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Endogenous Gibberellins and Cytokinins in Spear Tips of *Asparagus officinalis* in Relation to Sex Expression

Thomas M. Ombrello[1] and Stephen A. Garrison[2]
Department of Horticulture and Forestry, Rutgers University, Cook College, New Brunswick, NJ 08903

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Abstract. **Endogenous gibberellins (GA) and cytokinins (CK) were extracted from asparagus (*Asparagus officinalis* L.) spear tips, purified, and determined by lettuce hypocotyl and amaranthus bioassays, respectively. There was no quantitative difference in GA-like activity between heterogametic male and female spears. The major GA fraction in asparagus spears has 1 OH group. Asparagus spears contain 3 major fractions of CK-like activity. Fraction 1 eluted from Sephadex LH-20 and C_{18} HPLC columns with or before zeatin-riboside. Fractions 2 and 3 eluted in a similar pattern to IPA-riboside and IPA, respectively. There were higher levels of CK fraction 2 and trends toward higher levels of fraction 1 and total CK in female than in heterogametic male spears. There were also higher CK:GA ratios in female than in heterogametic male spears. The data support the hypothesis that sex in asparagus is controlled in part by CK levels or by CK:GA ratios.**

A. officinalis is a dioecious plant in a genus that contains both monoecious and dioecious species (1). Flowers from female plants of *A. officinalis* have rudimentary stamens and show little variation in flower morphology (23). Male plants have flowers with rudimentary ovaries and exhibit variations both within and among plants for the relative development of ovaries and stamens (18, 23, 30). Andromonoecious plants occur in low frequencies and are being used by plant breeders to develop all-male cultivars of asparagus (18), because male plants produce higher yields (24, 29) and have greater longevity in the field (10, 32). The sexes also differ in foliar morphology, which may influence plant productivity (2).

The mechanism of sex determination at the metabolic level is not understood. However, application of plant growth regulators have been shown to modify sex expression in asparagus. Lazarte and Garrison (17) showed that a cytokinin, *N*-(phenylmethyl)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine (PBA), promoted the development of styles and ovaries in male plants when applied to emerging spears at the time flower buds were differentiating. Gibberellin A_3 promoted the development of the rudimentary stamen of the female flower.

The literature contains many examples of the relationship between endogenous plant growth substances and sex expression in plants (4, 6–8, 25). Endogenous GA was higher in male plants while endogenous CK was higher in female plants of *Cannabis sativa* and in *Spinacia oleracea* (16).

There is increasing evidence that the ratios of endogenous plant growth regulators are as important in the control of plant development as the absolute levels of hormones. For example, the CK of in vitro plant tissue cultures interacts with auxins in controlling root and shoot differentiation (26, 31); the mechanism of apical dominance apparently is tied to an antagonism between auxins and CK (28); a current hypothesis on the bud dormancy of temperate woody plants is based on a GA–abscisic acid balance (19); and there appears to be an auxin–ethylene relationship to stem and root growth (19).

The ability to control the sex expression of *A. officinalis* or other dioecious species would facilitate the incorporation of desirable traits into breeding lines and allow for the routine development of inbred lines.

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[1]Present address: Dept. of Biology, Union College, Cranford, NJ 07016.
[2]Associate Professor.

The objectives of this study were to measure the levels of endogenous GA and CK fractions at flower bud development in male and female asparagus spears and to determine possible relationships between hormone levels and sex expression.

