Planta 148, 124–129 (1980)

Planta

Different Endogenous Cytokinins between Male and Female *Mercurialis annua* L.

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Abstract. The endogenous pool of cytokinin metabolites during sexual differentiation of *Mercurialis annua* L. was studied with a computerized gas-chromatography-mass spectrometry system. Certain metabolites were common to both sexes: ribosides (isopentenyl-adenosine, ribosylzeatin) and the nucleotide of I_6-Ade. Zeatin could be detected only in females while its nucleotide was present in males. The results were obtained with differentiating apices and whole plants. The high Z concentration and the low level of its nucleotide are related to the absence of two dominant complementary genes, determining maleness. Study of the regulation of cytokinin metabolism now seems possible.

Key words: Cytokinin metabolism – *Mercurialis* – Sexual differentiation.

Introduction

In the dioecious plant *Mercurialis annua* L., sex conversion may be provoked by the action of exogenous hormones. Thus, cytokinins induce the appearance of female flowers on male individuals and auxins lead to the appearance of male flowers on genetic females (Kahlem et al. 1975). Concomitant syntheses of specific macromolecules accompany these transformations. A group of anodic isoperoxidases constitutes a specific and early marker of stamen differentiation (Kahlem 1976), and a group of specific isoesterases, two sub-species of chloroplast tyrosine tRNAs and one leucyl tRNA synthetase, are specific for the differentiation of the carpel (Bazin et al. 1975).

The synthesis of these markers depends on the hormone balance which appears to be different in each sex. This is suggested by the initial bioassays, showing that cytokinin levels are higher in females, while auxin levels are higher in males.

The purpose of the present work was to determine the nature of these differences, to discover if they are purely quantitative or if they qualitatively affected certain metabolites. If a metabolic difference were demonstrated, one could presuppose its eventual relationship with the genes for sexual determinism, since this balance is sex genotype-dependent (Louis et al. 1978). Finally, knowledge of cytokinin metabolism in each sex type may contribute information concerning the active form of these hormones. In this work, we compared the endogenous cytokinin pools of male and female *Mercurialis annua* L. ($2n=16$).

Abbreviations: IPA = isopentenyl adenosine; I_6-Ade = isopentenyl adenine; Z = zeatin; RZ = ribosylzeatin

Materials and Methods

Strains

Male and female apices were taken simultaneously from a greenhouse culture composed of an artificial population. This population included a majority of males of AB_1b_2 genotype (Louis et al. 1978); highly sensitive (Ab_1B_2) and resistant (AB_1B_2) (to exogenous cytokinins) males were in the minority.

Assays on whole plants were performed with similar populations of the same origin, but were cultivated in constant condition growth chambers at 15° C nightime and 20° C daytime, a photoperiod of 16 h light and 8 h darkness, and an illumination intensity of 25,000 lx.

Extraction and Purification Procedures

Apex lots of 250 and 800 g and whole plant lots of 400 g were pooled and immediately homogenized in liquid nitrogen in a Waring blendor.

The extraction method, previously described (Dauphin et al. 1977), was briefly the following: the powder was taken up in 80% ethanol containing 120 mg of NaOH per 1. The extract was loaded onto a column of Sephadex SPC 25 and was eluted with 0.05 M NH_4OH. Nucleosides and free bases were extracted from the eluate with water-saturated butanol (Miller 1968). The extracted

0032-0935/80/0148/0124/$01.20

nucleosides and free bases were then separated by chromatography on a column of Sephadex LH 20 with 35% ethanol (Amstrong et al. 1969).

The remainder of the NH_4OH eluate contained nucleotides. Cytokinin nucleotides can be fixed on SPC 25 cation exchange Sephadex, since the NH group, even though located on a benzene ring, is sufficiently charged as a result of the isopentyl chain. Optimum fixation was obtained after equilibrating the column at pH 5.5. Recovery, determined with a standard nucleotide of I_6-Ade, was greater than 80%. (We thank M. Gawer for furnishing this standard.)

