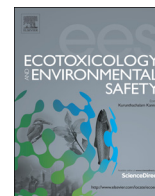




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The long-term effect of zinc soil contamination on selected free amino acids playing an important role in plant adaptation to stress and senescence

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ABSTRACT

Increased endogenous plant cytokinin (CK) content through transformation with an isopentenyl transferase (*ipt*) gene has been associated with improved plant stress tolerance. The objective of this study is to determine amino acid changes associated with elevated CK production in *ipt* transgenic tobacco (*Nicotiana tabacum* L., cv. Wisconsin 38). Nontransformed (WT) and transformed tobacco plants with *ipt* gene controlled by senescence-activated promoter (SAG) were exposed to zinc soil contamination (tested levels Zn1=250, Zn2=500, Zn3=750 mg kg⁻¹ soil). The Zn effect on plant stress metabolism resulted in changes in levels of selected free amino acids playing an important role in adaptation to stress and plant senescence (alanine, leucine, proline, methionine and γ -aminobutyrate) and differed for transformed and nontransformed tobacco plants. Analyses of amino acids confirmed that SAG tobacco plants had improved zinc tolerance compared with the WT plants. The enhanced Zn tolerance of SAG plants was associated with the maintenance of accumulation of proline, methionine and γ -aminobutyrate. The concentrations of leucine and alanine did not show significant differences between plant lines.

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1. Introduction

The presence of toxic elements in a soil affects plant metabolism of many cellular compounds such as glutathione, organic acids, stress proteins and amino acids. A number of organic acids and amino acids in plant tissues can act as possible ligands for metal complexation, thereby conferring metal tolerance (Hall, 2002). Regulation of accumulation of amino acids and metabolism of their products may play an important role in the plant development, growth and stress resistance. The significance of polypeptides and proteins with high contents of amino acids proline, hydroxyproline, glycine, cysteine, leucine and methionine has been proved to play important role in plant cell wall growth and in stress adaptation as osmotic adjustment *via* accumulation of compatible osmolytes (Bradley et al., 1992; Cheung et al., 1995; Davies and Robinson, 2000; Pavlíková et al., 2008; Zemanová et al., 2013). Amino acids alanine and γ -aminobutyrate are especially discussed in relation to intracellular pH regulation (Crawford

et al., 1994; Singh, 1999; Pavlíková et al., 2008; Xu et al., 2012). Solanki and Dhankhar (2011) reported that when trace element toxicity exceeds the threshold limit, the protein level decreases and this might be due to the breakdown of protein synthesis mechanism at toxic concentration level of trace elements and/or due to reduced incorporation of free amino acid into protein.

Cytokinin (CK) levels have been found to change significantly in plants under a variety of stress conditions including trace elements. A decline in CK production has been associated with leaf senescence and the growth inhibition of shoots induced by abiotic stresses. Molecular techniques such as transformation with a gene encoding isopentenyl transferase (*ipt*) to increase endogenous CK content under stress conditions have been found to be effective means to improve stress tolerance in various plant species (Peleg et al., 2011; Merewitz et al., 2012). CK are connected with biosyntheses of sterols and isoprenoids *via* isopentenyl and with the metabolism of lipids and citrate cycle *via* acetyl CoA and hence with the formation of N-transport amino acids (e.g. Gln, Glu, Asp, Asn). Plants with introduced SAG12:*ipt* gene construct, which increases CK biosynthesis in response to stress and thus these plants have longer life span (Gan and Amasimo, 1995, 1996), showed better tolerance against abiotic stresses compared to nontransformed plants (Merewitz et al., 2010; Procházková et al., 2012). Their

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resistance was associated with the maintenance of greater antioxidant enzyme activities (Merewitz et al., 2011). According to Atici et al. (2005) and Xu et al. (2013) high Zn concentration can have stronger effects on CK metabolism and induced a decrease in CK content in plants, while optimum Zn concentration increased their content. Previous studies have highlighted the close correlation between nitrogen and CK in several plant species (Cline et al., 2006; Takei et al. 2001, etc.). The majority of nitrate-specific responsive genes are involved in nitrate assimilation, synthesis of N-transport amino acids, and the related pathways supporting assimilation. By contrast, CK affects other hormones metabolism and signaling, cell wall expansion and protein synthesis (Sakakibara et al., 2006). Therefore, the objectives of this study were to evaluate if the increased CK level in SAG12:ipt transgenic tobacco has some important role in plant adaptation to stress and in senescence induced by zinc contamination, which also influences CK levels, with emphasis on the possible role of amino acids.

2. Material and methods

2.1. Plant material and cultivation conditions

For pot experiment, tobacco plants (*Nicotiana tabacum* L., cv. Wisconsin 38) transformed with a construct consisting of SAG12 promoter fused with *ipt* gene for CK synthesis (SAG plants) were planted into plastic pots containing the soil as specified below. The seeds were kind gift from Prof. R. Amasino, University of Wisconsin, USA. As a control its wild type (WT plants) was used. After 30 days of in vitro precultivation, plants (three plants per pot) were cultivated for 90 days in the soil from the non-polluted site Prague-Suchdol (Chernozem – pH=7.2, CEC=258 mol₍₊₎/kg, C_{org.}= 1.8 percent, Zn_T=106.0 mg kg⁻¹).

