DIFFERENTIAL CYTOKININ BINDING TO DIOECIOUS PLANT RIBOSOMES

S. R. CHUNG, R. DURAND and B. DURAND

Laboratoires de Botanique et Biologie Végétale, Domaine Universitaire de La Sourse, Université D'Orleans, 45045 Orleans Cédex, France

Received 18 April 1979

1. Introduction

It has been reported that the administration of auxins, gibberellins and cytokinins to plants affects nuclear transcription [1–7]. Other observations suggest that cytokinins also have an effect at the translational level [8]. Cytokinin binding to the ribosomes of Chinese cabbage [9], wheat germ and tobacco callus [10] has been described and isolation of the specific receptors has been attempted.

Based on our knowledge of sexual differentiation of the dioecious plant *Mercurialis annua* [11], we believe that genetically male meristem is a specific target organ of cytokinins. Exogenous cytokinins provoked obvious changes of these male meristems, including the appearance of certain tRNA species and enzymes specific to female flowers [12].

The purpose of the present study was to detect differential cytokinin binding to ribosomes in male and female target cells. The binding of BAP, FAP, 2IP and Z, as well as their less active ribosides, were compared in order to detect any relation between physiological action and binding efficiency.

2. Materials and methods

[8-^{14}C]BAP (spec. radioact. 13.4 mCi/mmol, 60 μCi/mg) was purchased from the Radiochemical Centre, UK. BAP, RBAP, FAP, RFAP, Z, RZ, 2IP were purchased from Sigma (USA). All other products were Reagent Grade.

Abbreviations: [8-^{14}C]BAP, radioactive 6-benzylaminopurine; BAP, 6-benzylaminopurine; FAP, 6-furfurylaminopurine; 2IP, 6-γ,γ-dimethylallylaminopurine; Z, zeatin; STMP, 0.1 M Tris–HCl (pH 7.0), 0.4 M sucrose, 0.05 M $MgCl_2$, 0.05 M 2-mercaptoethanol, 50 μg polyvinyl sulfate/ml; TMP, 0.01 M Tris–HCl (pH 6.8), 1 mM $MgCl_2$, 50 μg polyvinyl sulfate/ml

2.1. *Cell disruption, fractionation and purification*

Young male and female flower buds of the dioecious plant *Mercurialis annua* L. ($2n = 16$) (*Euphorbiaceae*) were collected in the field, rapidly frozen in liquid nitrogen and stored at –20°C. The purification of subcellular components [13–15] was performed at 0–4°C. Plant tissue (60 g) in 90 ml STMP buffer was homogenized in a Waring blendor, the homogenate was pressed through 4 layers of cheesecloth and was centrifuged at 700 × g for 15 min. A 15 min centrifugation of the above supernatant (1 g plant tissue = 1.75 ml) at 18 000 × g sedimented organelles. The 18 000 × g supernatant of this step was then centrifuged at 150 000 × g for 2 h and the ribosomal pellet thus obtained was stored at –20°C until the assay. Ribosomes prepared according to this technique remain fully active for 3 months at –20°C [16].

2.2. *[8-^{14}C]BAP binding to ribosomes*

Stored ribosomal pellets (1 vol.) were suspended in TMP buffer (1 vol.) immediately prior to assay. After a gentle homogenization in a Kontes glass homogenizer, the suspension was clarified by centrifugation at 23 500 × g for 5 min. The ribosome concentration was estimated by A_{260}. The A_{260}/A_{235} and A_{260}/A_{280} ratios were found to be 1.30–1.60 and 1.70–1.90, respectively. These values agree fairly well with corresponding figures for plant [9,17] and animal ribosomes [15–17]. The final yield of these ribosomes

(1 g plant material = 1.50 ml ribosome suspension) was 13.7 A_{260} units/ml.