After concentrating the aqueous phase in vacuo to 1 ml, it was treated with alkaline phosphatase in a reaction mixture containing 1 ml of the extract, 0.8 ml of Tris-HCl, pH 8, and 0.05 ml of alkaline phosphatase (Sigma, No. 4,502). Incubations were allowed to continue for 4 h at 37° C and were stopped by chilling to −80° C. Nucleosides were then extracted with ethyl acetate (the yield of nucleoside separation, determined by mass spectroscopy with standards, was higher than with butanol). The assay of nucleosides after hydrolysis was considered to reflect the levels of the corresponding nucleotides.

The gas-chromatography, mass-spectrometry, computer-assay system has been previously described (Dauphin et al. 1977, 1979). Each metabolite is assayed according to the intensity of the molecular ion or of the molecular ion minus 15 in relation to the intensity of a standard consecutively chromatographed.

During the chromatographic run, a spectrum was recorded on a magnetic disk every 4 s. The curve of frequency and the intensity of the characteristic ions of a metabolite, given in the different spectra, traces a chromatographic peak, leading to identification, whose surface is proportional to quantity (Figs. 1–5). Assays may thus be performed by cutting peaks and weighing in comparison to standard peaks.

For the identification of IPA, characteristics were chosen from the group of ions M/E 508, 348, 536, 551 and 232, with increasing intensity in the spectrum. The molecular ion M/E 551 was chosen for the assay.

The series of ions with increasing intensity M/E 638, 624, 549 and 536 was chosen for the identification of RZ; ion M/E 624 was chosen for the assay.

For the identification of zeatin, the group of ions M/E 363, 348, 273, 260 was considered for monotrimethylsilyl zeatin; the ion M/E 348 was chosen for the assay.

In the case of ditrimethylsilyl zeatin, ions M/E 435, 420, 346, 304 were used for identification and M/E 420 was used for assay.

Absolute intensities were given directly by the computer. As previously indicated (Dauphin et al. 1979) the efficiency of the recovery of standards (IPA, RZ, Z) added to plant extracts is about 50%, without plant extracts it is greater than 80%. The error during GC run and fragmentation did not exceed 5%.

Results

As shown in the curves, IPA (Fig. 1), RZ (Fig. 2), and the nucleotide of I_6-Ade (Fig. 4) are present in both sexes; zeatin, on the other hand (Fig. 3), could be detected only in females, while its nucleotide (Fig. 5) could be detected only in males, under our experimental conditions, i.e., 250 to 800 g of fresh material. Analogous results were obtained with differentiating apices and with whole plants in which the apex represented only a small fraction of the total weight. The purity of the extracts was verified by