Materials and Methods

Plant material. Three unrelated dioecious single cross hybrids of *A. officinalis* (Md 10 x 14, 44 x 22, and Md 1 x 277S) were selected for endogenous GA and CK analysis. The male parents (14, 22, and 277S) of these hybrids are heterogametic males and thus produce ≈50% females and 50% heterogametic males in the F_1 population. (J.H. Ellison, personal communication). The sex of each plant was identified, and spears from 4.5-year-old greenhouse-grown plants of Md 10 x 14 and 44 x 22, and field-grown plants of Md 1 x 277S were collected when they were 10–15 cm high. Spears of male and female plants of each hybrid were placed in separate polyethylene bags, immediately frozen, and maintained at −70°C until extraction. Just prior to extraction, the upper 5-cm section of each spear was excised and cut in half longitudinally. Fifty grams of spear tip halves were used for each GA extraction, and a 10-g composite of the other spear tip halves was extracted for CK. In each replication, GA and CK were extracted and determined within a cross on tissues from the same spears.

Extraction, purification, and detection of endogenous GA. Frozen spear tips were homogenized thoroughly for 1 min in cold 80% aqueous methanol (v/v) using 3 ml solvent/g of tissue. The slurry was stirred for 2 hr at 0° to 1°C, then vacuum-filtered. The tissue was re-extracted by stirring the residue for 2 hr with more cold 80% aqueous methanol (v/v) (3 ml solvent/g of tissue) at 0° to 1° and then vacuum-filtering the slurry. The methanol–water extracts were bulked along with 20 ml of 0.5 M phosphate buffer at pH 8.5 and evaporated to the aqueous phase under reduced pressure at 30°. The aqueous extract was adjusted to pH 8.5 and partitioned three times against half-volumes of hexane. The hexane phase was discarded and the aqueous phase was stirred with 100 mg·ml^{-1} of polyvinylpyrrolidone (PVP) at 0° to 1° for 30 min. The PVP was vacuum-filtered and washed with 0.5 M phosphate buffer at pH 8.5. The PVP was discarded and the aqueous extract was adjusted to pH 2.5 and partitioned three times against half-volumes of ethyl acetate. The aqueous phase was discarded and the ethyl acetate phase was washed with 1 half-volume of water. The ethyl acetate then was filtered through anhydrous sodium sulfate and evaporated to dryness under reduced pressure at 30°.

The residue was taken up in 5 ml 20% aqueous acetone (v/v) and loaded on a column of charcoal–celite (1:2, by weight). The column then was washed with 20% aqueous acetone (v/v), which was discarded, followed by 80% aqueous acetone (v/v), which was collected and evaporated to the aqueous phase under reduced pressure at 30°C. The aqueous phase was adjusted to pH 2.5 and partitioned three times against half-volumes of ethyl acetate. The ethyl acetate phase was washed with 1 half-volume of water, filtered through anhydrous sodium sulfate, and evaporated to 1 to 2 ml under reduced pressure at 30°.

The extract then was subjected to modified silicic acid column chromatography (21). The fractions were collected and dried under a stream of air and bioassayed directly using a modified version of the Frankland and Wareing (11) lettuce hypocotyl test employing *Lactuca sativa* 'Butter King'.

GA_3 was used to establish a standard curve, and GA activity of the extracts was expressed in nanograms of GA_3 equivalents.

Some extracts were fractionated further by HPLC and then assayed. The HPLC system consisted of a DuPont 841 Liquid Chromatograph with an air-operated hydraulic pump, fitted with a Waters Associates 3.9 mm (i.d.) × 30-cm μBondapak C_{18} reverse-phase analytical column. Appropriate fractions collected from the silicic acid column were bulked, air-dried, and taken up in 30% methanol in 1% aqueous acetic acid (v/v). The solutions were filtered through a Millipore AP prefilter and FH filter (0.5 μm) and then injected into the HPLC. Elution was accomplished by means of a gradient of methanol (30% to ≈90%) in 1% aqueous acetic acid (v/v) with a flow rate of 2 ml·min^{-1} and a pump pressure of ≈1.5 × 10^4 kPa. Two-milliliter fractions were collected, dried under a stream of air, and then bioassayed by the lettuce hypocotyl test.