The binding assay was a modification of the assay of auxin binding to particulate corn fractions [18,19]. The ribosome suspension (10 ml) was added to nitrocellulose centrifuge tubes with the same capacity and 0.1 vol., i.e., 1 ml test solution (6×10^{-5} M) was then added. The test solution consisted of 6×10^{-8} M of [8-^{14}C]BAP in the absence or in the presence of unlabeled BAP added in increasing quantities from 1.4×10^{-7} M to 1.6×10^{-5} M. After a 5 min incubation, the tubes were centrifuged at 150 000 × g for 2 h in a Beckman T 50 rotor. After removal of the aqueous phase, the ribosome pellet was carefully washed with 1 ml ice-cold TMP to remove residual material, e.g., protein, was drained of excess moisture and was left overnight at 4°C. Bound BAP/mol ribosomes was calculated as in [6], assuming that 1 mg ribosomes/ml was $A_{260} = 16$ and that 1 unit of A_{260} corresponded to 15 pmol/ribosomes, based on a ribosomal mol. wt 4.0×10^6.

Determination of radioactivity: a 0.3 ml aliquot of the supernatant, as well as the entire ribosome pellet, obtained by cutting the bottom of the centrifuge tube, were added to scintillation vials containing 10 ml Bray's solution [20]. A Beckman LS 3133 P liquid scintillation counter at room temperature was used with a ^{14}C counting efficiency >90%. Total and specific binding were calculated as in [18,21].

The binding constant was determined by the Scatchard method [22]. The binding constant of the other cytokinins was calculated by plotting the quantity of [8-^{14}C]BAP bound versus the logarithm of the increasing quantities of the free hormones, BAP, FAP, 2IP, Z (fig.5). The linear distance along the X-axis between the [8-^{14}C]BAP curve and the other curves was measured at the optimum ($f = 4 \times 10^{-7}$, 642 cpm) calculated from Scatchard plots to saturate 50% of the binding sites. This distance furnishes an approximation of log K_a. Standard BAP is then defined with K_a BAP = 1 [23].

3. Results

3.1. *Characterization of cytokinin binding*

Binding of BAP was very rapid (table 1), a phenomenon also observed for auxin binding [18] and for chloramphenicol binding to *Bacillus megatherium* ribosomes [24]. The rate of binding was temperature dependent, being most rapid at 4°C, and was pH dependent (fig.1). Specific and non-specific binding were both pH dependent, but specific binding was optimal at pH 6.2, suggesting a specific interaction.

Table 1
Kinetics of BAP binding to *M. annua* ribosomes

Incubation with	Incubation time (min)		
[8-^{14}C]BAP	2	5	20
	751	748	737
	785	760	773
[8-^{14}C]BAP + 8.6×10^{-7} M unlabeled BAP	589	590	597
	591	558	578

Numbers represent cpm bound/reaction

We next studied comparative binding between male and female ribosomes, because the specific binding only appeared to be reliable in the ribosome fraction and since the properties of our ribosome suspensions were in good agreement with published criteria [9,15–17].

3.2. *Binding to male and female ribosomes*

Binding of [8-^{14}C]BAP to male and female ribo-

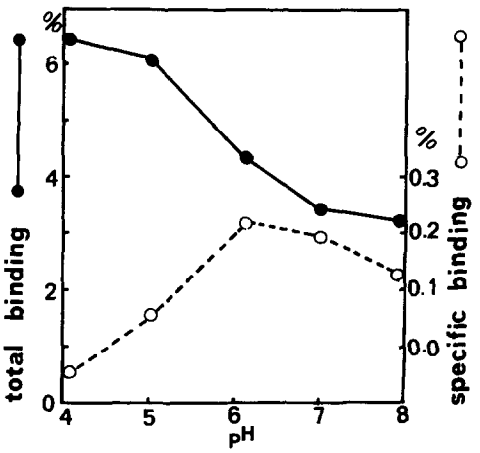

Fig.1. [8-^{14}C]BAP binding to *M. annua* ribosomes as a function of pH. Homogenates were adjusted with HCl or NaOH to pH 4–8 then total and specific binding were assayed. Binding (%) = (bound cpm/free cpm) ×100. Specific binding = % binding of [8-^{14}C]BAP alone minus binding % of the highest assay in the presence of unlabeled BAP. The data are the average values of 4 independent determinations.