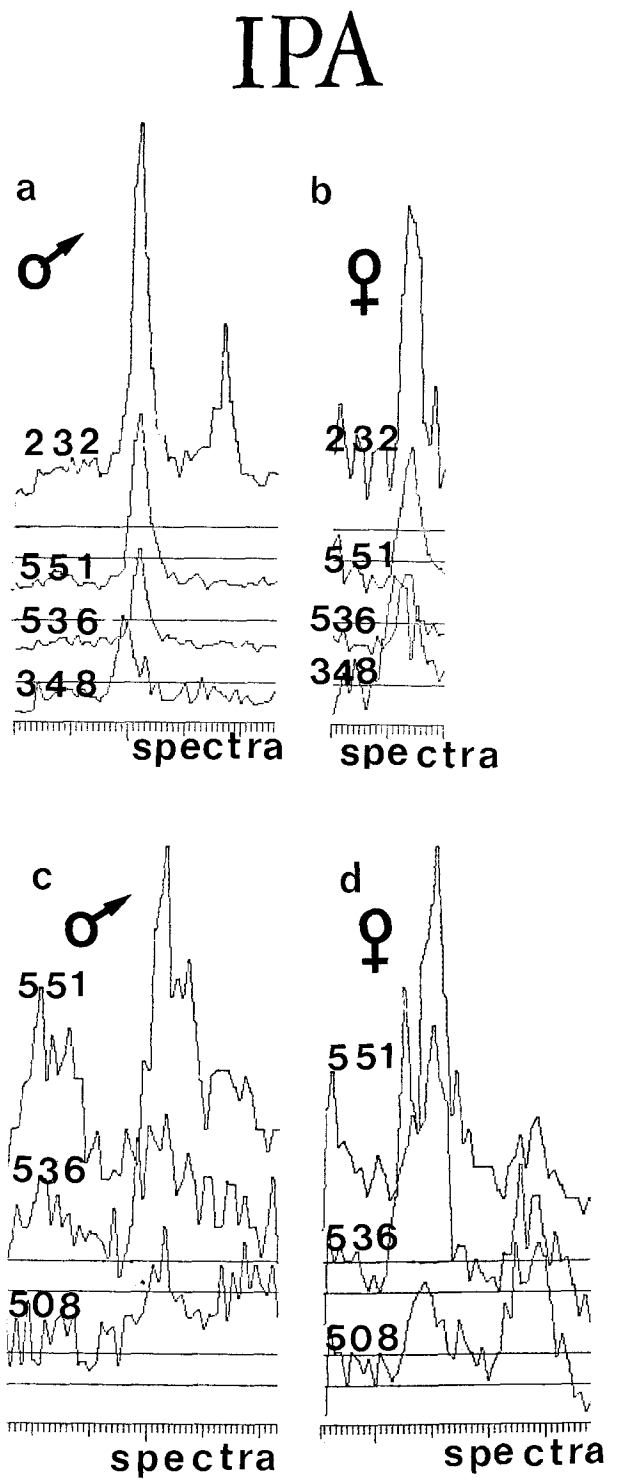


Fig. 1a–d. IPA detection. The curves shown are those of intensity of the characteristic ions of IPA in the different spectra recorded. **a** 250 g of ♂ apex, detection by the typical ions M/E 232, 551, 536 and 348, of decreasing intensity. **b** 250 g of ♀ apex, detection by the same ions. **c** 400 g of whole ♂ plants, detection by the ions M/E 551, 536 and 508. **d** whole ♀ plants, detection by the same ions. In the chromatographic peaks obtained, the surface is proportional to the quantity of substance extracted: peaks of male extracts are larger and more regular than those of female extracts

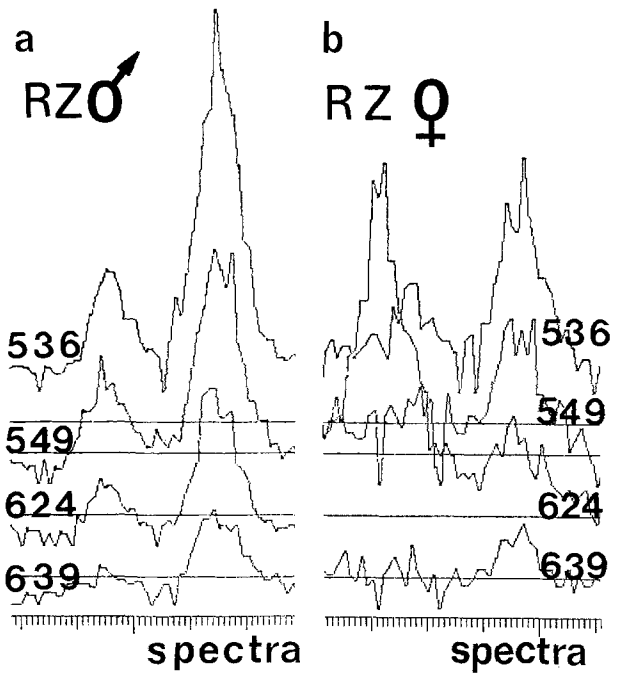


Fig. 2a and b. RZ detection. **a** 800 g of ♂ apex, ions of decreasing intensity M/E 536, 549, 624 and 639 (molecular ion). **b** 800 g of ♀ apex, detection by the same ions. As in the case of IPA, peak surfaces are greater in male extracts

