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Effect of temperature stress on the endogenous cytokinin content in *Arabidopsis thaliana* (L.) Heynh plants

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Abstract

The levels of three endogenous cytokinin equivalents: zeatin (Z), *iso*-pentenyladenine (iP) and dihydrozeatin (dZ) in two *Arabidopsis thaliana* (L.) Heynh genotypes – wild type (wt) and ethylene-insensitive mutant (*eti5*), were compared using enzyme immunoassay (ELISA). Cytokinin content was measured after exposure to low (4 °C for 24 h in darkness) or high temperature (38 °C for 24 h in darkness). Measurements were performed immediately and 24, 48 and 120 h after treatments. It was found that at normal growth conditions *eti5* plants contained more endogenous cytokinins compared to the wild type. At both temperature treatments mutant plants had decreased total cytokinin levels. Wild-type plants treated with high temperature (HT) exhibited reduced total cytokinins (with the exception of rates at 48 h), while low temperature (LT) treatment resulted in elevated total amount of the studied equivalents (except at 24 h). The obtained results suggested that HT had greater effect on cytokinin levels than LT since it caused more profound changes in the total content. We assume that this was due to the natural chilling tolerance of *Arabidopsis* plants.

List of abbreviations: dZ – dihydrozeatn, dZR – dihydrozeatin riboside, ELISA – enzyme linked immunosorbent assay, HT – high temperatute, iP – *iso*-pentenyladenine, iPA – *iso*-pentenyladenosine, LT – low temperature, SE – standard error, wt – wild type, Z – zeatin, ZR – zeatin riboside

Introduction

Senescence as programmed ageing process leads to plant death (Dangl *et al.* 2000). According to Nooden *et al.* (1997) leaf senescence resembles processes occurring at oxidative stress. Levels of reactive oxygen species increased during senescence likewise after environmental stress (Merzlyak and Hendry 1994). The plant responses to different environmental stresses are specifically mediated by plant hormones. Cytokinins play an important role in several aspects of plant growth, metabolism and development at normal growth conditions. The mechanisms by which environmental changes affect cytokinins are still not clear but their adaptive function is undoubted. It is suggested that cytokinins

may prevent oxidation and delay senescence (Musgrave 1994) and, probably the antisenescence properties of these phytohormones are related to their antioxidant activity (Pauls and Thompson 1982).

There are data on endogenous cytokinin levels at different stress conditions. In general, the cytokinin titers decreased at abnormal growth conditions like drought, excess salinity, low and high temperature, changes in nutrient solutions (see Letham 1978; Goodwin *et al.* 1978; Van Staden and Davey; 1979, Hare *et al.* 1997), upon micropropagation (Zaffari *et al.* 1998) and herbicide treatment (Sergiev and Karanov, unpublished). There are also data on increased cytokinin levels at stress conditions – such as heat shock (Veselov *et al.* 1995), water stress (Lopez-Carbonell *et al.* 1996), rust (Kiraly *et al.* 1967) and viral (Whenham 1989) infections as well as treatment with exogenous human interferon 2 and 2'-5'oligoadenylates (Kulaeva *et al.* 1992).

Mutants with altered sensitivity/production of plant hormones are a convenient model system to investigate the role of phytohormones in different physiological processes (Scott 1990). Earlier it was established that ethylene-insensitive *Arabidopsis* mutant - *eti5* had characteristics of delayed senescence (Harpham *et al.* 1991) accompanied by higher amount of leaf pigments and soluble proteins (Sergiev *et al.* 2003) as well as elevated endogenous polyamines (Todorova *et al.* 2002) and higher level of cytokinins (Kudryakova *et al*. 2001). These data lead us to the suggestion that wild type and *eti5* mutant of *Arabidopsis thaliana* might differ in tolerance to environmental stresses and temperature stress in particular. It is known that *Arabidopsis* is a chilling-tolerant plant (Jarillo *et al.* 1993). On the other hand, there is little information about the effect of high temperature on *Arabidopsis*. Regarding this preliminary information it was of interest to study the cytokinin levels in wild type and ethylene-insensitive mutant *eti5* of *Arabidopsis* subjected to extreme temperatures. We attempted to discuss changes in cytokinin content in the context of plant tolerance to low and high temperature stresses.

