrobacterium tumefaciens* B_6 cannot be proved by observations on only eight different mosses, although the data are suggestive. All eight mosses give marked developmental responses when treated with *trans*-zeatin and i^6Ade. Only the three in the first group respond to ribosyl-*trans*-zeatin and to *A. tumefaciens* B_6. In this study *P. selwynii*, *E. seductrix*, and *H. haldaneanum* produce callus or abnormal gametophores when treated with i^6Ade or the *cis* or *trans* isomers of zeatin or ribosyl-zeatin. *A. undulatum*, *P. commune*, *T. delicatum*, *C. americanum*, as well as *F. hygrometrica*, were much less responsive to *cis*-zeatin or to *cis* or *trans* isomers of ribosyl-zeatin, the few structures resulting being normal gametophores.

Because so little is known about the specific details of the role of cytokinins in the biochemistry and physiology of the plant cell or in regulation of development of the moss, it is difficult to speculate on the differences in the responses of these mosses to various cytokinins or any correlation between the morphological features described and the responses to cytokinins.

It would be easy to explain the response to *A. tumefaciens* B_6 by moss in the first group (sensitive to ribosyl-*trans*-zeatin) and the lack of response by mosses in the second group (insensitive to this cytokinin) if *A. tumefaciens* B_6 was known to produce ribosyl-*trans-zeatin*. Ribosyl-*trans*-zeatin has been found in crown gall tumors resulting from an infection by *A. tumefaciens* B_6 (5, 17). *A. tumefaciens* B_6 bacteria themselves produce i^6Ade (18) although Morris and Chapman (6) have shown recently that several different cytokinins may be found in the tRNA of different strains of *A. tumefaciens*. Normal gametophores result from inoculation of *P. selwynii*, *E. seductrix*, or *H. haldaneanum* with *A. tumefaciens* and not callus or abnormal gametophores as would be expected if a cytokinin produced by the bacteria was

Table I. *Developmental Response of Moss Group 1*

Letters following numbers: a = abnormal gametophore; b = normal gametophore; c = callus.

	Pylaisiella selwynii	Entodon seductrix	Heterophyllum haldaneanum
Control	0.013[1]	0.12	0.35
trans-Zeatin[2]	3.05[c]	5.00[c]	3.12[a]
ribosyl-trans-Zeatin	2.35[c]	4.57[c]	4.00[a]
cis-Zeatin	1.92[c]	4.00[c]	...
Ribosyl-cis-Zeatin	0.64[c]	1.25[c]	...
i^6Ade	3.30[c]	5.00[c]	5.00[a]
A. tumefaciens B_6	2.90[b]	1.93[b]	2.00[b]

[1] Number of structures produced per plant. Minimum of 30 plants examined for each treatment.

[2] Cytokinins used at concentration of 10^{-6} M.

Table II. *Developmental Response of Moss Group 2*

Letters following numbers: a = abnormal gametophores; b = normal gametophores; c = callus.

	Atrichum undulatum	Polytrichum commune	Funaria hygrometrica	Thuidium delicatum	Climacium americanum
Control	0.00[1]	0.00	0.20	0.00	0.00
trans-Zeatin[2]	6.40[b]	5.00[a]	5.32[c]	10.00[c]	10.00[c]
ribosyl-trans-Zeatin	0.30[b]	1.00[b]	0.37[b]	0.00	0.00
cis-Zeatin	1.66[b]	...	0.66[b]	0.00	0.00
ribosyl-cis-Zeatin	0.25[b]	...	0.50[b]	0.00	0.00
i^6Ade	4.90[b]	8.00[a]	8.75[c]	25.00[c]	7.00[c]
A. tumefaciens B_6	1.00[b]	0.125[b]	0.093[b]	0.20[b]	0.175[b]

[1] Number of structures produced per plant. Minimum of 30 plants examined for each treatment.

[2] Cytokinins used at concentration of 10^{-6} M.

solely responsible for development. Rhizobium species which produce zeatin or ribosyl-zeatin (7) also induce normal gametophores on *P. selwynii* and not callus (15). The fact that *cis*-zeatin, i^6Ade, and *Corynebacterium fascians* which produces both *cis*-zeatin and i^6Ade (3, 8) all induce callus on *P. selwynii* may indicate that the cytokinin produced by these bacteria is responsible for the development change on the moss.

Experiments by Spiess *et al.* (16) show that *P. selwynii* does not respond to *A. tumefaciens* B_6 or to *Rhizobium leguminosarum* unless the bacteria are in physical contact with the moss. Once the bacterium binds or interacts with a specific site on the plant cell, the plant host's metabolism may be changed so as to produce amongst other regulators a specific cytokinin such as ribosyl-*trans*-zeatin. Other regulators may modify the response to the cytokinin so that normal gametophores result.

Funaria hygrometrica and other moss in the second group described in this paper may not respond to *A. tumefaciens* B_6 because of their inability to utilize ribosyl-*trans*-zeatin or they may lack the binding sites for the bacteria for initiation of the developmental response on the plant. Further studies on the association of bacteria with *P. selwynii* or *F. hygrometrica* should be useful in the understanding of the role of cytokinins in moss development and the problem of tumorigenesis by bacteria on higher plants.

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