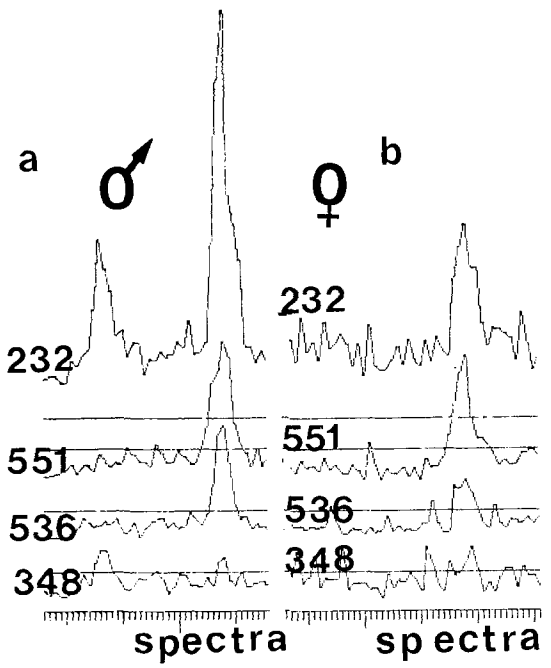
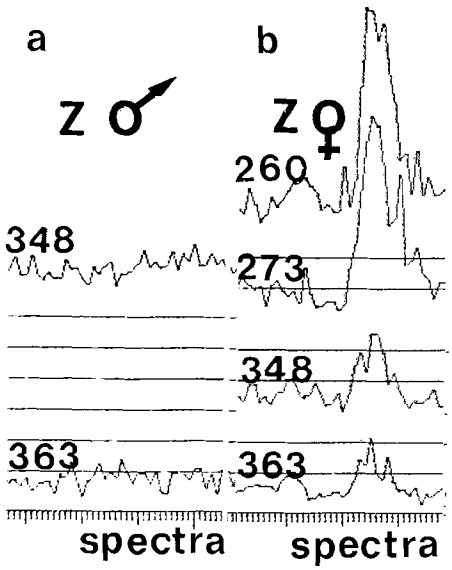


Fig. 4a and b. Detection of IPA in hydrolysates of I_6-Ade nucleotide. This was done by screening for IPA in the aqueous phase of the butanol extract after alkaline phosphatase treatment. **a**: sample of whole male plants, 400 g. **b** sample of whole female plants, 400 g. IPA was detected in all assays. Both male and female plants contain the nucleotide of I_6-Ade



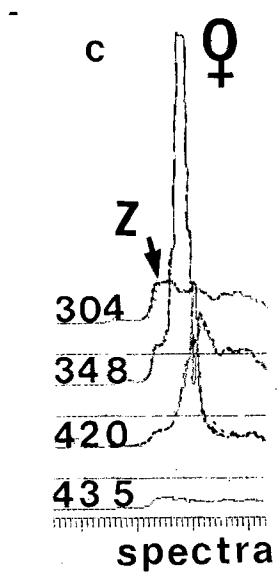


Fig. 3a–c. Identification of Z. **a** 250 g of ♂ apex. **b** 250 g of ♀ apex. The search for monotrimethylsilyl-zeatin by ions M/E 363 and 348 was negative in male extracts, and positive in female ones where it was performed by the ions M/E 260, 273, 348 and 363, of increasing intensity. The search for ditrimethylsilyl-zeatin by the ions M/E 420 and 435 (molecular ion) was negative. **c** 400 g of whole female plants. Only the search for ditrimethylsilyl-zeatin by ions M/E 304, 348, 420 and 435 was positive. Zeatin could not be detected in whole ♂ 0 plants

conventional graphical representation of the mass spectra.

