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Cytokinins and Differentiation Processes in *Mercurialis annua*

Genetic Regulation, Relations with Auxins, Indoleacetic Acid Oxidases, and Sexual Expression Patterns

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ABSTRACT

Cytokinins in apices of eight isogenic lines of *Mercurialis annua* were compared (high performance liquid chromatography-gas chromatography mass spectroscopy-computer system). These apices develop normal staminate or pistillate differentiation processes (sex series lines) or empty (sterile), semiempty (semisterile), and full anthers (restored fertile male) in the sterility series in which a pistillate line was constructed. Both series developed two different cytokinin pathways: *trans*-cytokinins characterized the sex series, whereas the *cis* pathway characterized the sterility series. Drastic changes in the *trans* pathway (0/250 nanograms *trans*-zeatin and 166/0 nanograms zeatin nucleotide) induced staminate/pistillate differentiations. Less drastic quantitative changes in the *cis* pathway induced sterility or restored fertility compared to normal fertile anthers (192 or 669 nanograms/traces). The action of the complete *cis*-pathway was morphologically effective in the sterility series when the ratio of *cis* to *trans* pathways was 1:2 or 1:1 instead of 1:3. A final diagram shows the action of each sex or sterility allele on the enzymes controlling specific metabolites in both pathways. The discussion provides insights on the regulation of cytokinin-auxin balances specific for each kind of reproductive differentiation.

Cytokinins, in combination with auxins, are generally believed to play regulatory roles in several differentiation processes. One accepted developmental effect of cytokinins is the induction of pistillate flowers on genetically male individuals of several dioecious plants, such as certain species of *Vitis* (24), *Mercurialis* (8), *Spinacia* (1), or *Cannabis* (9). This differentiation process results from external applications of synthetic hormones, so that numerous endogenous steps, uptake mechanisms, transport, metabolic transformations, or tissue responses to the exogenous compound are incompletely or not at all understood. To avoid these difficulties, and with the possibility offered by cloned phytohormone biosynthetic genes of *Agrobacterium tumefaciens,* it has been possible to transfer cytokinin genes to alter directly the endogenous cytokinin metabolism of transformed plants and to observe the effects of added genes on growth and differentiation. The transfers only resulted in increased levels of endogenous cytokinins of transgenic species, with no alteration in the programmed differentiation pattern (22). A major interest of the experiments reported here was to identify clearly the cytokinin-dependent phenomenon.

Direct and unambiguous measurements of cytokinin metabolites at the developmental site of a cytokinin-dependent process may allow the determination of the key metabolite necessary for activation of the process. This analytical strategy is complicated by the fact that numerous cytokinin metabolites have been recognized. Data furnished by the different species widely separated in the plant kingdom for which cell growth, rather than organogenesis, was studied are variable (15, 21). If a cytokinin-dependent differentiation process can be correlated with genes controlling it or, in other words, if studies are performed on mutant plants affected in their response or requirement for cytokinins, new information about mode of action and regulation of biosynthesis pathways can be obtained from comparative measurements.

In *Mercurialis,* several lines of evidence have demonstrated the involvement of cytokinins in the final floral phenotype of flowers. Exogenous b^6Ade[1] or furfurylaminopurine (f^6Ade) supplemented to genetically male individuals induced feminization of newly appearing floral *initia*, resulting in the formation of pistillate instead of staminate flowers (8). Direct evidence of the role of cytokinins in inducing floral phenotype has been furnished by cytokinin measurements at the site of staminate and pistillate floral differentiations: the shoot apices of male and female plants. Zeatin, the specific metabolite for expression of femaleness, could not be detected in measurable quantity as a free base in male apices. Zeatin nucleotide rather than the free base accumulated in male apices (7).

To correlate various cytokinin metabolites with flower phenotypes and to attribute to the genes controlling these phenotypes their probable role in cytokinin pathways, cytokinins from the apices of several strains from *M. annua* were identified and measured using HPLC/GC/MS and deuterated standards. These constructed lines (19) vary in sexual phe-

[1] Abbreviations: b^6Ade, benzyladenine; f^6Ade, furfurylaminopurine; i^6Ade, isopentenyladenine; c-io^{6}Ade and t-io^{6}Ade, *cis*- and *trans*-zeatin; i^6Ado, isopentenyladenosine; c-io^{6}Ado and t-io^{6}Ado, *cis*- and *trans*-zeatin riboside; i^6AMP, isopentenyl nucleotide; io^{6}AMP, zeatin nucleotide; GC/MS/SIM, gas chromatography, mass spectrometry, selected ion monitoring.