Extraction, purification, and detection of endogenous CK. Frozen spear tips were homogenized thoroughly for 1 min in cold 70% aqueous ethanol (v/v) (3 ml/g of tissue). The slurry was stirred for 2 hr at 0° to 1°C, then vacuum-filtered. The tissue was re-extracted by stirring the residue for 2 hr with more cold 70% aqueous ethanol (v/v) (3 ml/g of tissue) at 0° to 1° and then vacuum-filtering the slurry. The ethanol–water extracts were bulked, adjusted to pH 2.5, and loaded on a column of Dowex-50W acidic cation exchange resin. The acidified extract was passed through the column followed by 200 ml of 70% aqueous ethanol (v/v), then 200 ml of water. The CK were eluted from the column with 100 ml 2 N ammonium hydroxide, followed by 100 ml 2 N ammonium hydroxide in 70% aqueous ethanol (v/v). The two ammonium hydroxide fractions were collected, bulked, and evaporated to dryness under reduced pressure at 50°. The residue was taken up in 2 ml of 35% aqueous ethanol (v/v) and loaded on a column [1.8 (i.d.) × 50 cm] of Sephadex LH-20. The column was eluted with 35% aqueous ethanol (v/v) and 10-ml fractions were collected. The fractions were dried under a stream of air and then bioassayed using a modified version of the *Amaranthus* betacyanin test (3, 22), employing seeds of inbred *Amaranthus caudatus* selected for seedling color uniformity. Zeatin was used to establish a standard curve and cytokinin activity of the extracts was expressed in nanograms of zeatin equivalents.

Some extracts were fractionated further by HPLC, then assayed. The HPLC system used in the CK separations was the same as that described above for the GA. Appropriate fractions eluted from the Sephadex LH-20 column were bulked, air-dried, taken up in a solution of 30% methanol in 1% aqueous acetic acid (v/v), filtered, injected, and eluted with the same solvent and gradient as described above for the GA. After air-drying, the fractions were bioassayed directly by the *Amaranthus* betacyanin test.

Extractions and determinations were replicated three times for each sex within each cross. The data were transformed prior to an analysis of variance (ANOVA), then converted to the antilog for presentation. Means were compared using the highest significant difference test.

Results and Discussion

Gibberellins. Four peaks of GA activity were observed and were numbered 1 to 4 in order of their elution from the silicic acid column. A representative elution pattern of endogenous and authentic GA from this column is presented in Fig. 1.

Attempts to fractionate the GA further by HPLC yielded results similar to that obtained from a silicic acid column. The order of GA elution from the HPLC was reversed, however, since the column was a reverse-phase type. Peaks 1 and 4 from the silicic acid column did not elute from the HPLC in sufficient