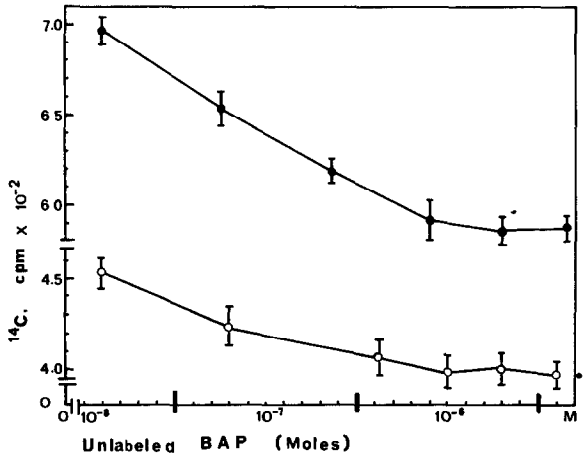

Fig.2. Inhibition profiles of [8-^{14}C]BAP binding to male (solid circles) and female (open circles) ribosomes in the presence of increasing concentrations of unlabeled BAP. Each point is the mean value of 5 independent determinations. 17 000 cpm/ml supernatant.

somes in the presence of increasing concentrations of unlabeled BAP is shown in fig.2,3. The initial amount of 6×10^{-8} M was chosen because it is suitable for specific binding assays in a wide concentration range (S.R.C., unpublished results). It appears from the data that BAP in increasing concentrations effectively compete with the labeled compound up to 2×10^{-6} M on either male or female ribosomes. Female ribosomes initially bound about half as much BAP as male ribosomes and were less sensitive to competition. In the related Scatchard plot (fig.4) the number of binding

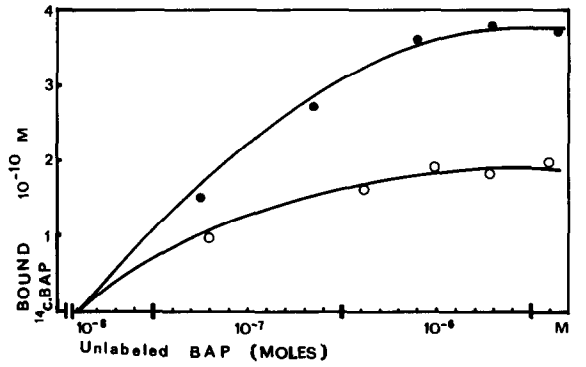

Fig.3. Saturation kinetics of specific binding to male (solid circles) and female (open circles) ribosomes.

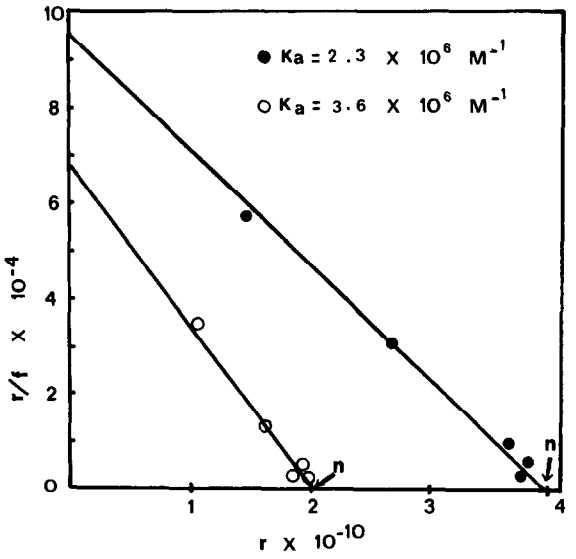

Fig.4. Scatchard plot of BAP binding to male (solid circles) and female (open circles) ribosomes. Data obtained from fig.3. r = mol cytokinins, f = mol free cytokinin, K_a = binding constant, n = quantities bound.

sites, expressed as mol BAP fixed, was estimated by the distance between the origin and the X-intercept and was found to be 3.9×10^{-10} mol in male ribosomes and 2×10^{-10} mol in female ribosomes. Molar binding ratios were calculated as 1.85 mol/male ribosome and 0.94 mol/female ribosome, with respective binding constants of 2.36×10^6 M^{-1} and 3.6×10^6 M^{-1}. Only one of the two binding sites described [10] could be demonstrated, since the concentration of [8-^{14}C]BAP was too low to enable weak binding sites to be detected. Thus, only the site with the highest affinity was expressed.