Table I. *Selected Genotypes for Cytokinin Measurements (HPLC/GC/SIM/MS)*

Strains	Phenotype	Genotype for		Cytoplasm[b]	Effect of
		Sex	Male sterility[a]		
Sex series[c]					
Strong male K_{2-2}	Staminate-fertile Resistant to f^6Ade b^6Ade feminization	A/A, B_1/b_1, B_2/b_2	?	N	3 genes for maleness A + B_1 + B_2
Moderate male O_{-4}	Staminate-fertile 30% feminized by f^6Ade	A/a, B_1/B_1, b_2/b_2	?	N	2 genes for maleness A + B_1
Weak male L_{6-6}	Staminate fertile 100% feminized by f^6 Ade	A/A, b_1/b_1, B_2/B_2	?	N	2 genes for maleness A + B_2
Female N_1	Pistillate-fertile Masculinized by IAA (*in vitro* cutting)	A/A, b_1/b_1, b_2/b_2	?	N	Gene A for femaleness
Male sterility series[d]					
Total sterile male 10_{-2}	Staminate-empty anthers	A + B	I/I, r_1/r_1, r_2/r_2	S	Gene I for sterility
Semisterile male 2	Staminate 30% fertile pollen	A + B	I/i, R_1/r_1, r_2/r_2	S	Gene R_1 for partial restoration
Restored fertile male 12_{-6}	Staminate 100% fertile pollen	A + B	I/I, R_1/R_1, R_2/R_2	S	Genes R_1 and R_2 for total restoration
Female 19_{-5}	Only pistillate-fertile, masculinized by IAA *in vivo*	A/A, b_1/b_1, b_2/b_2	I/I, R_1/R_1, R_2/R_2	S	I + R_1 + R_2 + S compared to N_1 A compared to restored male 12_{-6}

[a] ?, unknown. [b] N, normal cytoplasm; S, sensitive cytoplasm necessary for sterility expression. [c] All the strains with same genetic background. [d] All the strains with same genetic background, the male strains of this series are entirely feminized by b^6Ade, a strong cytokinin, f^6Ade, or kinetin, a moderate cytokinin.

notype (fertile staminate, pistillate, sterile or semisterile staminate, etc.) and in response to exogenously supplied cytokinin and auxin (3). The results show that these lines also vary qualitatively and quantitatively in their cytokinin content as a function of regulator genes controlling the sexual phenotypes. These lines represented 'mutant plants' in which mutations clearly affect cytokinin metabolism. The work also provides a more complete view on the cytokinin-auxin regulation of sexual differentiation processes in these isogenic genotypes.

MATERIALS AND METHODS

Material

The various lines of *Mercurialis annua* selected for the experiments were constructed by Louis (19). The descriptions of genotypes and corresponding phenotypes as well as their phytohormonal requirements are summarized in Table I. The shoot apices (0.5 cm) were excised and then frozen immediately in liquid nitrogen.

Chromatographic material was purchased from Merck (Lichrosorb-diol column, particle size 5 μm diameter), solvents (degassed before use) from Carlo Erba, and alkaline phosphatase from Sigma.

Methods

Extraction and Purification Procedures

Extraction and purification procedures were first described by Dauphin-Guerin *et al.* (7) for i^6Ado, io^6Ado, io^6Ade, and i^6Ade and by Palni *et al.* (26) for nucleotides. Free cytokinins were removed by water-saturated butanol from ethanol (80%) extracts of fresh apices (60 g) previously purified by chromatography on Sephadex SPC 25 and then separated on Sephadex LH20. Although free i^6Ade was never detected in *Mercurialis,* the method proved to be effective in detecting this free base metabolite in *Fragaria.* Deuterated io^6Ado and io^6Ade were added to the crude extract. Following the separation of fractions on Sephadex LH20, another purification step by HPLC was added, using a Spectra-Physics S.P. 8700

ternary solvent delivery system equipped with a model 7125 Rheodyne injector valve. Reverse-phase HPLC was performed on lichrosorb-diol columns (250 × 4.5 mm); mobile organic phase, isocratic system: methylene chloride/methanol/cyclohexane (i^6Ado: 34/6/60; io^6Ado: 28/12/60, io^6Ade: 40/10/50); flow rate: 1 mL/min^{-1}; injection: 70 μL; pressure: 2200 psi; detection: A (A_{254}) (Analytical UV detector Altex 153). After the separation step in butanol/aqueous phase, the nucleotides remained in the aqueous phase. After nucleotide concentration to 1 mL, alkaline phosphatase hydrolyses were performed and the liberated nucleosides were also separated on Sephadex LH20. The hydrolysates were purified by HPLC.

