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# Changes in Cytokinin Distribution in the Ovule and Ovary of *Taraxacum officinale* Web. in Early Stages of Embryogenesis

**M. A. Gussakovskaya* and A. N. Blintsov**

*Department of Plant Physiology, School of Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; E-mail: radames@mail.ru*

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**Abstract**—Quantitative changes in the hormonal status of the ovules and ovaries were first studied in *Taraxacum officinale* Web. at the early stages of embryogenesis. The plant material was analyzed by ELISA using labeled anti-rabbit antibodies. A new procedure for differential and quantitative determination of the main endogenous cytokinins based on the estimation of the effective zeatin and zeatin riboside concentrations from calibration curves constructed using zeatin and zeatin riboside as standard antigens was developed. It was shown that, at the three initial stages of embryogenesis examined, the concentration of zeatin uniformly increased in *T. officinale* ovules. The concentration of zeatin riboside, conversely, uniformly decreased. However, their total concentration changed insignificantly. A gradual increase in the concentration of the active and storage hormone forms from the ovary to the ovule was shown.

*Key words*: *Taraxacum officinale*, ovule, ovary, endogenous hormones, zeatin, zeatin riboside, ELISA, immunochemical micro method for differential and quantitative determination of zeatin and zeatin riboside

We earlier showed that in the parthenogenetic species *Taraxacum officinale* Web. the concentration of zeatin uniformly increased from the complete maturation of the embryo sac (0 h) in the ovary to the beginning of the first division of the ovule (24 h), while the concentration of zeatin riboside (ZR) increased threefold during the first 12 h and then decreased to the initial level in the next 12 h [1-3]. Since no data on the hormonal regulation of the initial stages of embryogenesis in apomicts and amphimicts are available in the literature, the interpretation of these results was difficult. The initial stages of embryogenesis are extremely important for all subsequent events in early development. The absence of a model of hormonal regulation of early embryogenesis is an essential gap in the whole problem of regulation of embryogenesis in angiosperms.

To develop such a model, we determined the hormonal status of the ovaries and ovules of *Taraxacum officinale* at the initial stages of embryogenesis. This required significant modification of an earlier developed procedure of immunochemical analysis of hormones in *T. officinale* ovaries. A new micro procedure for the differential and quantitative determination of the main endogenous forms of cytokinins described in this work may be used in different biochemical and molecular biological studies for the analysis of low-molecular-weight compounds similar in structure but differing in biochemical activity.

The objectives of this work were as follows: 1) to describe a new ELISA micro procedure for the differential and quantitative determination of the major endogenous forms of cytokinins; 2) using this method, to analyze the dynamics of cytokinins in the ovules and ovaries of *T. officinale*; 3) to compare the results with the data obtained earlier for ovaries, and 4) to make some preliminary conclusions.

## MATERIALS AND METHODS

In this work, we investigated ovaries and ovules of *T. officinale* at three initial stages of embryogenesis: mature embryo sac (stage 1), interphase of a parthenogenetically

*Abbreviations*: $Ab_2$-HRP) anti-rabbit antibodies conjugated with horseradish peroxidase; BSA-ZR) BSA conjugated with zeatin riboside; ZR) zeatin riboside; $[Z]_{eff}$ and $[ZR]_{eff}$) effective concentrations of zeatin and zeatin riboside (M); ELISA) enzyme-linked immunosorbent assay; OV) ovalbumin; OV-ZR) ovalbumin conjugated with zeatin riboside; HRP) horseradish peroxidase; TPB) 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.01 M sucrose, and 0.1% Triton X-100.

* To whom correspondence should be addressed.

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developing ovule (stage 2), and first division of the ovule (stage 3). Plant material isolated from flowers was treated with solution containing 0.1% $NaN_3$ and 0.1% ascorbic acid. The material was analyzed by competitive enzyme-linked immunosorbent assay (ELISA) using anti-rabbit antibodies conjugated with horseradish peroxidase ($Ab_2$-HRP, EC 1.11.1.7).