3.3. *Inhibition of [8-^{14}C]BAP binding to male ribosomes by free cytokinin bases (FAP, 2IP, Z) and their corresponding ribosides*

It can be seen from the data shown in fig.5 that all 3 bases competed actively with [8-^{14}C]BAP with Z being the most effective, as shown by saturation kinetics for specific binding sites. The greatest inhibition at the lowest concentration was exhibited by Z. The binding constants determined are 2.3×10^6 M^{-1} for BAP, 3.9×10^6 M^{-1} for FAP, 1.6×10^7 M^{-1} for 2IP and 2.0×10^7 M^{-1} for Z. Thus, the highest

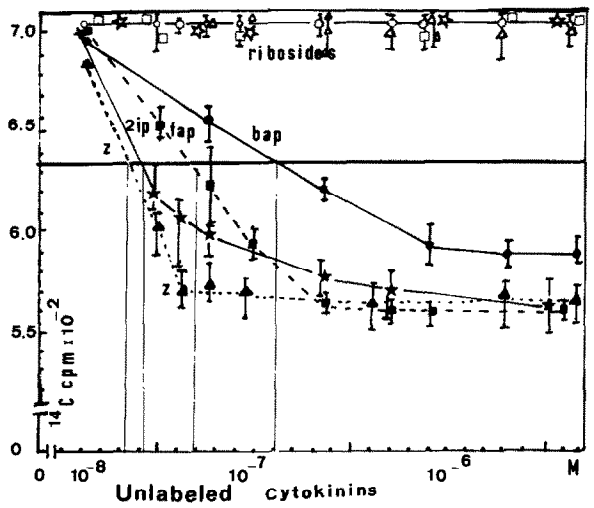

Fig.5. Inhibition of [8-^{14}C]BAP binding to male ribosomes by increasing concentrations of unlabeled free bases (solid figures) and their corresponding ribosides (open figures).

affinity corresponds to the greatest physiological effectiveness expressed by bioassays, i.e., the activity of Z. The ribosides of these bases exhibited no inhibition of BAP binding.

4. Discussion

The amount of BAP bound by male ribosomes was twice that bound by female ribosomes. Analysis of the endogenous cytokinin contents of buds with a computerized gas chromatography–mass spectrometry system showed that the levels of free Z were higher in female apeces than in either male of monoecious apeces [26–29]. Endogenous Z cannot be removed from its ribosomal sites as a result of its high natural binding constant. The observed difference in site numbers thus agrees with the saturation of more female sites by higher Z concentrations.

Even though genotypically male cells contain very low quantities of Z, they nonetheless contain specific receptors. If confirmed, these results would suggest that the receptors evidenced here in both male and female apical cells would be expressed in a manner similar to that of the steroid receptors involved in mammalian sexual development [30].

The binding constant is apparently sex dependent. Certain non-histone proteins purified from chromatin of *M. annua* specifically bind BAP and similar differences of site numbers and binding constant have been shown by the same method [31]. This suggests a structural difference between male and female receptors and so sexual differentiation might imply the synthesis of sex-specific receptors.

A differential sex-specific RNase activity that would partially change female ribosomes does not appear capable of explaining the observed differences. Results remained constant after several repeated extractions and binding assays, ultraviolet absorption spectra remained unchanged and there were no detectable differences in the properties of male and female ribosomes from one extraction to another.

The ribosides of the hormones tested were inactive. Only the free bases effectively bound to ribosomes and among these synthetic or natural compounds, the most actively bound was Z which was also the most active in specific bioassays. IPA and RZ, ribosides which exist in almost equal quantities in male and female apeces [28,29] did not compete for BAP binding sites, which is consistent with their lower physiological activities in specific bioassays. Thus, free bases may have a primordial role in the manifestation of physiological effects.

