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SALT STRESS-INDUCED CHANGES OF CYTOKININS IN MAIZE AND PEA PLANTS RNA

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Summary. Maize and pea plants were grown on liquid nutrient medium. NaCl was applied to roots for 10 days. Salt treatment reduced growth of roots and shoots of both plants by more than 20% and 50%, respectively. Total RNA content isolated from growing root parts of both plants was lower under stress. Total RNA content of stressed maize apical parts did not change significantly compared to the controls but in the case of pea the content of RNA decreased. Alkaline-phosphatase hydrolyzed RNA preparations were tested by ELISA for iPA and ZR content. Under stress their content in roots of both species increased to 130%. In treated apical parts both nucleosides altered slightly with a tendency to reduction which was more obvious for iPA of pea RNA.

Key words: *Zea mays, Pisum sativum,* salt stress, RNA, cytokinins, ELISA

Abbreviations: iPA – N^6-(Δ^2-isopentenyl)adenosine; ZR – *trans*-zeatin riboside

Introduction

The occurrence of cytokinin molecules in RNA of higher plants has been known for many years but its biological significance is not well understood. Cytokinins were shown to be incorporated in RNA or to result from RNA degradation. Besides in tRNA, cytokinins have been indicated in rRNA, polyA-chains of the mRNAs and

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adenine-containing oligonucleotides from RNA digestion (Hall, 1970; Maaß and Klämbt, 1981; Jouanneau et al., 1982; Klämbt, 1983; Taller et al., 1987; Taller, 1994). In prokaryotic tRNA the levels of some cytokinins were found to be affected by environmental and physiological factors (Björk et al., 1987). A proposition was made that these alterations act as a regulatory mechanism allowing cells to respond to environmental changes. Cytokinin response in plant RNAs to different growth and environmental conditions yet is scarcely known.

At this stage of our study on cytokinin nucleotides in RNA a research on the effect of salt stress on the levels of N^6-(Δ^2-isopentenyl)adenine and *trans*-zeatin in total RNA from maize and pea plants was carried out. We found that the stress factor which induced fast and obvious effects on the growth and on the endogenous free levels of both cytokinins (Atanassova et al., 1996) affected their levels in RNA as well.

Materials and Methods

Plant material

Maize plants (*Zea mays* L., cv. Knezha) were grown on Hoagland–Arnon nutrient solution at natural conditions in June and July. NaCl of 100 mM was added to the nutrient solution when plants expanded their 4th leaf. Pea plants (*Pisum sativum* L., cv. Ran-1) were grown on the same solution in climatic chamber at 23–25 °C, light intensity 100 $\mu mol.m^{-2}.s^{-1}$ and a day/night photoperiod 14/10 hours. Plants with developed 2nd leaf were placed on nutrient solution, containing 150 mM NaCl. Control maize and pea plants were grown on nutrient solution.

At the 10th day of the treatment with NaCl, roots and shoots of 10–12 control and stressed maize and pea plants were taken for analysis. Growth was evaluated by lenght and biomass production of roots and shoots. The samples for the isolation of RNA were taken from the growing apex parts of roots and shoots, fixed with liquid nitrogen and kept at -70 °C.

Isolation of total cell RNA

RNA were isolated by the method of Czomczynsky and Sacchi (1987) with several modifications made by Kayser and Klämbt (1995). The frozen material (5 g) was ground to a fine powder and homogenised in a denaturating solution containing guanidinium thiocyanate, sodium citrate, N-lauroylsarcosine and β-mercaptoethanol. The pH was kept in acid range by adding 3 M sodium acetate, pH 4.8. The homogenised material was extracted twice with 24:24:1 of phenol:chloroform:isoamyl alcohol, followed by chloroform. The organic and water phases were divided by centrifugation for 10 min at 10 000 × g. RNA was precipitated with isopropanol overnight

at -20°C and centrifuged 10 min at 10000×g. The pellet was washed with 75% ethanol, resedimented for 5 min at 10000×g and dried carefully on air. RNA was dissolved in sterile water and stored frozen till hydrolysis. The concentration and purity of the RNA were determined by spectrometry. Their A_{260}/A_{280} absorbance ratio was about 2.0. RNA content was calculated assuming that 50 µg/ml RNA has an optical density of 1 at 260 nm.