Materials and Methods

Plant material and treatment

Wild type (wt) and ethylene-insensitive mutant (*eti5*) plants of *Arabidopsis thaliana* (L.) Heynh were grown in a growth chamber at the following conditions: 16/8 h day/night photoperiod, 67 μmol. m^{-2} s^{-1} photon flux density, 26/22 °C day/night temperature and 60 % air humidity. All plants (38 day-old) were transferred into darkness for 24 h, including the controls, and at the same time some of them were subjected to low 4 °C (LT) or high 38 °C (HT) temperature treatments. The normal growth conditions were restored after treatments and samples were collected at every subsequent stage of recovery period – 0, 24, 48 and 120 h. Measurements were performed using material derived from 3-7 leaf nodes.

Cytokinin extraction

Cytokinins were extracted with 80 % CH_3OH for 12 h at 4 °C in the dark. After that the methanol extracts were passed through a C_{18} cartridge (Sep-Pak, Waters Ass., Milford, MA). Losses of the cytokinins after the purification were tested in advance (Genkov *et al.* 1996) and varied from 4 to 8 %. The eluates were evaporated in vacuum at 45 °C and cytokinins were analysed in the aqueous phase by ELISA.

ELISA

Cytokinin contents were measured using polyclonal antibodies against iPA, ZR and dZR. The polyclonal antibodies were preliminary characterised for their specificity by cross-reactivity studies with different structural cytokinin analogues (Genkov *et al.* 1996) according to Weiler and Zenk (1976) and the detected values were insignificant (below 5 %). Antibodies were highly reactive with their corresponding free bases (64 % for anti-iPA, 85 % for anti-ZR and 86 % for anti-dZR) (Genkov *et al.* 1996).

For the assay, polystyrene wells were uniformly coated with polyclonal antibodies against iPA, ZR and dZR in 50 mM $NaHCO_3$, pH 9.6 and the microtitration plates were left at 4 °C for 16 h. After washing, the wells were filled with 0.25 % solution

of bovine serum albumin in phosphate buffered saline and incubated at a room temperature for 30 min. After washing, the wells were filled with equal volumes of plant extract or cytokinin standard solutions (0.1-100 pmol) and incubated at 37 °C for 1h. After the addition of enzyme tracer the plate was incubated at 37 °C for 1h and washed. The phosphatase activity was measured by adding of a p-nitrophenylphosphate solution (1 mg/ml in 50 mM $NaHCO_3$, pH 9.6) to each well and after incubation at 37 °C for 1h the reaction was stopped by 2 N NaON. Absorbance was measured at 405 nm. The values were corrected for non-specific binding and the content of the endogenous cytokinin equivalents was calculated, using the linearized standard curve by Logit-transformation (Weiler *et al.* 1981).

Replication

All experiments were repeated three times with three to five replicates in each. The data presented in the figure are means ± SE.

Results and Discussion

The experiment was conducted with 38 day-old (5-6-week-old) plants. It was established that at normal growth conditions *eti5* plants contained higher levels of endogenous cytokinins (197.2 pmol/g FW) comparing to the wild type (wt) (105.6 pmol/g FW). The transfer of plants in darkness for 24 hours did not influence significantly control cytokinin levels (see Table).

According to Kudryakova *et al.* (2001) young leaves (3 weeks old) of ethylene-insensitive *eti5* mutant contained approximately 17 times more endogenous cytokinins (zeatin and its riboside) than wt plants. This difference decreased with plant age. Five-week-old plants exhibited only three times higher cytokinin amount than wt and this difference was negligible in 7-week-old plants. Cytokinin titers detected in our study confirmed Z+ZR contents reported elsewhere (Kudryakova *et al.* 2001). Additionally, increased dZ+dZR and iP+iPA levels in 5-6-week-old mutant plants were found.