These results may help to explain how cytokinins exert their physiological effects. It appears that the nature of hormonal response is conditioned by the specific nature of the binding site in target cells as well as by the related hormone. The difference usually pointed out between the actions of plant and animal hormones now appears to be less clear-cut.

References

[1] Letham, D. S. (1969) Bioscience 19, 309–316.
[2] Matthysse, A. G. and Phillips, C. (1969) Proc. Natl. Acad. Sci. USA 63, 897–903.
[3] Mondal, J., Mandal, R. K. and Biswas, B. B. (1972) Nature New Biol. 240, 111–113.
[4] Davies, P. J. (1973) Bot. Rev. 69, 139–171.
[5] Jacobsen, J. V. and Zwar, J. A. (1974) Proc. Natl. Acad. Sci. USA 71, 3290–3293.
[6] Ho, D. T. H. and Varner, J. E. (1974) Proc. Natl. Acad. Sci. USA 71, 4783–4785.
[7] Higgins, T. J. V. et al. (1976) Nature 260, 166–169.
[8] Berridge, M. V. and Ralph, R. K. (1969) Biochim. Biophys. Acta 182, 266–269.

[9] Berridge, M. V., Ralph, R. K. and Letham, D. S. (1970) Biochem. J. 119, 75–84.
[10] Fox, J. E. and Erion, J. L. (1975) Biochem. Biophys. Res. Commun. 64, 694–700.
[11] Kahlem, G., Champault, A., Louis, J. P., Bazin, M., Chabin, A., Delaigue, M., Dauphin, B., Durand, R. and Durand, B. (1975) Physiol. Vég. 13, 763–779.
[12] Bazin, M., Chabin, A. and Durand, R. (1975) Devel. Biol. 44, 288–297.
[13] Bonner, J. and Varner, J. E. (1965) Plant Biochemistry, pp. 7–61, Academic press, New York.
[14] Mahler, H. R. and Cordes, E. H. (1971) Biological Chemistry, 2nd edn, pp. 441–455, Harper and Row, New York.
[15] Florini, J. R. and Breuer, C. B. (1966) Biochemistry 5, 1870–1876.
[16] Wool, J. G. and Cavicchi, P. (1967) Biochemistry 6, 1231–1242.
[17] Petermann, M. L. (1974) in: The Physical and Chemical Properties of Ribosomes, pp. 1–258, Elsevier, Amsterdam.
[18] Hertel, R., Thomson, K. S. and Russo, V. E. A. (1972) Planta 107, 325–340.
[19] Normand, G., Hartmann, M., Schuber, F. and Benveniste, P. (1975) Physiol. Vég. 13, 743–761.
[20] Bray, G. A. (1960) Anal. Biochem. 1, 279–285.
[21] Thomson, K. S., Hertel, R., Müller, S. and Tavares, J. E. (1973) Planta 109, 337–352.
[22] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[23] Cebra, J. (1971) in: Methods in Immunology, vol. 3, pp. 58–66, Academic Press, New York.
[24] Vasquez, D. (1964) Biochem. Biophys. Res. Commun. 15, 464–468.
[25] Gardner, G., Sussman, M. R. and Kende, H. (1976) 9th Int. Conf. Plant. Growth Subst., Lausanne, coll. abst. pp. 103–104.
[26] Chung, S. R., Durand, R. and Durand, B. (1977) CR Acad. Sci. Paris 284, 1417–1420; (1977) FEBS Meet. B 3-5, 513.
[27] Dauphin, B., Teller, G. and Durand, B. (1977) Physiol. Vég. 15, 747–762; abst. Int. Mass Spectrom. Symp. Natural products, Rehovot, August 1977.
[28] Durand, B., Teller, G., Dauphin, B. and Chung, S. R. (1978) Fed. Eur. Soc. Plant Physiol. abst. Meet. Edinburgh.
[29] Dauphin, B., Teller, G. and Durand, B. (1979) Planta 144, 113–119.
[30] Ohno, S. (1976) Cell 7, 315–321.
[31] Lhermitte, A. (1979) Third Cycle Thesis Molecular Biophysicochemistry, Orleans University.