To produce antisera, the BSA-ZR conjugate at molar BSA/ZR ratio of 55 obtained by periodate oxidation of the carbohydrate component of the phytohormone followed by binding of the aldehyde groups with amino groups of the protein was used as the immunogen. For this purpose, 50 mg of sodium periodate were added to 10 mg of ZR in 10 ml of 50% ethanol. The mixture was incubated in the dark for 1 h at room temperature. Twenty-five milligrams of BSA (or 200 mg of OV) in 0.5 M potassium carbonate buffer, pH 9.5, were added to the mixture. The solution was incubated for 2 h under stirring, then 10 mg of sodium boron hydride was added and incubated for 2 h at room temperature. The conjugate was purified by dialysis against distilled water. Protein concentration was determined according to Lowry.

For immunization of animals, the BSA-ZR conjugate with Freund's complete adjuvant mixed at a 1 : 1 ratio was used. To prepare the immunizing solution, the immunogen was diluted with 0.01 M potassium phosphate buffer containing 0.1 M NaCl, pH 7.0, to a protein concentration of 1 mg/ml, then an equal volume of Freund's adjuvant was added and thoroughly stirred until a stable emulsion was formed. The immunization was conducted as follows: for five weeks (once a week), 1 ml of the mixture of the antigen with Freund's complete adjuvant was injected subcutaneously at several sites along the back of mature rabbits (body weight 2.5-3.0 kg). After a four-week interval, daily for three days, rabbits were injected intravenously with 1.0 ml of the antigen at a concentration of 1 mg/ml in 0.01 M potassium phosphate buffer containing 0.1 M NaCl, pH 7.0. Repeated cycles of immunization were conducted every four weeks. Blood was taken seven days after the last injection. Antisera with a titer of 1 : 1000 and higher were used.

OV-ZR containing from three to five molecules of hormone per molecule of protein was used as an antigen immobilized on a solid phase.

The ZR/OV and ZR/BSA ratios in the conjugates were determined by titration of free amino groups of the proteins with trinitrobensenesulfonic acid [4].

The $Ab_2$-HRP conjugate was produced by oxidation of the carbohydrate components of the peroxidase with sodium periodate while protecting the amino groups of the enzyme by protonation under acid conditions [5]. To 1.0 ml of HRP (4 mg/ml, $RZ = A_{403}/A_{280} = 2.7\text{-}3.1$) in cold distilled water, 0.2 ml of freshly prepared 0.1 M sodium periodate in distilled water was added. The mixture was incubated at room temperature for 20 min with constant stirring. During HRP oxidation, the pH in the reaction mixture was held constant (4.4-4.5). The modified HRP was dialyzed against 1 mM potassium acetate buffer, pH 4.4, for 12 h at 4°C. The pH of the HRP solution was adjusted to 9.0-9.5 by the addition of 0.2 M sodium carbonate buffer, pH 9.5, and immediately 8 mg of immunoglobulins isolated from goat anti-rabbit serum were added. The reaction mixture was incubated for 2 h at constant shaking and room temperature and dialyzed against 0.01 M potassium phosphate buffer containing 0.1 M NaCl, pH 7.4, for 12 h at 4°C. To this solution, 0.1 ml of a freshly prepared solution of sodium borohydride (4 mg/ml) in distilled water was added. After incubation for 2 h, the conjugate was dialyzed against 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl. Unbound enzyme was removed by gel filtration on an Ultragel AcA-44 column (1.5 × 100 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl. The same buffer was used for elution at a flow rate of 18 ml/h. The concentration and composition of the immunoenzyme conjugate in the fractions were analyzed spectrophotometrically at 280 and 403 nm using the molar absorption coefficients $\varepsilon_{403}$ of $9.5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for HRP and $\varepsilon_{280}$ of $2.2 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ for IgG and *RZ* value of the HRP used. After the addition of an equal volume of glycerol, the conjugate solution was stored at −10°C.

The adsorption of the OV-ZR conjugate onto the polystyrene surface of Maxi-Sorb polystyrene plates (Nunc, Denmark) proceeded overnight at 4°C in 0.2 ml of 0.02 M sodium carbonate buffer, pH 9.5, at a protein concentration of 0.5 μg/ml. After the wells were washed three times with 0.3 ml of TPB, 0.1 ml of the sample (extracts of ovaries and ovules, a standard hormone preparation, etc.) and 0.1 ml of the antiserum solution diluted 1 : 1000 or more with TPB were applied. The competitive binding of analyzed and immobilized antigens occurred overnight at 4°C, whereupon the components not bound to the polystyrene surface were removed by three washes in 0.3 ml TPB. Then 0.15 ml of the $Ab_2$-HRP solution in TPB (at a conjugate concentration of $1.5 \cdot 10^{-9}$ M HRP) was added and incubated for 2 h at room temperature, whereupon the wells were washed three times with 0.3 ml of TPB.