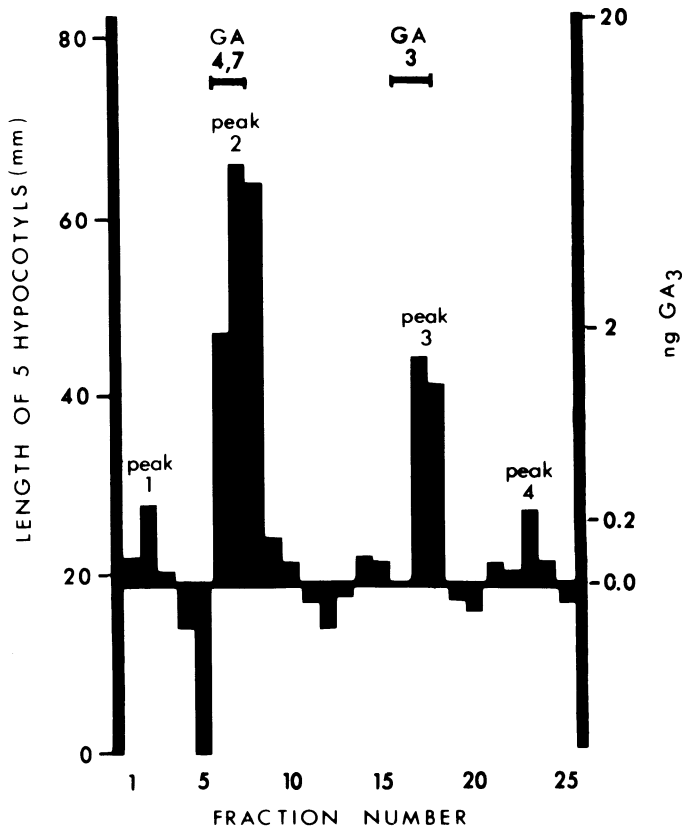

Fig. 1. Representative levels of gibberellins from 50 g (fresh weight) of female spear tips of *A. officinalis* (44 x 22). The elution patterns of authentic GA_3 and $GA_{4,7}$ and the bioassay response to known quantities of GA_3 are included.

quantities to allow the bioassay to respond. A representative elution pattern of endogenous GA and authentic GA from a C_{18} reverse-phase HPLC column is presented in Fig. 2.

Based on the known elution patterns of GA from silicic acid columns, peak 1 represents GA with no hydroxyl groups, peak 2 represents GA with one hydroxyl group, peak 3 represents GA with two hydroxyl groups, and peak 4 represents GA with three hydroxyl groups. It appears that a majority of the GA in the asparagus spear tips have one hydroxyl group and are similar in nature to GA_4 and GA_7. There are reduced levels (on the order of 6 times less) of GA with two hydroxyl groups that are similar in nature to GA_3. Only trace amounts of GA with either zero or three hydroxyl groups could be detected.

The results of the measurement of endogenous GA levels in the spears of three crosses of *A. officinalis* studied are presented in Table 1. Peaks 1 and 4 were quite small, while peak 3 was larger and peak 2 contained most of the GA activity. The three crosses did not differ in GA levels for peaks 1, 3, and 4. Peak 2 (the largest peak) did differ among crosses with 44 × 22 greater (at $P = 0.08$) than Md 10 x 14, with Md 1 x 277S intermediate and not differing from either of them. The total GA level did not differ significantly among crosses.

The differences in GA levels between heterogametic males and females were not significant in *A. officinalis;* this was true for the total GA content and for each of the four peaks. There were no significant interactions among crosses and sexes for GA levels.

Cytokinins. Three peaks of CK activity were noted and numbered 1 to 3 in order of their elution from the Sephadex LH-20 column. A representative elution pattern of endogenous CK and authentic CK from a Sephadex LH-20 column is presented in Fig. 3. Attempts to fractionate CK further by HPLC did not yield results different from those obtained from the Sephadex LH-20 column. The elution patterns were essentially the same, and this would be expected based on the work of Carnes et al. (5). A representative elution pattern of endogenous and authentic CK from a C_{18} reverse-phase HPLC column is presented in Fig. 4.

