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Effects of Cytokinin and Anticytokinin on the Initial Stage of Adventitious Bud Differentiation in the Epidermis of *Torenia* Stem Segments

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In the epidermis of *Torenia* stem segments cultured in vitro, meristematic zones (MZ) were initiated prior to adventitious bud differentiation. Application of benzyladenine (BA) stimulated MZ and bud formation, and the average number of MZ per epidermal strip increased linearly with rising concentrations of BA. The presence of naphthaleneacetic acid together with BA in the medium suppressed MZ formation. Some derivatives of 4-substituted-2-methylpyrrolo[2,3-*d*]pyrimidine which have anticytokinin activity inhibited BA-promoted MZ formation. Interactions of various cytokinins and anticytokinins in MZ and adventitious bud formation were also examined. The number of MZ formed by treatment with 5 μM BA was reduced 50% by the simultaneous application of one of the anticytokinins at the same concentration.

Key words: Adventitious bud differentiation — Anticytokinin — Cytokinin — Meristematic zone — Pyrrolo[2,3-*d*]pyrimidine — *Torenia fournieri*.

Hormonal control of organogenesis in plant tissues and organs cultured in vitro has been reported by many researchers (review by Murashige 1974, Thorpe 1980). The promotion of adventitious bud differentiation by a high ratio of cytokinin to auxin in the medium has been shown in tobacco tissue cultures (Skoog and Miller 1957). In organ fragments of *Torenia* (Kamada and Harada 1979), *Perilla* (Tanimoto and Harada 1980) and *Rudbeckia* (Tanimoto and Harada 1982a), however, the application of cytokinin alone was able to induce bud differentiation. The mechanisms of cytokinin action in growth and organ differentiation in intact plants, as well as in cultured tissues and cells, are still not understood at present. In order to investigate in detail the action of cytokinin in organogenesis, it is necessary to develop sensitive and simple experimental systems. Chlyah (1974) reported that meristematic cell divisions occurred prior to bud differentiation in the epidermis of *Torenia* stem segments. Using this plant material, we showed that cytokinin was required only during the early period of adventitious bud differentiation (the first 6 days of culture) (Tanimoto and Harada 1982b). During this period, MZ were initiated in the epidermal layer of stem segments, which then developed into adventitious buds with no further application of growth regulators.

Much effort has been devoted to synthesizing substances with anticytokinin activities, a number of which are now available. They were often tested using a tobacco callus bioassay (Hecht et al. 1971, 1975, Iwamura et al. 1974, 1975, Skoog et al. 1975). Iwamura et al. (1979)

Abbreviations: BA, N^6-benzyladenine; MZ, meristematic zone; NAA, naphthaleneacetic acid; 4PU, *N*-phenyl-*N'*-(4-pyridyl)urea; 4PU-Cl, *N*-phenyl-*N'*-(2-chloro-4-pyridyl)urea; AA-P, 4-*n*-amylamino-2-methylpyrrolo[2,3-*d*]-pyrimidine; BA-P, 4-*sec*-butylamino-2-methylpyrrolo[2,3-*d*]pyrimidine; CB-P, 4-cyclobutylamino-2-methylpyrrolo-[2,3-*d*]pyrimidine; CH-P, 4-cyclohexylamino-2-methylpyrrolo[2,3-*d*]pyrimidine; CP-P, 4-cyclopentylamino-2-methylpyrrolo[2,3-*d*]pyrimidine; HE-P, 4-(2-hydroxyethylamino)-2-methylpyrrolo[2,3-*d*]pyrimidine.

reported that some derivatives of 4-substituted-2-methylpyrrolo[2,3-*d*]pyrimidine exerted a significant anticytokinin activity against simultaneously applied kinetin in the tobacco callus assay. Only a few reports have appeared concerning the interactions between anticytokinins and cytokinins in organogenesis. Skoog et al. (1973) showed that one of the derivatives of 7-substituted-3-methylpyrazolo[4,3-*d*]pyrimidine inhibited bud formation of tobacco callus tissue. In this article, we report some correlative effects of cytokinins, anticytokinins (derivatives of 4-substituted-2-methylpyrrolo[2,3-*d*]pyrimidine) and NAA on MZ and adventitious bud formation in the epidermis of *Torenia* stem segments cultured in vitro.