GC/MS/Computer System

The mass spectrometer was an LKB 9000 S instrument coupled with a Digital 11-24 computer by LKB system 2130 (EI: 70 eV; filament current: 60 μ A; HV: 3500 V; source temperature: 250°C; Ross injector, moving needle). GC was performed on a semicapillary column (diameter 0.53 mm, length 10 m) (Macrobore [2 μm thickness] grafted SE 30 silicon); vector gas: helium; temperature program: from 180 to 230°C for io^6Ade, 2°C/min rate; 220 to 270°C for io^6Ado, 3°C/min rate; 205 to 270°C for i^6Ado, 3°C/min and 160 to 250°C, 2°C/min rate for i^6Ade). During the chromatographic run, a spectrum was recorded onto a magnetic disk every 4 s. The frequency curve and intensity of the characteristic ions of each metabolite lead to identification and measurement. Values were obtained by the classical method (comparison with the intensities of characteristic ions of a standard consecutively run) for io^6Ado and i^6Ado from nucleotide hydrolysates. The isotopic dilution method was used in the case of free cytokinins of isogenic lines (see ref. 10 for calculation). All measurements performed with these more effective methods agree with prior ones (7) taking into account the losses due to further HPLC purification. These data prove the reliability of purification procedures and strengthen our conclusions on cytokinin levels in the various lines.

RESULTS

In all the lines used in these experiments (Table I), comparative measurements of free bases (i^6Ade, c-io^6Ade, and t-io^6Ade) and cytokinin ribosides (i^6-Ado or c-io^6Ado and t-io^6Ado) were performed. Because t-io^6AMP and i^6AMP had been previously measured (7), i^6Ado and *cis* and *trans* forms of io^6Ado resulting from alkaline phosphatase hydrolysis of corresponding nucleotides (26) were also compared in wild male and wild female fertile plants. These last measurements were performed to confirm the specificity of zeatin nucleotide for male shoot apices.

Fertile Staminate Shoot Apices: Correlation with *trans*-Zeatin Nucleotide Content

The most important characteristic of fertile male lines (Table II) was the absence of measurable quantities of free bases *t*-io^6Ade or i^6Ade. The specific presence of *trans*-zeatin nucleotide in wild male apices confirmed results already obtained with less effective methods such as GC with noncapillary columns and without prior purification by HPLC (7). On the selected ion chromatograms of wild males, small quantities (one-third) of c-io^6AMP appeared along with large quantities of *trans*-compounds (166 ng/100 g fresh weight). i^6AMP was also present (10 ng).

Free nucleosides (i^6Ado; *cis*- and *trans*-io^6Ado) were always

Table II. *Measurement of Current Cytokinin Metabolites in Shoot Apices of the Various Lines Selected in Mercurialis annua (ng/100 g Fresh Weight)*

Metabolites	Fertile Staminate Flowers				Staminate Flowers 'Sterile Series'[a] A + B + S			Pistillate 'Sterile Series'[a] A + S	Normal Pistillate Flowers	
	Wild ♂	Strong[b]	Moderate[b]	Weak[b]	Restored fertile	Semisterile	Sterile	Female 19_{-5}	Female N_1[b]	Wild ♀[c]
	A + B	$A + B_1 + B_2$	$A + B_1 + b_2$	$A + b_1 + B_2$	$I + R_1 + R_2$	$I + R_1 + r_2$	$I + r_1 + r_2$	$I + R_1 + R_2$	A	A or B
ng/100 g fresh shoot apices										
i^6Ado	.[d]	170	112	103	106	51	32	14	33	.
i^6AMP	10	.	.	.	.	.	.	.	.	35
c-io^6AMP	66	.	.	.	.	.	.	.	.	.
t-io^6AMP	166	.	.	.	.	.	.	.	.	0[e]
c-io^6Ado		48	30	35	225	95	106	12	15	.
t-io^6Ado		147	105	92	404	118	133	33	43	.
c-io^6Ade		+[f]	+	+	669	178	192	18	+	.
t-io^6Ade	0	0	0	0	30	0	0	214	250	.
IAA[c]	107	175	156	94	200	117	81	132	31	31
IAA-oxidases[g]		309	395	456	370	509	627	457	751	