The authentic samples of zeatin riboside (Z-R), zeatin (Z),

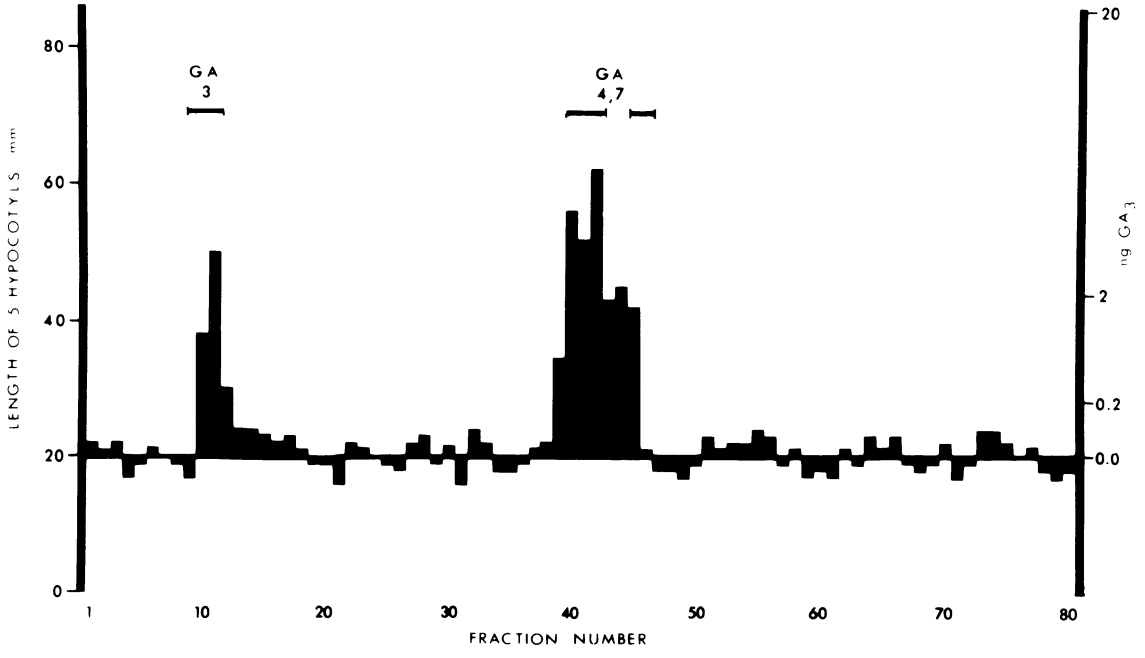

Fig. 2. Representative elution pattern of gibberellins from 50 g (fresh weight) of female spear tips of *A. officinalis* (44 x 22) from a C_{18} reverse-phase HPLC column. The elution patterns of authentic GA_3 and $GA_{4,7}$ and the bioassay response to known quantities of GA_3 are included.

Table 1. Endogenous gibberellin levels in *A. officinalis* spears.

Cross	ng GA_3 equivalents/100 g fresh wt of spear tips				
	Peak 1[z]	Peak 2	Peak 3	Peak 4	Sum of the four peaks
Md 10 x 14	0.5 a[y]	12.8 a	4.5 a	0.5 a	18.9 a
44 x 22	0.4 a	30.6 b	4.0 a	0.6 a	36.4 a
Md 1 x 277S	0.5 a	17.2 ab	3.9 a	1.0 a	22.6 a
Probability	>0.10	0.08	>0.10	>0.10	>0.10
Sex					
Female	0.5 a	15.7 a	3.7 a	0.7 a	21.3 a
Male	0.5 a	22.7 a	4.6 a	0.6 a	29.3 a
Probability	>0.10	>0.10	>0.10	>0.10	>0.10

[z]Individual peaks numbered in order of elution from a silicic acid column.

[y]Mean separation in columns by HSD test.