Materials and Methods

Plants of *Torenia fournieri* Lind. were raised in a growth room at a temperature of 25±2°C under a 16-hr light photoperiod. The second stem internodes (counting from the top) of 6 to 8 week old plants, which most readily differentiated adventitious buds in vitro (Tanimoto and Harada 1979), were excised and surface-sterilized. Several 5 mm long segments were cut out from each second internode and cultured under sterile conditions. The basal culture medium was comprised of the mineral salts of Murashige and Skoog's medium (Murashige and Skoog 1962), sucrose (2%) and Difco Bacto agar (0.8%). In one series of experiments, BA, kinetin, zeatin, 4PU and 4PU-Cl were added individually to the basal medium. In another series of experiments, NAA or one of the derivatives of 4-substituted-2-methylpyrrolo[2,3-*d*]pyrimidine including AA-P, BA-P, CB-P, CH-P, CP-P and HE-P was added in combination with one of the above cytokinins. Solutions of zeatin and the derivatives of 2-methylpyrrolo[2,3-*d*]pyrimidine were sterilized through a Millipore filter (0.45 μm).

After 7 days of culture, epidermal layers (ca. 5×2 mm) were stripped off the cultured stem segments, stained with aceto-carmine, and immediately observed under a microscope. The numbers of MZ were counted on at least 200 epidermal strips for each treatment, and the data were recorded as the average number of MZ per epidermal strip. To evaluate the rate of bud formation, at least 75 explants were examined macroscopically at the end of a 6 week culture period; the results were expressed as the percentage of cultures producing adventitious buds and also evaluated by the degree of bud development.

Results and Discussion

The effects of BA on MZ and bud formation are summarized in Table 1. The addition of 0.5 μM BA to the medium was sufficient to induce bud differentiation in almost all the treated explants. The average number of MZ per epidermal strip was increased by raising the concentration of BA in the medium. Although some fluctuation was noted in the number of MZ produced among similarly treated epidermal strips, a linear correlation was found between the concentration of BA and the number of MZ produced in each experiment. Therefore, this experimental system seems to be suitable for a cytokinin bioassay on bud formation.

Auxin (at a relatively low concentration) applied together with cytokinin generally stimulates adventitious bud differentiation (Murashige 1974, Thorpe 1980); however, such an application somewhat inhibits bud formation in *Torenia* stem segments (Kamada and Harada 1979, Tanimoto and Harada 1981a). In the epidermis of *Torenia* stem segments, MZ formation was not stimulated by treatment with auxin alone (Tanimoto and Harada 1982b), and cytokinin-induced MZ formation was clearly suppressed by simultaneously applied NAA (Table 2). When auxin alone at a low concentration was added to the culture medium, adventitious bud formation was promoted, but with a high concentration, it was suppressed (Table 2, Tanimoto and

Table 1 Effects of BA on meristematic zone (MZ) and bud formation in the epidermis of *Torenia* stem segments cultured in vitro

BA (μM)	No. of MZ per strip of epidermis [a]	Cultures with buds [b] (%)	Mean no. of buds per explant	Degree of bud development [c]
0	0.5 (0.2– 0.9)	7± 6.1	1.7	+
0.05	2.1 (1.2– 3.1)	38±16.5	2.3	++
0.5	6.5 (4.4– 8.9)	95± 4.8	15.4	+++
1.5	9.9 (5.9–12.1)	96± 3.8	28.2	+++
5.0	12.5 (8.1–17.1)	98± 1.9	>40	++

[a] Values represent the average of 12 experiments, each of which consisted of at least 70 epidermal strips per treatment. Figures in parentheses indicate the minimum and maximum numbers of MZ per epidermal strip. Data was recorded 7 days after the beginning of culture.