Hydrolysis of RNA

RNA was hydrolysed with 0.5 M KOH overnight at 38°C followed by neutralization with $HClO_4$. The nucleotide mixture was dephosphorylated at pH 9.3 with alkaline phosphatase for 3 hours at 38°C. The hydrolysed preparations containing nucleosides were immunotested for iPA and ZR.

Determination of cytokinins in RNA by ELISA

N^6-(Δ^6-isopentenyl)adenosine (iPA) and *trans*-zeatin riboside (ZR) were conjugated to bovine serum albumin (BSA) by the periodate procedure of Weiler (1980). Sera against these conjugates were raised in rabbits. Anti-iPA- and anti-ZR-immunoglobulins (IgG) were purified by immunoaffinity chromatography of BSA-Sepharose.

The hydrolysed RNA-preparates were tested by indirect competitive ELISA. The immunoassay was a slight modification (Atanassova et al., 1996) of the procedure developed by Von Schwartzenberg (1989). The labelling of the immunocomplex was made by conjugate of goat anti-rabbit-IgG and alkaline phosphatase. The conjugate was prepared by Zlatka Dimitrova and Emilia Ivanova (National Centre of Infections and Parasitic Diseases, Sofia). The standard curves for authentic iPA and ZR and the results of immunotesting of the samples were calculated by the computer programme of Ts.Tsonev (Institute of Plant Physiology, Sofia) on the basis of formulae for percentage calculation of maximum binding (B/B_0) and log-logit transformation.

The results are mean averages of three ELISA determinations from two experiments.

Results

The treatment with 100 mM NaCl resulted in gradual decrease of size and accumulation of biomass of maize plants. At the 10th day of stress (Table 1) the whole root and shoot biomasses compared with the control plants were reduced with 30 and 50%, respectively.

The content of total RNA isolated from stressed growing root and shoot parts declined in roots but it retained, even slightly increased, in apical shoot parts (Table 1). RNA preparates following alkaline-phosphatase hydrolysis were tested by ELISA for

Table 1. Production of biomass, content of total cell RNA and RNA-cytokinins in roots and shoots of control (-) and salt stressed (+) maize plants

Variant	Biomass	Total RNA	iPA	ZR
	(g FW/ plant)	(mg/g FW)	(pmol/mg RNA)	
Roots				
(-)	6.75 ± 1.04 (100%)	0.94 (100%)	25.1 (100%)	5.0 (100%)
(+)	4.70 ± 1.50 (70%)	0.70 (74%)	31.2 (124%)	6.7 (134%)
Shoots				
(-)	11.40 ± 0.75 (100%)	1.36 (100%)	20.7 (100%)	4.0 (100%)
(+)	5.60 ± 1.40 (50%)	1.49 (110%)	22.0 (107%)	3.5 (88%)

iPA and ZR content. The levels of both nucleosides in mg total RNA compared in roots to shoots of control plants were similar. Under stress their content in roots was increased to 130%. In apical parts the level of both nucleosides did not change significantly.

The same parameters were measured in pea plants (Table 2). During the 10 days of treatment with NaCl the biomass of the whole plant was reduced by 40–50%. The total RNA contents of growing root and apical parts were lower in stressed plants. In control plants higher immunoreactivities against both anti-cytokinin-antibodies, hence

Table 2. Production of biomass, content of total cell RNA and RNA-cytokinins in roots and shoots of control (-) and salt stressed (+) pea plants

Variant	Biomass	Total RNA	iPA	ZR
	(g FW/ plant)	(mg/g FW)	(pmol/mg RNA)	
Roots				
(-)	1.43 ± 0.06 (100%)	0.80 (100%)	41.7 (100%)	2.5 (100%)
(+)	1.06 ± 0.07 (74%)	0.61 (76%)	53.3 (128%)	3.3 (132%)
Shoots				
(-)	2.06 ± 0.12 (100%)	2.60 (100%)	146.7 (100%)	7.2 (100%)
(+)	1.12 ± 0.01 (54%)	1.53 (63%)	111.7 (76%)	6.0 (83%)

more cytokinin nucleosides, were determined in shoot RNA than in the root. In stressed roots both RNA-nucleosides increased with about 30%. The levels of both cytokinins in apical shoot parts of the stressed plants were slightly reduced in comparison to the controls.