Assays performed by comparing the intensities of a characteristic ion with that of a standard are shown in Tables 1–4. The quantities of IPA (Table 1) are always slightly greater in males, with a ratio varying from 3 for the apex to 1.5 for the whole plant. We

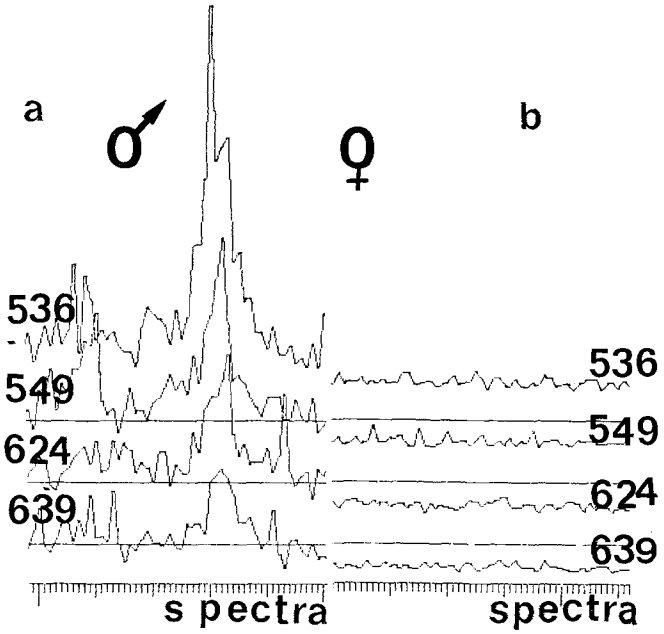


Fig. 5a and b. Detection of the nucleotide of Z. This was done by screening for RZ in the purified hydrolysates of the aqueous phase of the butanol extract. RZ was only detected in male extracts (**a** 400 g of entire male plants) but was undetectable in females (**b** 400 g of entire female plants)

Table 1. Assay of IPA

Material	Fresh weight	Absolute intensity of the M/E 551 ion		Calculated nmol/100 g fresh weight
		Extract	Standard	
Apex ♂	800 g	201	639 for	0.58
Apex ♀	800 g	68	5 μg	0.19
Apex ♂	250 g	906	3,438 for	0.62
Apex ♀	250 g	335	2 μg[a]	0.22
Whole plants ♂	400 g	767	4,149 for	0.34
Whole plants ♀	400 g	488	2.5 μg	0.21

[a] The sensitivity of ion source has been improved in the mean time

Table 2. Assay of RZ

Material	Fresh weight	Absolute intensity of the M/E 624 ion		Calculated nmol/100 g fresh weight
		Extract	Standard	
Apex ♂	800 g	55	182 for 5 μg	0.53
Apex ♀	800 g	19		0.18

detected slightly more RZ (Table 2) in males. These differences can be seen in the detection curves, where the riboside peaks are higher and more distinct in male extracts. The quantities of nucleosides measured with this method oscillated around 0.5 nmol/100 g fresh weight in males and 0.20 nmol/100 g fresh weight in females.

Table 3. Assay of Z

Material	Fresh weight	Absolute intensity of the M/E 348 ion		Calculated nmol/100 g fresh weight
		Extract	Standard	
Apex ♂	250 g	261	776 for	1.22
Apex ♀	250 g	0	2 μg	0

Material	Fresh weight	Weight of ion 348 peak after cutting		Calculated nmoles/100 g fresh weight
		Extract	Standard	
Apex ♂	800 g	390	450 mg	0.99
Apex ♀	800 g	0	for 2 μg	0
Whole plants ♀	400 g	Positive detection was obtained but impurities rendered assay impossible (Fig. 3c′)		
Whole plants ♂	400 g	Detection negative		

We could never detect nor assay zeatin in males. Its concentration in females was found to be about 1 nmol/100 g fresh weight (Table 3). The I_6-Ade nucleotides are present in both sexes at lower levels than the corresponding ribosides. Their concentration oscillated around 0.1 nmol/100 g fresh weight. Finally, the Z nucleotide was detected only in males at a similar concentration (Table 4).