The optical density $A_{405}$ of the product of enzymatic oxidation, 2,2′-azino-bis(3-ethyl-2,3-dihydrobenzthiazoline-6-sulfonic acid) (ABTS) (2.0 mM), by hydrogen peroxide (2.5 mM) in 0.1 M sodium acetate buffer, pH 4.2, was measured at 405 nm on an AIFR-01 Uniplan analyzer (Pikon, Russia) [6].

## RESULTS

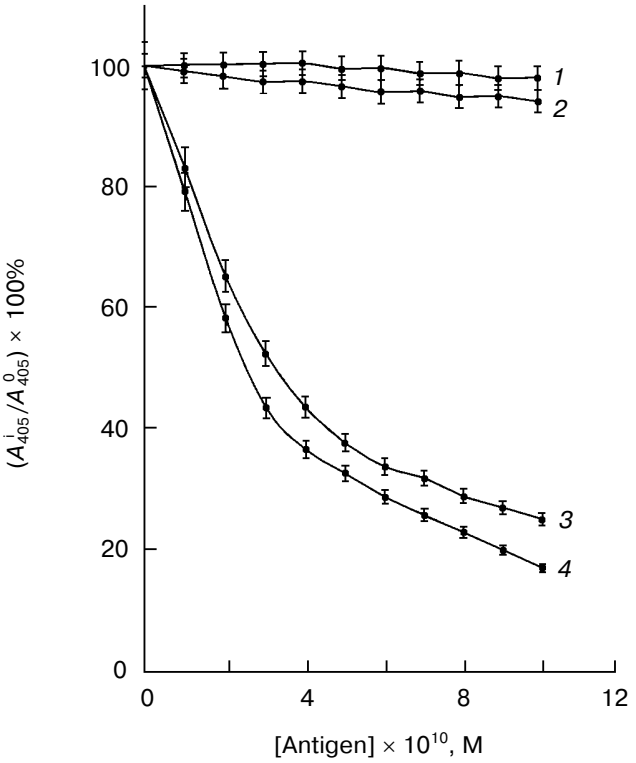
Analysis of the dynamics of endogenous cytokinins in ovules of angiosperms at early stages of embryogenesis necessitates the development of new approaches for the

immunochemical detection of phytohormones present in extremely low amounts. Earlier, we described several methods for quantitative determination of endogenous cytokinins, and these were used in studies of Z and ZR dynamics in wheat and dandelion ovaries [1-3, 7, 8]. These methods were highly reproducible and sensitive in determination of ZR, this allowing us to use them for small amounts of generative organs and tissues. However, these methods had certain disadvantages that limited their application to exceedingly low amounts of the biological material, whose collection and isolation were laborious and time-consuming. The main limitation of the previously used methods consisted in the necessity to optimize two immunochemical test systems, in each of which the concentration of endogenous cytokinins was measured [1-3]. Using effective concentrations of ZR estimated by different analytical methods, the actual concentrations of Z and ZR in the samples were calculated. Such investigations require independent development (production of immunochemicals) and optimization of a whole complex of parameters for two separate immunochemical systems, this limiting the usefulness of the approach. Furthermore, the sample is used twice, this being highly undesirable if extremely low amounts of generative tissues, such as ovules, embryo sacs, etc., are examined. We developed a new immunochemical micro procedure for the analysis of the endogenous cytokinin concentration in ovules. In this method, like in those described earlier, ZR-specific antibodies are used. However, in contrast to previous methods, only one competitive ELISA procedure is necessary. Both Z and ZR are used as standards for the construction of calibration curves. This simplifies considerably the production of immunochemicals and optimization of the analytical system since there is no longer need in the additional immunochemical system. Furthermore, the plant material is used only once (in a necessary number of analytical replicates), this allowing us to consider our new quantitative method of determination of cytokinin concentration as a micro-analytical procedure that can be employed for the analysis of small amounts of biological material (depending on the developmental stage, the wet weight of one dandelion ovule varies from 10 to 20 μg), excluding the preliminary stage of fractionation of the sample. High sensitivity of the developed method in determination of Z and ZR concentrations ($0.8 \cdot 10^{-10}$ and $0.5 \cdot 10^{-10}$ M, respectively; figure, curves *3* and *4*) makes it possible even to dilute the aqueous extract of the ovule a thousand times or more with TPB, this actually eliminating the effect of other endogenous components present in the ovule extract and providing high specificity of the measurements. Thus, our new method is based on determination of effective concentrations of Z ($[Z]_{eff}$) and ZR ($[ZR]_{eff}$) from calibration curves constructed using Z and ZR as standard antigens (figure, curves *3* and *4*). It should be noted that: 1) the formation of the complex between the OB-ZR conjugate and ZR-specific antibodies is inhibited by free and bound Z to a different degree; 2) cross-reactivity of the antiserum calculated for Z from ZR was 80%, while for dihydrozeatin and isopentyladenine it was less than 0.1% (this means that ZR-specific antiserum was more specific to ZR than to other endogenous cytokinins); 3) the obtained values of concentrations are effective parameters and do not reflect actual Z and ZR concentrations since the contributions of free and bound Z to the estimated parameter $A_{405}$ used for determination of effective concentrations of cytokinins in the sample are different. Variation in specificity of the analytical system used with respect to Z and ZR allows one to calculate actual concentrations of free and bound Z from the measured effective concentrations of endogenous cytokinins $[Z]_{eff}$ and $[ZR]_{eff}$:

$$[Z]_{eff} = a \cdot [ZR] + b \cdot [Z] , \quad (1)$$

$$[ZR]_{eff} = c \cdot [ZR] + d \cdot [Z] . \quad (2)$$

Note that the system of equations (1) and (2) is valid for the linear region of the calibration curve, where




Relationship between chromophore response and the concentration of dihydrozeatin (*1*), isopentyladenine (*2*), zeatin (*3*), and zeatin riboside (*4*) in TPB determined by ELISA using ZR-specific antibodies. The OV-ZR conjugate was used as a immobilized antigen. Abscissa, phytohormone concentration in TPB; ordinate, $A^i_{405}/A^0_{405}$, where $A^i_{405}$ is the optical density of phytohormone solution of a standard concentration and $A^0_{405}$ is the optical density in the absence of phytohormone.

the coefficients *a*, *b*, *c*, and *d*, which characterize the contribution of different Z forms to the measured effective concentrations of cytokinins, are constant and do not depend on the concentration of the calibration standards. In our case, the initial regions of the calibration curves were linear at Z concentrations below $4.0 \cdot 10^{-10}$ M and ZR concentrations below $3.0 \cdot 10^{-10}$ M (figure, curves *3* and *4*). Therefore, all measurements were conducted in the antigen concentration range of $(0\text{-}3.0) \cdot 10^{-10}$ M. For this purpose, the analyzed solution was consecutively diluted 1 : 2 with TPB, i.e., for each sample a series of consecutive twofold dilutions was made. The calculations of effective concentrations of Z and ZR from the absorption at 405 nm were made only for the linear regions of the curves. A narrow range of the standard antigen concentrations $(0\text{-}3.0) \cdot 10^{-10}$ M at absorption values ranging from 100 to 45% provided high resolution of the measurements: it was possible to significantly differentiate antigen concentrations in the standards from 0.1 to 0.3 nM for Z and ZR. The variation coefficient did not exceed 11-13% for the entire linear region of the calibration curves. The latter observation is extremely important since physiological concentrations of endogenous cytokinins in the reproductive organs may change very slightly, and, consequently, remain undetected due to low resolving power of the method used despite its high sensitivity. The determination of effective concentrations of Z and ZR in the standards, which differed in Z/ZR ratios, allowed us to estimate the contribution of various natural forms of phytohormones to the effective parameters (Table 1). Since in the absence of Z in the standard only the bound Z form is estimated, namely at [Z] = 0, Eq. (2) transforms into: $[ZR]_{eff} = c \cdot [ZR]$, from which we find *c* as follows: $c = [ZR]_{eff}/[ZR] = 1$, namely, the measured value of effective ZR concentration coincides with its true concentration in the standard. Similarly, at [ZR] = 0, when only free Z in the standard sample is measured, Eq. (1) may be written as follows: $[Z]_{eff} = b \cdot [Z]$. From this equation we find $b = [Z]_{eff}/[Z] = 1$. Accordingly, in the system of equations (1) and (2) for Z and ZR effective concentrations, the coefficients *b* and *c*, which reflect the contribution of a corresponding form to its effective concentration, are equal to unity. From the above-mentioned considerations, the original system of equations (1) and (2) may be written as follows:

$$[Z]_{eff} = a \cdot [ZR] + [Z] , \quad (3)$$

$$[ZR]_{eff} = [ZR] + d \cdot [Z] . \quad (4)$$

The coefficients *a* and *d* were determined experimentally using a series of standards containing different amounts of Z and ZR at different total concentrations and Z/ZR ratios in the linear range of the calibration curves (Table 1). The results indicate that, in the selected range of Z and ZR concentrations, *a* and *d* values are constant at different Z/ZR ratios. Calculated mean values of the coefficients *a* and *d* are: $d = 0.85 \pm 0.05$ and $a = 1.3 \pm 0.1$, at a variation coefficient below 9%. Thus, the original system of equations can ay be finally presented as follows:

$$[Z]_{eff} = 1.3 \cdot [ZR] + [Z] , \quad (5)$$

$$[ZR]_{eff} = [ZR] + 0.85 \cdot [Z] . \quad (6)$$

The expressions (5) and (6) for the relationship between the effective concentrations of Z and ZR and their actual concentrations were used to test the validity of the method for determination of Z and ZR concentrations in standard solutions, which differed in hormone ratio and concentrations (Table 2). In all instances, good correspondence between estimated and actual hormone concentrations in solution was observed, i.e., the ratios of concentrations calculated from the equations (5) and (6) to actual concentrations in standard solutions varied from 90 to 120%. The results of this experiment, shown in Table 2, demonstrate that Z and ZR concentrations estimated using the immunochemical approach developed were highly significant. The concentrations of Z and ZR in different solutions (TPB and buffered homogenates of leaves, roots, and inflorescences of dandelion diluted 1 : 10 and dialyzed against distilled water for three days at 4°C) did not differ considerably.

Since in the above-mentioned test system, cross-reactivity with dihydrozeatin and isopentyladenine was extremely low (less than 0.1%, see the figure, curves *1* and *2*), and the concentration of these cytokinins was much more lower than that of free Z and ZR, their effect on the measurements can be neglected. The effect of other forms of cytokinins, mainly of O- and 7N-glucosides of Z [9], is also small. Since we used antiserum obtained by immunization of rabbits with BSA conjugated with 9N-ZR, the functional groups of adenine located opposite to the protein molecule act as antigenic determinants, because in this case the steric shielding of the hormone functional groups by the protein globule is minimal. The antibodies interact primarily with those antigenic determinants that are available for protein–ligand interactions; therefore, their chemical modification should decrease binding of O- and 7N-glucosides of Z to antibodies. This phenomenon is known in immunochemistry of low-molecular-weight compounds [10]. Therefore, it was reasonable to suggest that O- and 7N-glucosides of Z will not interfere with the determination of the main forms of endogenous cytokinins, such as Z and 9N-ZR, due to decreased specificities of the antisera used to all O- and 7N-derivatives of Z. In addition, the main storage form of Z is

**Table 1.** Determination of Z and ZR in TPB at different hormone ratios in standard solutions ($N = 10$, number of analytical replicates)

| Original Z concentration × $10^{10}$, M | Original ZR concentration × $10^{10}$, M | $[Z]_{eff} \times 10^{10}$, M | $[ZR]_{eff} \times 10^{10}$, M | Estimated Z concentration × $10^{10}$, M | Estimated ZR concentration × $10^{10}$, M | $[Z]_{eff}$/original concentration, % | $[ZR]_{eff}$/original concentration, % |
|---|---|---|---|---|---|---|---|
| 3.0 | — | 2.9 | 2.5 | 2.9 | — | 97 | — |
| 2.0 | — | 1.9 | 1.6 | 1.9 | — | 95 | — |
| 1.0 | — | 1.0 | 0.9 | 1.0 | — | 100 | — |
| 3.0 | 1.0 | 4.7 | 3.8 | 3.1 | 1.2 | 103 | 120 |
| 2.0 | 1.0 | 3.4 | 2.8 | 2.0 | 1.1 | 100 | 110 |
| 1.0 | 1.0 | 2.4 | 1.9 | 1.1 | 1.0 | 110 | 100 |
| 3.0 | 2.0 | 5.9 | 4.8 | 3.2 | 2.1 | 107 | 105 |
| 2.0 | 2.0 | 4.8 | 3.9 | 2.1 | 2.1 | 105 | 105 |
| 1.0 | 2.0 | 3.7 | 2.9 | 1.2 | 1.9 | 120 | 95 |
| 3.0 | 3.0 | 7.3 | 5.9 | 3.3 | 3.1 | 110 | 103 |
| 2.0 | 3.0 | 6.2 | 5.0 | 2.3 | 3.0 | 115 | 100 |
| 1.0 | 3.0 | 5.0 | 3.9 | 1.2 | 2.9 | 120 | 97 |
| — | 3.0 | 3.8 | 2.9 | — | 2.9 | — | 97 |
| — | 2.0 | 2.7 | 2.1 | — | 2.1 | — | 105 |
| — | 1.0 | 1.4 | 1.1 | — | 1.1 | — | 110 |