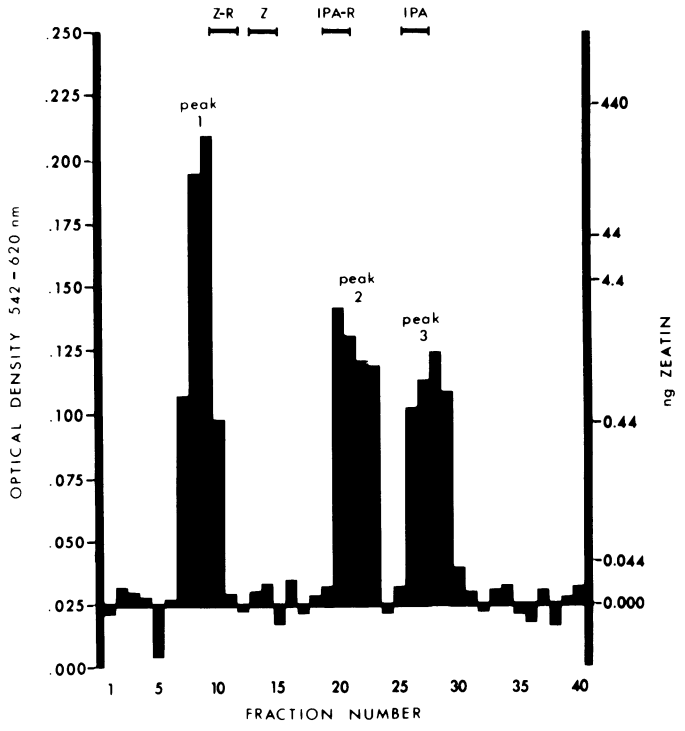

Fig. 3. Representative levels of cytokinins from 10 g (fresh weight) of female spear tips of *A. officinalis* (44 x 22). The elution patterns of authentic zeatin riboside (Z-R), zeatin (Z), N^6-(2-isopentenyl)adenosine (IPA-R), N^6-(2-isopentenyl)adenine (IPA), and the bioassay response to known quantities of zeatin are included.

N^6-(2-isopentenyl)adenosine (IPA-R), and N^6-(2-isopentenyl)adenine (IPA) that were passed through the Sephadex LH-20 column and the C_{18} reverse-phase HPLC column served as a basis for interpreting the elution pattern of the endogenous CK in asparagus. At best, the interpretation can be based only on the relative polarities of the unknown peaks in comparison to the standards. The comparison for CK is not as clear as for GA, where the elution pattern from silicic acid columns can be used to determine the number of hydroxyl groups on the endogenous unknowns and compare them to authentic GA.

The authentic CK eluted from both columns in the following order: Z-R, Z, IPA-R, IPA. This order was expected since, under the solvent and column conditions used, both the LH-20 and C_{18} columns resolve by reverse phase partitioning, with the more polar compounds eluting first. By comparing these standards to the elution of the endogenous CK, it can be concluded that peak 1 represents a CK more polar than Z-R. Peak 2 represents a CK similar in polarity to IPA-R, and peak 3 represents a CK similar in polarity to IPA. The methods used do not allow for more specific interpretations of the results.

It is interesting to note that other workers have found only a few plants with endogenous CK more polar than Z-R (5, 13, 15). Based on chemical analyses, Hewett (13) identified the CK more polar than Z-R in *Populus* as a CK glucoside. He suggested that this CK could be zeatin glucoside, which has been found in *Oryza sativa* stem exudate.

The levels of endogenous CK in the spears of the three crosses of *A. officinalis* studied are presented in Table 2. Without exception, peak 1 was the largest of the three peaks of CK activity, with peaks 2 and 3 being smaller and about equal to each other. The three crosses showed differences in CK levels for each individual peak and for the sum of the three peaks. Crosses 44 x 22 and Md 1 x 277S had the same levels of CK peak 1, but both had more peak 1 CK than Md 10 x 14 ($P = 0.07$). However, 44 x 22 and Md 10 x 14 had the same level of CK peak 2, but Md 1 x 277S had significantly more CK peak 2 than both of them ($P = 0.03$). Cross Md 1 x 277S had more CK peak 3 than 44 x 22, but both crosses had the same CK peak 3 levels as Md 10 x 14 ($P = 0.5$). The total CK levels (sum of three peaks) among the crosses differed the same as that for peak 1, with 44 x 22 and Md 1 x 277S the same, but both greater than Md 10 x 14 ($P = 0.08$). This pattern was expected, since peak 1 was the largest and contributed the most to total CK. These results indicate that there are important differences in CK levels among crosses of *A. officinalis*.

Females had significantly more CK than heterogametic males for CK peaks 1 and especially 2 ($P = 0.10$ and 0.05, respectively). There were no differences in CK levels between heterogametic males and females for peak 3. The total CK levels (sum of three peaks) were greater in the females than the heterogametic males ($P = 0.07$).