[a,b] Isogenic background. [c] Ref. 10. [d] Not determined. [e] Not detected. [f] Nonmeasurable traces. [g] Ref. 11. Measurement of methylene oxindole appearance (ng min^{-1} μg^{-1} protein). Although the method allows the detection and measurement of i^6Ade, this metabolite has not been detected in *Mercurialis*.

present in the three male lines with decreasing quantities in the order: strong male (3 genes for maleness A + B1 + B2), moderate male (2 dominant alleles A + B1), and weak male (A + B2, B2 weaker than B1). The ratios between the *cis* and *trans* forms was 1:3 for the three lines. These results, now obtained with more efficient methods, also showed the detection of traces of *cis*-zeatin in the three males lines while *trans*-zeatin was never measurable. Present data confirm that io^{6}AMP is the specific metabolite for maleness (0 ng in females) and show the role of alleles controlling male strength B1 and B2, in modulating riboside quantities.

Fertile Pistillate Shoot Apices (Female N_1): Correlation with *trans*-Zeatin Content

As shown in Table II, no *trans*-zeatin nucleotide was measurable in the wild female, whereas 35 ng/100 g fresh weight of i^{6}AMP were obtained. The most important result from measurements on the female line was the great quantity of *trans*-zeatin (250 ng/100 g fresh weight); traces of the *cis* form were also present. *Trans*-zeatin, therefore, constitutes the specific cytokinin for femaleness, also confirming our previous results on the activity of the free base for pistillate differentiation (7). Ribosides were also present one-third i^{6}Ado and one-half to one-third *t*- or *c*-io^{6}Ado compared to male lines. The ratio of *cis* to *trans* forms remains 1:3 as in male lines.

The presence of the dominant A gene and the recessive state of b_1 and b_2 can be correlated with the presence of *trans*-zeatin and with the absence of t-io^{6}AMP.

Shoot Apices of Staminate Lines of the 'Sterility Series': Correlations with *cis*-Zeatin, *cis*-Zeatin Riboside, and *cis*-Zeatin Nucleotide Content

Table II shows that the absence of *trans*-zeatin in shoot apices of sterile (10_{-2}) and semisterile strains (-2-) conforms to results obtained for all normal fertile male lines. A low quantity (30 ng/100 g fresh weight) was present in the restored fertile male strain 12_{-6}. Surprisingly, the three staminate lines of the sterility series contained large variable amounts of *cis*-zeatin (Fig. 1A), a metabolite of which only traces were found in fertile male strains. *c*-io^{6}Ade quantities decreased in the order: restored fertile male (669 ng), sterile (192 ng), and semisterile male (178 ng). They are linked to the number of active restorer genes in the dominant state R_1+ R_2 in the restored fertile, to R_1 in the semisterile male, but to only sterility inducer gene I in the sterile male line in which genetics demonstrated the inactivity of R_1 when alone (17). Riboside quantities (i^{6}Ado and t-io^{6}Ado) follow approximately the same order. It is important to note that *c*-io^{6}Ado quantities are three- to fourfold higher in the staminate flowers of the sterile series than in the fertile series. The ratio of *cis*- to *trans* forms was completely changed compared to fertile lines. The restored fertile strain always contained the highest amount of c- or t-io^{6}Ado. In the restored male strain, a small quantity of *t*-io^{6}Ade (30 ng) metabolite generally specific for pistillate flowers was measured. This unexpected metabolite was greatly made up for by the very large quantity of c-io^{6}Ade (669 ng) probably explaining the absence of female flowers on this line.