It has been reported that under stress conditions such as high (Mauk and Langille 1978) and low (Tantau and Dorffling 1991) temperature the levels of endogenous cytokinins declined strongly. Our data showed that at the first measurement (point 0 h) in wt plants HT provoked a decline in the amounts of Z, dZ and iP equivalents compared to the data obtained in LT experiment where elevated levels of cytokinins were observed (Figure, A1). A similar observation was made for mutant plants but when compared to normal growth conditions (Figure, B1). Likewise, the reduced cytokinin levels in wt plants subjected to HT were due to the natural chilling tolerance of *Arabidopsis* (Jarillo *et al.* 1993). LT also provoked decrease in contents of all the cytokinins in *eti5*.

After 24 h (Figure, A2) the levels of dZ and iP in both low and high temperature-treated wt plants remained below the control rates. The Z amount, however, was almost equal to the control one. However, 24 h after temperature treatment a certain rise of all cytokinins was observed in the stressed mutant plants compared to the contents detected at first measurement (Figure, B2).

At 48 h the levels of dZ, Z and iP in the temperature-treated plants of wt *Arabidopsis* were above controls (Figure, A3). Similar data were reported by Veselov *et al.* (1995). The authors found that in heated wild-type and *ipt*-transformed tobacco plants the levels of Z equivalent were elevated on the second day after the heat-shock treatment.

Table. Total cytokinin content in wild type (wt) and ethylene-insensitive (*eti5*) mutant of *Arabidopsis thaliana*, subjected to 4 °C (LT) and 38 °C (HT)

Measurement point	wt			*eti5*		
	Control	LT	HT	Control	LT	HT
	pmol/g FW	% of control		pmol/g FW	% of control	
0 h	108.4	111	80	196.1	73	71
24 h	124.0	76	80	193.0	85	99
48 h	104.3	145	158	185.6	81	81
120 h	97.5	121	81	170.2	82	53