[b] Values represent the average of 6 experiments, each of which had 25 replicates per treatment. Data was recorded 6 weeks after the beginning of culture.

[c] +, low; ++, moderate; +++, high.

Harada 1981b). A large proportion of the adventitious buds formed under treatment with auxin did not originate from the MZ (i.e., not from the epidermis), but instead from callus formed from the inner tissues and/or at the cut ends of segments.

Iwamura et al. (1979) examined the cytokinin-agonistic and -antagonistic activities of various 4-substituted-2-methylpyrrolo[2,3-*d*]pyrimidines using several bioassay systems. Among them, CP-P and CB-P exhibited high anticytokinin activity against simultaneously applied kinetin (0.05 μM) in the tobacco callus assay, but the activity of BA-P, CH-P and AA-P was relatively low. In the case of *Torenia* stem segments, these five compounds applied at concentrations of 5 μM or higher suppressed BA(0.5 μM)-promoted MZ formation to about same extent (Fig. 1). At a low concentration (1.5 μM), however, the inhibition of MZ formation by the chemicals differed in degree as follows: CP-P caused the greatest inhibition (about 50% in terms of the number of MZ formed per epidermal strip); AA-P and CB-P caused moderate inhibition; BA-P

Table 2 Correlative effects of NAA and BA on meristematic zone (MZ) and bud formation in the epidermis of *Torenia* stem segments cultured in vitro

BA (μM)	NAA (μM)	No. of MZ per epidermis	Cultures with buds (%)	Degree of bud development [a]
0	0	0.5	8	+
	0.5	0.1	47	+
	5.0	0.1	2	+
0.5	0	6.2	95	+++
	0.05	2.0	42	++
	0.5	0.8	27	+
	5.0	0.1	4	+
5.0	0	13.2	98	++
	0.5	1.3	62	+++
	5.0	0.3	34	+

[a] +, low; ++, moderate; +++, high.

For each treatment, at least 200 epidermal strips and 75 explants were observed in order to calculate the average numbers of MZ and the percentages of cultures producing adventitious buds, respectively.

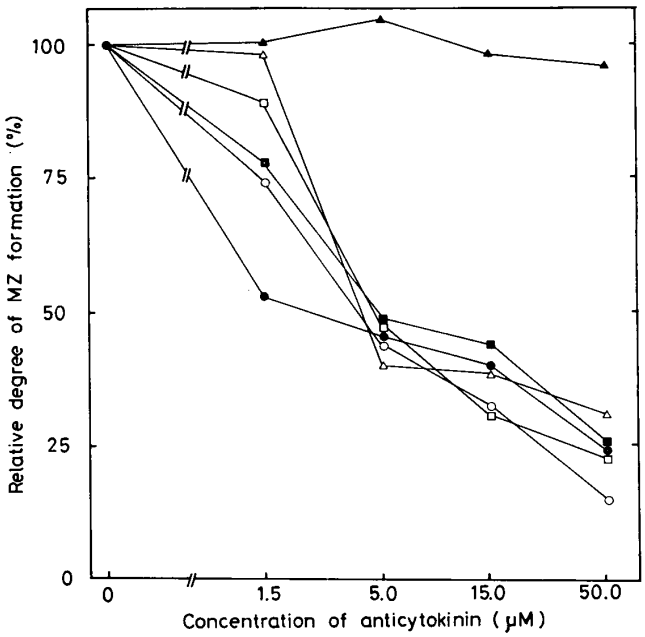

Fig. 1 Inhibitory effects of various anticytokinins on BA-promoted MZ formation in the epidermis of *Torenia* stem segments cultured in vitro. The control medium contained 0.5 μM BA without anticytokinin. The relative degree of MZ formation (ordinate) expresses the number of MZ formed per epidermal strip under BA plus anticytokinin treatment as a percentage of the number formed under treatment by 0.5 μM BA alone (which was 7.2 MZ per strip). Each point represents an average of at least 200 epidermal strips. -●-, CP-P; -■-, CB-P; -▲-, HE-P; -○-, AA-P; -□-, CH-P; -△-, BA-P.