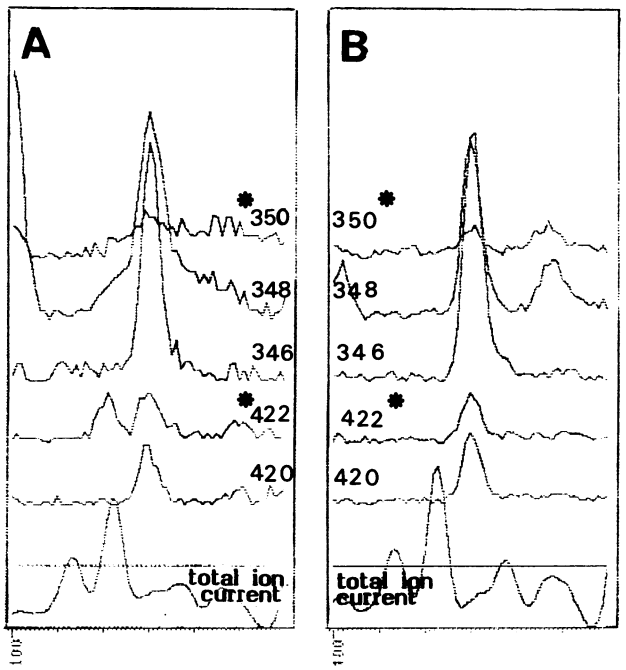

Figure 1. Selected ion chromatograms of *c-io*^{6}Ade of the restored fertile male line 12_{-6} (A) and of *t*-io^{6}Ade of the pistillate line 19_{-5} (B). The deuterated (marked by an asterisk) and nondeuterated ions characteristic for io^{6}Ade are present. The intensities of the characteristic ions are in agreement with the expected ratios of the fragmented standards. The intensities of nondeuterated ions correspond not only to io^{6}Ade extracted from plants but also to the nondeuterated io^{6}Ade arising from the standard.

Shoot Apices of the Pistillate Line 19_{-5} with Male Sterility Determinants: Presence of Free Bases *cis*- and *trans*-Zeatin

The female line 19_{-5} is an interesting isogenic line containing the gene A inducing femaleness as the normal female N_1, the male sterility inducer I, and both fertility restorers in S cytoplasm, as in the restored fertile male 12_{-6} of the preceding paragraph. Cytokinin metabolites of this line reflected the characteristic genes it contains: as a true female, it contains *t*-io^{6}Ade (Fig. 1B) with less quantities (214 ng *versus* 250 ng/100 g fresh weight), but it also contains a low quantity of *c*-io^{6}Ade as the restored male (18 ng *versus* 669 ng). Other metabolites, i^{6}Ado, *c*- and *t*-io^{6}Ado, were in the region of the true female strain N_1. We note that the ratio of *c*-io^{6}Ado to *t*-io^{6}Ado is 1:3 as in the normal fertile male lines.

DISCUSSION

Relation of Reproductive Differentiations to Specific Cytokinin Metabolic Pathway and to Specific Metabolite in Each Pathway

Two series of successive differentiation processes were examined in these experiments: (a) the staminate-pistillate differentiation and (b) the differentiation of full anthers on staminal filaments of normal and restored fertile male lines, or of empty anthers in male sterile flowers. Fertility (normal