Discussion

Salt stress reduced the size and the biomass production of maize and pea plants. The reduction was not lethal as maize plants only delayed growth and development of leaf mass and pea plants even reached the reproductive phase.

The results of the experiments with growing plant parts showed similar changes in the roots of maize and pea plants and some differences in the responses of their underground parts to salt stress. The reasons for such discrepancy usually are associated with the features of apical part growth in monocotyledonous and dicotyledonous plants on salinity (Aspinall, 1986; Barlow, 1986).

The total RNA content isolated from growing root parts of both plants was lower under stress. These results confirm previous observations demonstrating that one of the effects of salinity was the decreased stability of RNA machinery. *In vitro* Na^+ initiated RNA degradation but *in vivo* RNA stability depended on the relative concentration of the ion accumulated (Rauser and Hanson, 1966; Aspinall, 1986; Munns and Termaat, 1986).

As for shoots the apical RNA machinery of monocotyledonous plants, maize as their representative, has been considered to be more protected and less sensitive than that of dicotyledonous, such as pea (Aspinall, 1986; Barlow, 1986; Peterson et al., 1987). This feature explains to some extent the different metabolic status of RNA in apical parts of both species. More research on separate RNA-structures under salt stress is needed for obtaining a more satisfying answer.

Salinity induced changes in the levels of two cytokinin nucleosides included in RNA structures. The levels of iPA and ZR in stressed roots of both plants increased markedly. In apical regions, both nucleosides altered slightly with a tendency to reduction, which was more obvious for iPA of pea RNA. Much higher content of iPA in apical pea parts, which were green, may be associated with chloroplast tRNA (Vreman et al., 1978). Maize apical parts were practically non-green and the level of iPA in their RNA preparations was much lower.

In conclusion, it can be stated that after 10 days of salt stress the growth and total RNA content of maize and pea roots decreased but higher levels of isopentenylated nucleotides in RNA were induced. Presumably, changes in the growth conditions modified the metabolism of RNAs containing cytokinin nucleotides.

In studies of RNA metabolism in salinized roots, aberrations in tRNA, rRNA and mRNA metabolism, increase of the oligonucleotide pool as well as of the capacity

for nucleotide catabolism have been shown (Rauser and Hanson, 1966; Peterson et al., 1987; Peterson et al., 1988). There are enough data demonstrating the presence of cytokinins in each of the RNA structures mentioned. Largest variety and abundance of cytokinins occurred in tRNA preparations (Taller, 1994). In rRNA their concentrations were lower achieving 15–20% of the cytokinin RNA pool (Taller et al., 1987). PolyA-tails of mRNA and oligo(adenine)nucleotides were also demonstrated to contain cytokinin molecules as well as to act as good substrates for the specific Δ^2-isopentenyltransferase, the enzyme producing iPA in tRNA (Holtz and Klämbt, 1978; Maaß and Klämbt, 1981; Jouanneau et al., 1982; Klämbt, 1983). Regardless of the number of reports on this topic, the role of cytokinin presence in plant RNAs in cell metabolism regulation is still hypothetical. Growth conditions or stage of development have been shown to influence the relative abundance and the nature of cytokinin-active bases present in a given tRNA species from prokaryotes and yeast (Taller, 1994). These facts, as well as the present results, give us the reason to continue the research and in the following stage we'll try to find out what RNA structure is responsible for the changes of both cytokinins under salt stress.

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References

Aspinall, D., 1986. Metabolic effects of water and salinity stress in relation to expansion of the leaf surface. Austr. J. Plant Physiol., 13, 59–73.