and CH-P caused only a little. In the assay system utilizing *Amaranthus* betacyanin synthesis (Iwamura et al. 1979), CH-P and CB-P produced little effect, but HE-P showed suppressed the action of 1 μM 6-(3-methyl-2-butenylamino)purine. However, HE-P did not show any anticytokinin activity in either the tobacco callus assay (Iwamura et al. 1979) or in MZ formation (Fig. 1). These results indicate that BA-promoted MZ formation in the epidermis of *Torenia* stem segments can be suppressed by the same compounds which show anticytokinin activities in the tobacco callus assay.

In another series of experiments, BA and CB-P at various concentrations were incorporated into the medium. Epidermal strips of explants treated with 5 μM BA alone produced a large number of MZ (16.3 per epidermal strip). When 5 μM CB-P was added together with 5 μM BA,

Table 3 Effects of 5 cytokinins and CP-P on meristematic zone (MZ) and bud formation in the epidermis of *Torenia* stem segments cultured in vitro

Cytokinin	Conc. (μM)	No. of MZ per epidermis		Cultures with buds (%)	
		−CP-P	+CP-P (15 μM)	−CP-P	+CP-P (15 μM)
None		0.2	0.2 (77)	7	0
BA	0.5	7.9	3.0 (38)	95	69
	5.0	15.6	6.4 (41)	99	95
Kinetin	0.5	3.9	2.0 (53)	62	46
	5.0	4.8	2.3 (49)	74	56
Zeatin	0.5	8.6	4.9 (56)	100	79
4PU	0.5	4.9	3.0 (62)	83	82
4PU-Cl	0.4	17.5	3.3 (19)	100	97

For each treatment, at least 200 epidermal strips and 75 explants were observed in order to calculate the average numbers of MZ and the percentages of cultures producing adventitious buds, respectively. Numbers in parentheses indicate the percentage calculated on the basis of MZ numbers obtained with the application of respective cytokinins without CP-P.

the mean number of MZ per epidermal strip fell to 8.5. Similar results were obtained with serial combinations of BA and all the other anticytokinins tested except HE-P.

The effects of various cytokins and compounds with cytokinin activity in promoting MZ and bud formation, as well as the counteracting action of CP-P against those stimulating substances, were also examined (Table 3). At 0.5 μM, kinetin and 4PU were not as effective in MZ induction as the other compounds; 4PU-Cl was most effective in promoting MZ formation. Takahashi et al. (1978) reported that 4PU-Cl showed cytokinin activity 10 times higher than BA in the tobacco callus bioassay. Our results indicate that the number of MZ induced by treatment with 0.4 μM 4PU-Cl was greater than that produced by 5 μM BA (Table 3).

When CP-P was added to the medium simultaneously with one of the cytokinins, more or less similar inhibitory effects on MZ formation were noted regardless of which substance it was added with (Table 3). The strongest inhibitory effect of CP-P was shown against 4PU-Cl, decreasing the number of MZ to about 19%. Although CP-P clearly inhibited MZ formation, its suppressive effect on adventitious bud formation was less evident at the concentration examined (15 μM).

Previously we investigated the changes in endogenous levels of protein-incorporated and free amino acids during the early stages of adventitious bud differentiation in *Torenia* stem segments (Tanimoto and Harada 1982c). Another series of experiments is in progress to examine the histological, physiological and biochemical changes occurring in the epidermis during MZ formation. We hope that such studies of the interactions between cytokinin and anticytokinin or auxin in the epidermis of *Torenia* stem segments cultured in vitro may help elucidate the physiological and biochemical processes taking place during MZ formation and adventitious bud differentiation.

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