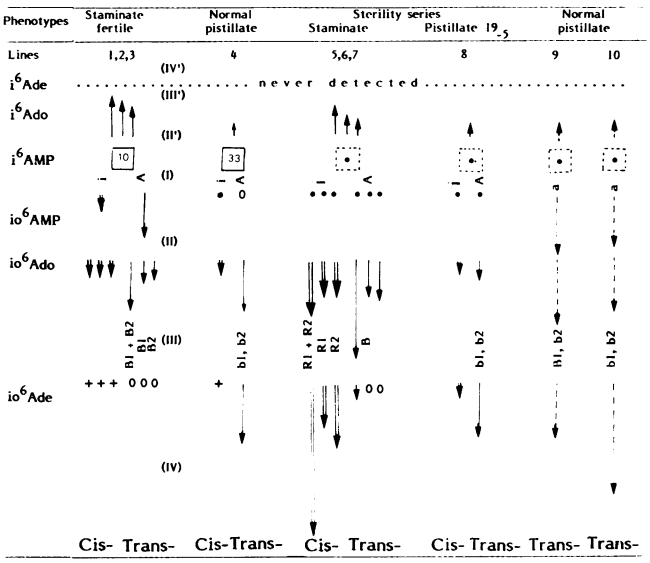

Figure 2. Diagrammatic interpretation of cytokinin metabolite measurements on the various strains of *Mercurialis annua*. Double-lines, *cis-pathway*; simple lines, *trans*-pathway; dotted lines, anticipation on the female strains containing one B gene or all the sex genes recessive; 1, 2, 3, strong, moderate, and weak fertile male lines; 4, female line with gene A in the dominant state; 5, 6, 7, staminate lines of the male sterility series (restored fertile, 5; semisterile, 6; and sterile, 7 males); 8, pistillate female line 19_{-5} containing the male sterility determinants; 9, 10, female lines a, B_1, b_2, and a, b_1, b_2; I, II, III, IV and II′, III′, IV′, enzymes for cytokinin interconversions; I, hydroxylation of isopentenyl sidechain; II, 5′-ribonucleotide phosphohydrolase; III, adenosine and io^{6}Ado deribohydrolase; IV, cytokinin oxidase. These activities, not measured or evidenced in this work, were only deduced from the general features of the endogenous cytokinin metabolism, rendering this diagram hypothetical. The action of the six loci for sex and sterility were also deduced from the results. (The lengths of the various arrows are proportional to metabolite quantities; ., not measured.)

or restored) is morphologically expressed by anthers producing 100% fertile pollen grains while sterility corresponds to anthers deprived of pollen.

Table II and Figure 2 clearly show that two different cytokinin pathways occur in both successive processes. The *trans*-cytokinin pathway is linked to male-female differentiation. The observation of staminate-pistillate columns of the sex series (1, 2, 3/4; Fig. 2) and staminate-pistillate 19_{-5} columns of the sterile series (5, 6, 7/8; Fig. 2) clearly lead to this conclusion. On the other hand, the *cis*-cytokinin pathway is found in anthers of staminate flowers of the sterility series as well as in normal staminate flowers of the sex series. The complete *cis*-pathway including *cis*-zeatin also exists in the pistillate line 19_{-5}, which contains all the sterility determinants (I R_1 R_2 + S) besides gene A for femaleness.

To obtain drastic changes in organs, *i.e.* stamen-pistil, the characteristic pathway has to develop drastic changes in its metabolites, *i.e.* 0 (staminate) to 250 ng (pistillate) *trans*-Z or 166 (male) to 0 ng (female) *trans*-zeatin nucleotide. Less drastic morphological changes, such as empty-full anthers, are induced by less important quantitative changes (669 [restored fertile] to 192 ng [sterile] or traces [normal fertiles] to 192 ng [sterile males] c-io^{6}Ade). In other words, *cis* forms of cytokinins (and particularly *c*-io^{6}Ade) are linked to anther sterility or restored fertility (or normal fertility) as a function of their quantity. The action of the complete *cis*-pathway is morphologically effective when the ratio of *cis* to *trans* becomes significant (normally 1:3 for io^{6}Ado in males *versus* 1:2 or 1:1 in sterility series). Pathway *cis*, however, remains in a background level in all strains.

The direct use of *c*- or *t*-io^{6}Ade sprayed on the weak male line $L_{6\text{-}6}$ confirms the physiological measurements of both pathways (Fig. 3). Used at the same concentration, for the same time, *c*-io^{6}Ade induced stamen sterility in anthers of newly developed primordia: the pistillate state is not reached, whereas *t*-io^{6}Ade induces pistillate instead of staminate floral primordia.

Genetic Regulation of Cytokinins: Sex Genes for *trans* Pathway Sterility Determinants for *cis* Pathway

From the physiological, morphological, and genetic data, the following conclusions can be drawn: (a) sexual differentia-