All attempts to detect glycosides, especially the I_6-Ade glycoside, by characteristic M/E ions were negative in both male and female extracts. The same was true for I_6-Ade.

Discussion

The following conclusions may be drawn from our analyses of the endogenous pool of cytokinin metabolites. There exist certain metabolites common to both sexes: ribosides, whose concentration is slightly greater in males, and the nucleotide of I_6-Ade.

It is interesting to note that the results of the IPA assay performed with mass spectra are the inverse of those performed with bioassay data and gas chromatography alone (Kalhem et al. 1975). This is caused by the co-migration of parasite molecules on Sephadex LH 20 and on the short chromatography column with DC 11 silicone as the stationary phase. An IPA spectrum free from parasites could be obtained only with a 3 m column. This is not particular to *Mercurialis* extracts since, as stated by Summons

Table 4. Assay of nucleotides

1. Hydrolysates of I_6-Ade nucleotide

Material	Fresh weight	Absolute intensity of the M/E 551 ion		Calculated quantities of I_6-Ade (ng)	Calculated nmol of nucleotides /100 g fresh weight
		Extract	Standard		
Whole plants ♂	400 g	236	4149 for 2.5 µg	142	0.10
Whole plants ♀	400 g	376		226	0.16
Whole plants ♂	400 g	655	3438 for 2.5 µg	381	0.28
Whole plants ♀	400 g	201		141	0.1

2. Hydrolysates of Z nucleotide

Material	Fresh weight	Absolute intensity of the M/E 624 ion		Calculated quantities of RZ (ng)	Calculated nmol of nucleotides /100 g fresh weight
		Extract	Standard		
Whole plants ♂	400 g	120	784 for 1 µg	156	0.1
Whole plants ♀	400 g	0		0	0
Whole plants ♂	400 g	177	767 for 1 µg	230	0.17
Whole plants ♀	400 g	0		0	0

et al., "Bioassay method... are at best a crude approximation and could well be completely misleading" (Summons et al. 1978).

The unequivocal differences observed in the present work involve a free-base zeatin, detected only in females, and the corresponding nucleotide, detected only in males. If IPA is the basic species of cytokinin metabolism (Hall 1973), we may speculate by considering that two higher levels of enzyme activity, a hydrolase in females and a phosphokinase in males, could explain the accumulation of Z in females and its nucleotide in males.

Although the differential accumulation of nucleotide or free base indicates a quantitative difference in common metabolites, it is also possible that males contain a base which is similar to, but not identical to zeatin.

Only females of *M. ambigua* ($2n=48$) also accumulate Z (Dauphin et al. 1979). Regardless of the true explanation, female sex differentiation is associated with a high concentration of the free-base zeatin. This high concentration and the low level of its nucleotide are also related to the absence of two dominant complementary genes, AB_1 or AB_2, which determine the male character (Louis et al. 1978). When one of the two genes, A or B, is no longer dominant, the quantity of zeatin decreases.

In order to determine the mechanism of this control, it will first be necessary to determine the endogenous levels of various homozygotic male or female clones obtained from different allelic combinations between AB_1 and B_2. We may nevertheless add that these genes appear to act in all the tissues of the plant, since there is no apparent difference in the direction of cytokinin metabolism between the floral apex and the rest of the plant. It seems that the same is true in calluses of each sex *in vitro* (Champault et al. 1978).

The first indications we obtained on specific receptors (Chung et al. 1979) (higher binding constant for zeatin, sites in females probably saturated by endogenous zeatin), combined with data on endogenous metabolism, lead to the hypothesis that the active form of cytokinins involved in female sex differentiation of *M. annua* seems zeatin.

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Received 16 July; accepted 9 October 1979