Cytokinin : gibberellin ratios. Using these data on endogenous GA and CK in *A. officinalis* spears at the stage of sex differentiation in the flowers, CK:GA ratios were calculated in two ways. First, the ratio of total endogenous CK content to the total endogenous GA content was generated, then the ratio of the largest endogenous CK peak (peak 1) to the largest endogenous GA peak (peak 2) was generated for each tissue analysis that was conducted. An ANOVA indicated the results of the two methods of calculating the ratios were the same (see Table 3).

The CK:GA ratios for the three crosses of *A. officinalis* showed a trend but did not differ significantly (HSD, 0.05) for either method of calculating the ratios. However, the CK:GA ratio in females was significantly higher than in heterogametic males (HSD, 0.05) for both methods of calculating the ratios. There were no significant interactions between crosses and sexes for either method of calculating the ratios.

These results indicate that the ratios of endogenous CK and GA in spears of *A. officinalis* vary according to the sex of the plant. Peak 1 of the CK and peak 2 of the GA made significant contributions to these different ratios. In view of the data presented in Tables 1 and 2, CK has more influence on the CK:GA ratios of male and female spears than does GA because of the higher CK content in spear tips and the greater difference in CK between males and females compared to GA. It is interesting to note that the crosses differed significantly in CK content (Table 2), but there was no interaction between crosses and

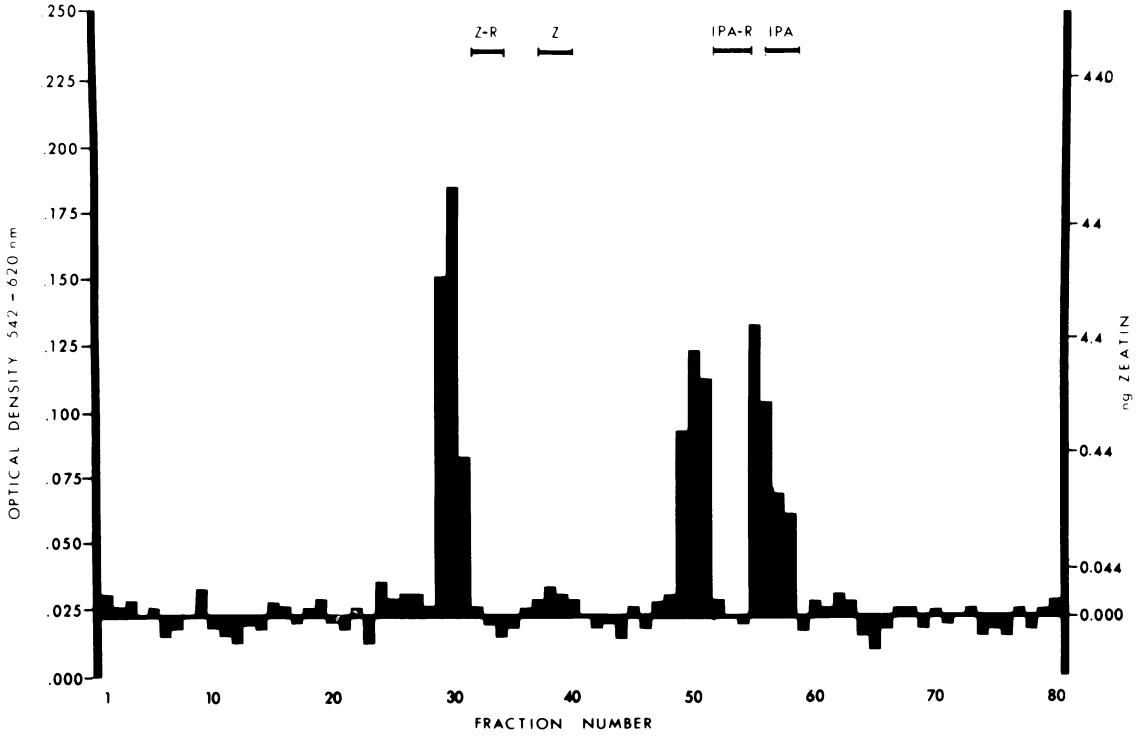

Fig. 4. Representative elution pattern of cytokinins from 10 g (fresh weight) female spear tips of *A. officinalis* (44 x 22) from a C_{18} reverse-phase HPLC column. The elution patterns of authentic zeatin riboside (Z-R), zeatin (Z), N^6-(2-isopentenyl)adenosine (IPA-R), N^6-(2-isopentenyl)adenine (IPA), and the bioassay response to known quantities of zeatin are included.