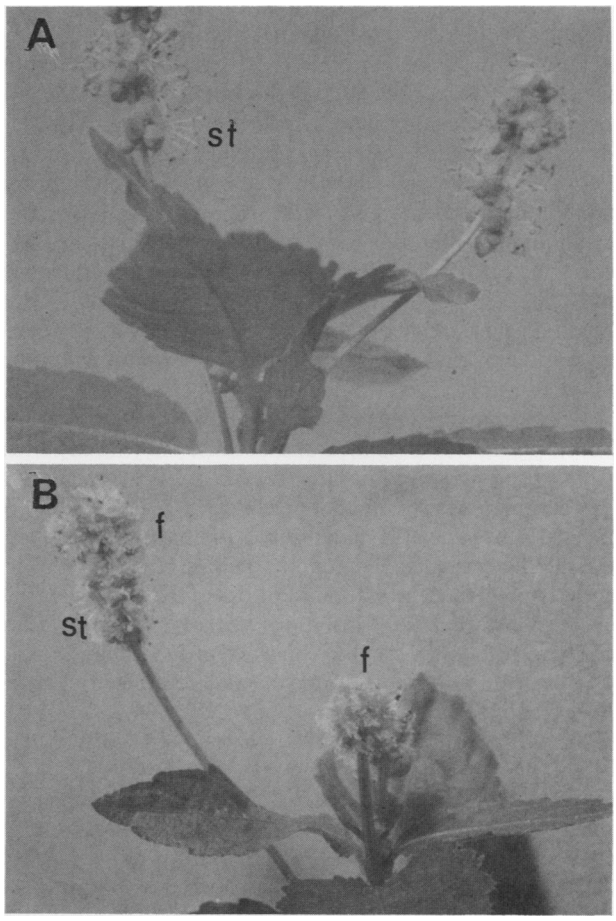

Figure 3. *c*-io^{6}Ade (A) and *t*-io^{6}Ade (B) used on the weak male line $L_{6\text{-}6}$. Sprayed *in vivo* for a same period, the *cis* form only induced sterile stamens (st) while the *trans* form induced sterile stamens at the base of the first inflorescence while the second is entirely female (f).

tion (stamen-pistil) determined by allelic combinations of sex genes is linked to *trans*-cytokinins, (b) the *cis*-pathway revealed by measurements of stamen fertility-sterility is regulated by genes acting on anther-sterility (sterility determinants). Only the allelic combinations of these six loci in both isogenic backgrounds differ between the various lines. Two requirements, however, must be fulfilled in alloting to these genes a role in their respective pathway. First, the resulting model must satisfy normal enzymic requirements. Second, it must be able to predict the morphologies of the 64 genotypes selected (19) and for which no measurements are available. For example, *t*-io^{6}Ade is considered as the specific metabolite for femaleness induction on the genotype A/A,b_1/b_1,b_2/b_2. The possibilities of obtaining pistils on female genotypes a/a,B_1/B_1,B_2/B_2 or a/a,b_1/b_1,b_2/b_2 must be anticipated.

The general feature of cytokinin metabolism (14, 16, 21, 28) can be summarized as follows: the synthesis of i^{6}AMP from adenine nucleotide is now considered as the first step of metabolism; this step is followed by an oxidation reaction forming io^{6}AMP. Cytokinin ribosides and free bases follow from nucleotides as plausible cytokinin pathways, zeatin being the active form. The enzymic interconversion of these metabolites with activities of adenosine nucleosidase (III and III′, Fig. 2; deribosylation of ribosides to free bases) and 5′-ribonucleotide phosphohydrolase (II and II′, Fig. 2; nucleotide dephosphorylation to ribosides) have been shown in crude plant extracts (4, 5). i^{6}AMP and the isomers of io^{6}AMP are known in higher plants as in *Mercurialis*. The step at which hydroxylation of the isopentenyl side chain occurs to produce the zeatin pathway, and whether the enzymes involved in the formation of zeatin *cis*- and *trans*-isomers are stereospecific, are still unclear but the conversion of i^{6}AMP to io^{6}AMP isomers (I, Fig. 2) has been demonstrated in several plant tissues (16, 23).

Based on this knowledge and our measurements, we therefore consider as plausible the genetic regulation of cytokinin pathways implied in reproductive organogeneses of *Mercurialis*. Because no enzyme acting in cytokinin metabolism has yet been purified and isolated and because enzyme activities have not been measured in our lines, the model presented in Figure 2 remains partly hypothetical.

I. i^{6}AMP to i^{6}Ado. We never found any trace of free i^{6}Ade. Although the existence of cytokinin oxidase (IV and IV′, Fig. 2) rapidly oxidizing the sidechain of bases or nucleosides has been demonstrated (20, 25), this type of oxidase appears unlikely to be active here: the existence of io^{6}Ado and i^{6}Ado or of traces of *c*-io^{6}Ade (example of male fertile strains) goes against this hypothesis.