Atanassova, L., M. Pissarska, I. Stoyanov, 1996. Cytokinins and growth responses of maize and pea plants to salt stress. Bulg. J. Plant Physiol., 22 (1–2), 22–31.

Barlow, E. W. R., 1986. Water relations of expanding leaves. Austr. J. Plant Physiol., 13, 45–58.

Barnes, M. F., Ch. L. Tien, J. S. Gray, 1980. Biosyntesis of cytokinins by potato cell cultures. Phytochemistry, 19, 409–412.

Björk, G. R., J. U. Ericson, G. E. D. Gustafsson, T. G. Hagervall, Y. H. Jönsson, P. M. Wikström, 1987. Transfer RNA modification. Ann. Rev. Biochem., 56, 263–287.

Chomczynski, P., N. Sachi, 1987. Single-step method of RNA isolation by acid guanidinum thiocyanate–phenol–chloroform extraction. Anal. Biochem., 162, 156–159.

Jouanneau, J. P., B. Teyssendier de la Serve, C. Peaud-Lenoël, 1982. Incorporation of cytokinins into tobacco cell RNA: studies in relation to their physiological activity. In: Plant Growth Substances, Ed. P. F. Wareing, Academic Press, London, 197–206.

Hall, R. H., 1970. $N^6(\Delta^2$-isopentenyl)adenosine. Chemical reactions, biosynthesis, metabolism and significance for the structure and function of tRNA. Progr. Nucl. Acid Res. Mol. Biol., 10, 57–86.

Holtz, J., D. Klämbt, 1978. Characterization of the isopentenylation reaction of tRNA, oligo(A) and other nucleic acids. Hoppe Seyler's Z. Physiol. Chem., 359, 89–101.

Kayser, B., D. Klämbt, 1995. Auxin–binding protein (ABP1) is not confined to the outer epidermis of maize (*Zea mays* L.) coleoptiles. Bot. Acta, 108, 365–373.

Klämbt, D. 1983. The biogenesis of cytokinins. In: Plant Growth Regulators. Ed. D. Lilov et al. Pbl. House of Bulg. Acad. Sci., Sofia, 157–162.

Maaß, H., D. Klämbt, 1981. On the biogenesis of cytokinins in roots of *Phaseolus vulgaris*. Planta, 151, 353–358.

Munns, R., A.Termaat, 1986. Whole-plant responses to salinity. Austr. J. Plant Physiol., 13, 143–160.

Peterson, T. A., R. H. Nieman, R. A. Clark, 1987. Nucleotide metabolism in salt-stressed *Zea mays* root tips. Plant Physiol., 85, 984–989.

Peterson, T. A., C. J. Lovat, R. H. Nieman, 1988. Salt stress acceleration of purine catabolism and inhibition of pyrimidine salvage in *Zea mays* root tips. J. Exp. Bot., 39, 1389–1395.

Rauser, W. E., J. B. Hanson, 1966. The metabolic status of ribonucleic acid in soybean roots exposed to saline media. Can. J. Bot., 44, 759–776.

Taller, B. J., 1994. Distribution, biosynthesis, and function of cytokinins in tRNA. In: Cytokinins, Chemistry, Activity and Function, Ed. D. W. S. Mok and M. C. Mok. CRC Press, Florida, 101–112.

Taller, B. J., N. Murai, F. Skoog, 1987. Endogenous cytokinins in the ribosomal RNA of higher plants. Plant Physiol., 83, 755–760.

Von Schwartzenberg, K., 1989. Der Cytokiningehalt in nadeln unterschiedlich stark von "Neuartigen Waldschaden" betroffener fichten (*Picea abies* [L.] Karst.), bestimmt mittels einer immuno-enzymatischen metode – ELISA. PhD Thesis. University of Bonn.

Vreman, H. J., R. Thomas, J. Corse, S. Swaminathan, N. Murai, 1978. Cytokinins in tRNA obtained from *Spinacia oleracea* leaves and isolated chloroplasts. Plant Physiol., 61, 296–306.

Weiler, E., 1980. Radioimmunoassays for *trans*-zeatin and related cytokinins. Planta, 149, 155–162.