Table 2. Endogenous cytokinin levels in *A. officinalis* spears.

	ng zeatin equivalents/10 g fresh wt of spear tips			
Cross	Peak 1[z]	Peak 2	Peak 3	Sum of the three peaks
Md 10 x 14	34.8 a[y]	4.6 a	5.4 ab	46.6 a
44 x 22	91.9 b	5.0 a	3.6 a	103.7 b
Md 1 x 277S	81.3 b	11.6 b	9.7 b	103.6 b
Probability	0.07	0.03	0.05	0.08
Sex				
Female	86.0 a	8.7 a	7.2 a	106.3 a
Male	47.4 b	4.7 b	4.5 a	59.3 b
Probability	0.10	0.05	>0.10	0.07

[z]Individual peaks numbered in order of elution from a Sephadex LH-20 column.
[y]Mean separation in columns by HSD test.

Table 3. Ratios of endogenous cytokinin content to endogenous gibberellin content of *A. officinalis* spears.

	CK:GA ratio	
Cross	Total hormone[z]	Largest peak[y]
Md 10 x 14	27.0 a[x]	33.9 a
44 x 22	42.2 a	45.1 a
Md 1 x 277S	59.8 a	65.1 a
Sex		
Female	63.2 a	72.5 a
Male	22.8 b	23.6 b

[z]Ratio = total cytokinin content in a sample ÷ total gibberellin content in the sample.
[y]Ratio = peak 1 cytokinin content in a sample ÷ peak 2 gibberellin content in the same sample.
[x]Mean separation in columns by HSD test, 5% level.

sexes for CK content, indicating that differences in CK content between males and females is similar even though crosses vary in CK content.

Studies on the characteristics of male and female asparagus plants have shown that within a population or the progeny of a cross, female plants produce fewer spears (12, 23) with larger spear diameters (9, 23) than male plants. The differences in CK content and CK:GA ratios observed in this study could account, in part, for the differences in growth habit of male and female asparagus plants. No data were obtained on spear number and diameter of the hybrids used in this study.

Although other studies have shown that male and female asparagus plants differ in ascorbic acid, β-carotene, reducing and nonreducing sugar, total N, chlorophyll (27), vitamin B_1 (14), and polyphenoloxidase and peroxidase (20) content, none of these substances has been related to sex expression or sex modification in plants.

Lazarte and Garrison (17) reported that exogenous applications of CK to spears of male asparagus plants promoted pistil development in subsequent flowers. The higher CK and CK:GA ratios in female compared to male spears observed in this study suggests that sex determination in asparagus could be controlled in part by CK or the ratio of CK to another plant hormone, such as GA.

These results are not as clear as those of Khryanin and Chailakhyan (16). In their work with *Cannabis sativa* and *Spinacia oleracea*, endogenous CK was significantly higher in females than males, while endogenous GA was significantly higher in males than in females. They did not, however, report the specific hormone levels or calculate ratios. It is possible that sampling spears over time or sampling roots, crown tissue, or possibly

flower buds during the early stages of development could reflect more accurately the differences in CK and GA between male and female plants. The results reported here support the hypothesis of Lazarte and Garrison (17) that CK promotes female sex expression in *A. officinalis;* however, our results suggest that GA may not have an active role in promoting sex expression, but may interact with CK to produce CK:GA ratios that could determine or influence sex expression.

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