II. i^{6}AMP to *c*- or t-io^{6}AMP then to *c*- or *t*-io^{6}Ado and finally to the corresponding free bases.

Concerning the question of where exactly the three sex genes and the three sterility genes act, the answer is that these genes probably act to produce the differential pathway linked to each successive differentiation process and that each of these genes acts in both pathways at the level of differential metabolites. Gene i (in cytoplasm N) and gene I (in S cytoplasm) probably act to produce the *cis*-pathway. The action of gene I is effective after the induction of stamens due to the joint action of A on enzyme (I) to produce the *trans*-zeatin nucleotide isomer, plus B_1, B_2 or B_1 + B_2 on enzyme (III) in fertile males (1, 2, 3; Fig. 2). In the recessive state in females (4, 8, 9, 10; Fig. 2) genes b produce a very active deribosylation of *t*-io^{6}Ado resulting in increased levels of *t*-io^{6}Ade and decreased levels of *t*-io^{6}AMP. In the *cis*-pathway, gene I probably forms *cis*-zeatin nucleotide and the action of R_1-R_2 (parallel level of action than B_1-B_2) actively transforms *c*-io^{6}Ado into *c*-io^{6}Ade. The similarity between *t*-io^{6}Ado and i^{6}Ado contents in strong, moderate, and weak males (1, 2, 3; Fig. 2) suggests that the 5′-ribonucleotide phosphohydrolase (II and II′) is not specific and indifferently converts both i^{6}AMP and io^{6}AMP to their respective ribosides at the same rate. In contrast, only *t*-io^{6}Ado is converted to *t*-io^{6}Ade in the female line (4, b_1, b_2; Fig. 2), whereas i^{6}Ade is never detected: the ribohydrolase(s) (enzymes III and III′) seem(s) very specific. The same is true for the same metabolic level in the sterile series: *c*-io^{6}Ade only appears in the sterile series, whereas the *c*- and *t*-io^{6}Ado contents are similar. Nevertheless, no one metabolic pathway is totally separate from another: the various morphogeneses result from ratios between isopentenyl nucleotide pathway, *cis*- and *trans*-pathways.

The diagram (Fig. 2) can explain the morphologies of all other genotypes. The measurements of *c*- and *t*-io^{6}AMP in the lines of the sterile series and those of metabolites in female lines possessing the gene a in the recessive state (now in our collection) would confirm these findings.

The present results, however, show the necessary intervention of new genes to produce the specific metabolites inducing organogeneses. This agrees with the results of Medford *et al.* (22): only transfer of these specific genes able to modify the endogenous cytokinin metabolites can induce hormone-dependent morphogeneses in transgenic plants.

Regulation of Auxin-Cytokinin Balances and Sexual Differentiation

When IAA contents of these isogenic lines were measured (10), only correlations had been shown between genes for maleness or for sterility and IAA. How can these correlations be explained? These IAA quantities and IAA-oxidase activities (11) are shown in Table II. The results clearly indicated that quantities of IAA and active forms of cytokinins (io^{6}Ade) are inversely proportional while IAA-oxidases and io^{6}Ade are directly related. Other data (12) suggested the action of io^{6}Ado on IAA-oxidase activities: when b^{6}Ade was used in feminization of male plants, IAA-oxidases increased; consequently, the decrease of IAA to the level found in female plants was observed. These results agree with those of Smigocki and Owens (29) who observed a decrease in IAA content when the cloned gene for isopentenyltransferase was transferred into tobacco.

These results show that sex genes can control cytokinin-auxin balances in reproductive organogeneses. These genes can be considered as major regulators controlling phytohormones in every cell of the whole plant and even in undifferentiated callus tissue strains of our genotypes (3). The various constructed strains constitute 'mutants' of phytohormone balances. The morphological differences between these lines run parallel to differences in the poly(A)$^+$ RNA populations closely related to the phytohormonal content (13, 27). Con-

sequently, the problem of mode of action of signals for specific organogenesis and specific genes remains to be elucidated.

Different cytokinin receptors with different binding constants have been identified in various cell compartments of male and female *Mercurialis* (nucleus, chloroplasts, ribosomes etc. [2, 6]). None of these receptors have been isolated, but their existence may explain the role of cytokinins in the coordinated expression of some nuclear and chloroplast genes (18) or the increase in transcription of some nuclear genes and the repression of some others in male plants feminized by b^6Ade (13, 27). If a few regulator genes act on hormone biosynthesis in all plant cells, and if the hormone acts as a signal controlling the expression of several genes by means of '*trans*-acting factor-receptors,' why only some cells react to these hormones in the developing plant still needs to be understood.

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