Note: Antigen concentration (%) = estimated concentration/actual concentration × 100%.

**Table 2.** Dependence of coefficients $a$ and $d$ on concentration and Z/ZR ratio in standard solutions ($N = 10$)

| Original Z concentration × $10^{10}$, M | Original ZR concentration × $10^{10}$, M | $[Z]_{eff} \times 10^{10}$, M | $[ZR]_{eff} \times 10^{10}$, M | $a_{mean}$ | Variation coefficient, $a$ | $d_{mean}$ | Variation coefficient, $d$ |
|---|---|---|---|---|---|---|---|
| 1.0 | — | 1.1 | 0.8 | — | — | 0.8 | 8.8 |
| 2.0 | — | 1.9 | 1.6 | — | — | 0.8 | 8.7 |
| 3.0 | — | 3.0 | 2.7 | — | — | 0.9 | 8.7 |
| 1.0 | 1.0 | 2.2 | 1.8 | 1.2 | 8.6 | 0.8 | 8.3 |
| 2.0 | 1.0 | 3.3 | 2.6 | 1.3 | 8.2 | 0.8 | 8.5 |
| 3.0 | 1.0 | 4.3 | 3.4 | 1.3 | 8.4 | 0.8 | 8.2 |
| 1.0 | 2.0 | 3.6 | 2.9 | 1.3 | 8.4 | 0.9 | 8.5 |
| 2.0 | 2.0 | 4.5 | 3.6 | 1.3 | 8.5 | 0.8 | 8.4 |
| 3.0 | 2.0 | 5.8 | 4.4 | 1.4 | 8.3 | 0.8 | 8.6 |
| 1.0 | 3.0 | 5.2 | 3.9 | 1.4 | 8.1 | 0.9 | 8.3 |
| 2.0 | 3.0 | 5.7 | 4.6 | 1.2 | 8.3 | 0.8 | 8.2 |
| 3.0 | 3.0 | 6.9 | 5.4 | 1.3 | 8.0 | 0.8 | 8.5 |
| — | 1.0 | 1.2 | — | 1.2 | 8.2 | — | — |
| — | 2.0 | 2.6 | — | 1.3 | 8.3 | — | — |
| — | 3.0 | 3.8 | — | 1.2 | 8.5 | — | — |

**Table 3.** Changes in the concentrations of free and bound Z forms in aqueous extracts of ovaries and ovules of *Taraxacum officinale* (1 : 10) at three stages of embryogenesis ($N = 20$, number of analytical replicates; number of biological replicates was 200, $p = 0.998$)

| Sample | ZR concentration $\times 10^8$, M | Z concentration $\times 10^8$, M |
|---|---|---|
| | Ovule | |
| 1st stage | (8.7 ± 1.5) | (4.1 ± 0.8) |
| 2nd stage | (4.8 ± 0.9) | (6.8 ± 1.3) |
| 3rd stage | (1.2 ± 0.3) | (9.7 ± 2.0) |
| | Ovary | |
| 1st stage | (2.2 ± 0.4) | (3.2 ± 0.6) |
| 2nd stage | (6.7 ± 1.3) | (5.5 ± 1.0) |
| 3rd stage | (3.4 ± 0.7) | (8.9 ± 1.7) |