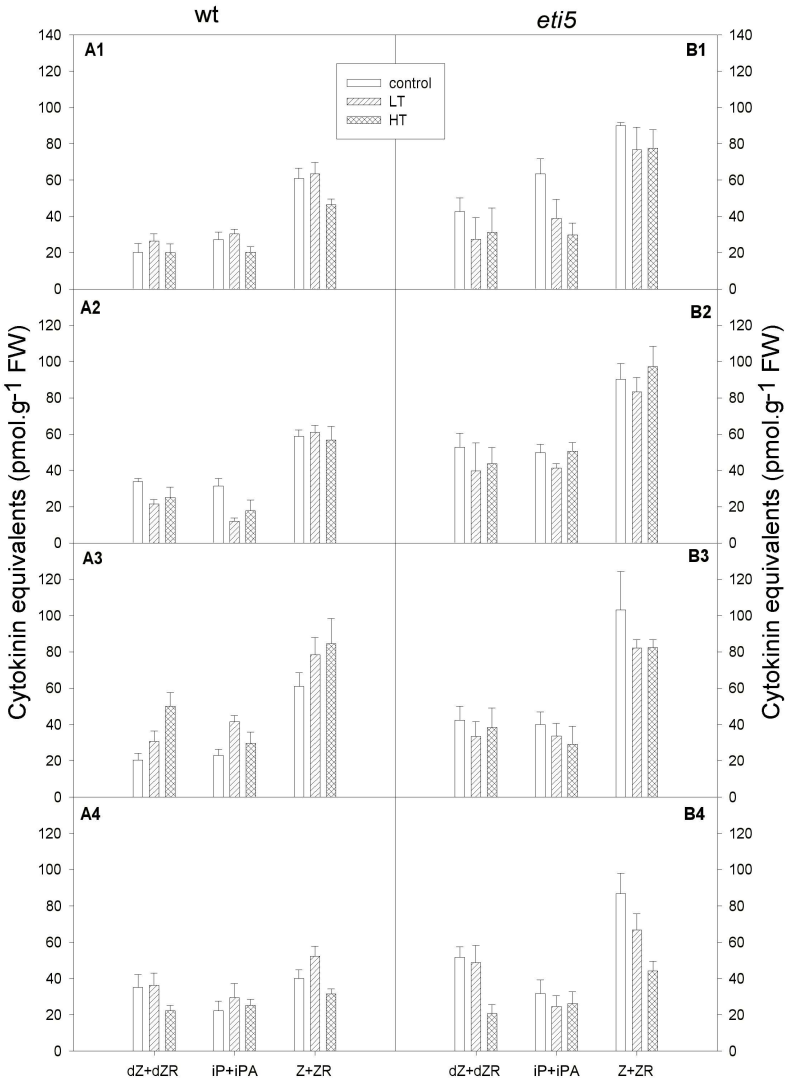

Figure. Cytokinin equivalents (pmol/g FW) in rosette leaves of wild type (wt) (**A**) and ethylene-insensitive (*eti5*) mutant (**B**) *Arabidopsis thaliana* measured after 4 °C (LT) and 38 °C (HT) treatments. Data are obtained from measurements done at 0 h (**1**), 24 h (**2**), 48 h (**3**) and 120 h (**4**) after temperature treatments.

However, in our model system the response of the mutant plants was the opposite – levels of the three cytokinins were below the control (Figure, B3).

At the end of the experiment (120 h), the only levels higher than the control were detected in LT-treated wt plants. All other cytokinin levels were below the controls in both genotypes with exception of iP detected in HT-stressed wt plants (Figure, A4). In *eti5*, HT provoked much more pronounced decrease in cytokinin levels than LT (Figure, B4).

Our data on the cytokinin content in the control plants are in contrast with the results of Tantau and Dorffling (1991), who observed remarkable differences in the levels of Z and ZR in chilling sensitive and chilling tolerant *Euphorbia pulcherrima* plants. In their experiments, the sensitive variety had about four times higher Z and ZR levels. However, LT stress strongly reduced the cytokinin content in chilling sensitive plants while in chilling tolerant – only a slight drop was found. Moreover, the authors suggested that the strong decrease in the cytokinin level in chilling sensitive plants could be considered as an indicator of the higher chilling stress sensitivity of this variety (Tantau and Dorffling 1991). However, we could not make the same conclusion. Previous data for the lower levels of some endogenous stress markers (H_2O_2, products of lipid peroxidation – MDA, products of oxidative protein modifications) in mutants compared to the wt *Arabidopsis* showed that *eti5* was less influenced by LT treatments. Additionally increased catalase (detoxifying H_2O_2 enzyme) and decreased superoxide-dismutase (producing H_2O_2 enzyme) activity in *eti5* was detected and this was in agreement with the above statement (Alexieva *et al.* 1998a, b).

Mauk and Langille (1978) reported lower ZR levels in potato plants, grown at higher temperature with 18-h photoperiod compared to those grown at lower temperature with reduced photoperiod (10 h). Data obtained in the present study showed that in general this trend was the same in both genotypes of *Arabidopsis*. The level of Z equivalents in plants subjected to HT was below the one studied in plants

treated with LT during all the experiment except 48-h (wt) and 24-h (*eti5*) stages. The same trends are valid for the total cytokinin content where the data are represented as % of the control (Table).

In a previous study with the same model system HT treatment caused more significant changes in Chlorophyllase activity and chlorophyll content than LT (Todorov *et al*. 2003). Our previous data indicated higher stress marker levels after HT compared to LT treatment in both genotypes. On the other hand HT compared to LT (Alexieva *et al*. 1998 a, b) more significantly inhibited stress defense enzyme activities. Generally, the greater reduction of hormone levels in the HT-treated plants at the end of the recovery period suggested that HT more effectively influenced the cytokinin content than LT. We assumed that these results originated from the natural chilling tolerance of *Arabidopsis* plants.

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