9N-riboside [9], therefore, quantitative determination of Z and ZR is possible in the presence of other endogenous Z forms, which are present at much lower concentrations. To prove these suggestions, we determined Z and ZR concentrations in standard solutions and in standards supplemented with some characterized biological extract. An aqueous extract of *T. officinale* ovary at different developmental stages was used. The concentrations of Z and ZR in the extract diluted 1 : 10 were $5.1 \cdot 10^{-8}$ and $4.6 \cdot 10^{-8}$ M, respectively. To this solution, different amounts of Z and ZR were added; afterwards, the hormone concentrations were determined again. The results presented in Table 2 show that the concentrations of Z and ZR in the standard solutions increased by the level of these hormones in the homogenate of dandelion ovaries. Accordingly, these data showed the validity and significance of the results obtained with the developed method of determination of Z and ZR concentrations in the reproductive organs of *T. officinale* that allowed us to focus on quantitative changes in Z and ZR in dandelion ovules at early stages of embryogenesis.

As seen from Table 3 (see also [1]), Z concentration in the *T. officinale* ovule uniformly increases during three initial stages of embryogenesis ($n = 1$, 2, and 3). In contrast, the concentration of zeatin riboside decreases. However, the total concentration $[Z]_n + [ZR]_n = 12.8$ ($n - 1$) changes insignificantly within the limits of experimental error (the multiplier $10^{-8}$ is omitted). It seems that an increase in $[Z]_n = 4.1 + 2.8$ ($n - 1$) and a decrease in $[ZR]_n = 8.7 - 3.8$ ($n - 1$) are correlated and due to the same phenomenon, namely the transition of cytokinins from the inactive (ZR) to the active form (Z) by cleaving the pentose group. If so, *de novo* synthesis of ZR in ovules at the initial stages of embryogenesis does not occur.

## DISCUSSION

The concentration of Z in the ovary and ovule at stage 1 is different. It is about 1.5 times higher in the ovule than in the ovary, i.e., there exists a positive gradient of Z concentration from the ovary to the ovule. Most likely, the active hormone exhibits its main functions in the ovule and not in the ovary.

In the linear approximation, in the ovary, $[Z]_n = 2.7 + 2.5$ ($n - 1$). The rate of accumulation of the active hormone in the ovary and ovule is similar. We suppose that the transition from the inactive to the active condition is controlled by spatial and temporal parameters associated with hormone activation by cleavage of the pentose group. If this is the case, the original total pool of active and inactive forms of cytokinins in the ovary is of exogenous origin. The hormone imported to the ovary from the outside passes to the ovule, where it is stored for a while and then activated.

The distribution of ZR in the ovary–ovule system at stage 1 is also different. The concentration of the inactive hormone in the ovule is about four times higher than in the ovary. Accordingly, the gradients of Z and ZR concentrations coincide; this is consistent with the suggestion that the ovule is the final target of the hormonal signal. The finding that ZR concentration in the ovule decreases indicates that the necessary hormone pool in the ovule has already been accumulated before the analyzed stages of embryogenesis. In the ovary, the concentration of ZR increases during the transition from stage 1 to stage 2. Consequently, in this instance changes in ZR concentration in the ovary and ovule are not correlated. The transition from stage 2 to stage 3 is accompanied by a decrease in ZR concentration both in ovaries and ovules. We suppose that, at stage 2, the active hormone performs its main functions, the signal is transmitted to the ovary by feedback regulation, and the ovary inhibits import of the exogenous inactive hormone. This finding is interesting to compare with the observations that the initial stages of embryogenesis *in vitro* are more effective in hormone-free media [11]. Hormones are usually supplied at the later stages.

In conclusion, it should be noted that the previously described ELISA procedure gives the model relationships for ovaries as follows: $[Z]_n = 2.7 + 2.5$ ($n - 1$), $[ZR]_n = 5.3 - 3.1\,|n - 2|$. Our new ELISA procedure used for the analysis of hormones in the ovules leads to the following model dependences for ovaries (see Table 3): $[Z]_n = 3.0 + 2.5$ ($n - 1$), $[ZR]_n = 6.0 - 3.1\,|n - 2|$, which are in good agreement with the results of this work and their interpretation. In addition, the similarity of the models indicates that the newly developed immunochemical procedure for

the determination of the main endogenous forms of Z is a highly accurate and valid tool in studies of cytokinin distribution in ovaries and ovules of *T. officinale* at early stages of parthenogenetic embryogenesis.

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