For the plant cultivation, 5 kg of soil was thoroughly mixed with 2 g N, 0.45 g P, and 1.1 g K applied in the form of NH₄NO₃ and K₂HPO₄ for control treatment and with the same amount of nutrients plus Zn (applied as Zn(CH₃COO)₂·2H₂O) in three concentrations (Zn1=250, Zn2=500, Zn3=750 mg kg⁻¹ soil) for treated variants. Each treatment was performed in five replications.

2.2. Analyses

2.2.1. Analysis of free amino acids in plant biomass

The amino acids from methanol+H₂O extracts from mature leaves were determined using EZ-faast amino acid analysis procedure (Phenomenex, USA). Samples were analyzed for amino acid contents by GC-MS using the Hewlett Packard 6890N/5975 MSD (Agilent Technologies, USA). Samples were separated on a ZB-AAA 10 m × 0.25 mm amino acid analysis GC column with the carrier gas (He) flow kept constant at 1.1 ml min⁻¹. The oven temperature program was the following: initial temperature 110 °C, 30 °C min⁻¹ ramp to 320 °C. The temperature of the injection port was 280 °C. Samples in volume of 1.5–2 μl sample were injected in split mode (1:15, v/v). MS conditions were as follows: MS source 240 °C, MS quad 180 °C, auxiliary 310 °C, electron energy 70 eV, scan m/z range 45–450 and sampling rate 3.5 scan s⁻¹ (Neuberg et al., 2010).

The complex of free amino acids was determined and for evaluation alanine (Ala), leucine (Leu), proline (Pro), methionine (Met) and γ-aminobutyrate (GABA) were chosen, because they play an important role in adaptation to stress and plant senescence.

Table 1
Total content of free amino acids (μmol g⁻¹ FW) in aboveground biomass of tobacco plants treated with different Zn concentrations in soil (Zn1=250, Zn2= 500, Zn3=750 mg kg⁻¹ soil). WT=nontransformed tobacco, SAG=tobacco with inserted gene *ipt* for CK biosynthesis under SAG promoter. The values represent the means of data obtained in the experiment (n=5, i.e. five replications per each treatment).

Treatment	Alanine (ALA)	Leucine (LEU)	Proline (PRO)	γ-Aminobutyrate (GABA)	Methionine (MET)
WT- 0	273.0 ± 8.4	20.7 ± 2.9	5757 ± 128	9.4 ± 0.9	170.2 ± 26.4
WT - Zn1	262.5 ± 14.6	34.1 ± 5.1	6672 ± 231	5.3 ± 0.7	110.1 ± 19.3
WT - Zn2	238.0 ± 10.9	37.0 ± 7.3	10172 ± 298	2.8 ± 0.4	98.8 ± 10.2
WT - Zn3	231.2 ± 9.2	40.4 ± 4.8	10637 ± 198	0.1 ± 0.02	89.6 ± 15.4
SAG - 0	289.3 ± 11.8	24.9 ± 2.5	10136 ± 254	4.0 ± 0.2	57.0 ± 9.1
SAG - Zn1	300.0 ± 15.4	35.8 ± 8.1	11679 ± 291	3.9 ± 0.5	105.9 ± 10.6
SAG - Zn2	276.4 ± 20.7	42.9 ± 6.4	13475 ± 169	3.7 ± 0.4	109.2 ± 11.7
SAG - Zn3	259.4 ± 21.3	42.9 ± 7.9	13690 ± 301	3.5 ± 0.6	113.3 ± 9.8

2.2.2. Analyses of zinc in plant biomass

Plant samples were processed using the dry ashing procedure as follows: an aliquot (~1 g) of the dried and powdered biomass was weighed into a borosilicate glass test-tube and mineralized in a mixture of oxidizing gases (O₂+O₃+NO_x) at 400 °C for 10 h in a Dry Mode Mineralizer Apion (Tessek, Czech Republic). The ash was dissolved in 20 ml of 1.5 percent HNO₃ (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until the analysis. Aliquots of the certified reference material RM NCS DC 73350 poplar leaves (purchased from Analytika, CZ) were mineralized under the same conditions for quality assurance. The Zn concentrations were determined by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia).

For the calculation of linear correlation (R²) Statistica for Windows version 7.0 CZ was used (StatSoft, Inc., Tulsa, USA).

3. Results

Results of the pot experiment revealed the different toxic effect of Zn on WT and SAG tobacco plants. The biomass yields of both WT and SAG plants were reduced by Zn2 and Zn3 concentrations (Zn2 – by fifteen (WT) and three (SAG) percent and Zn3 – by eighteen percent for both plant lines in contrast to control treatments). Both tobacco lines accumulated Zn proportionally to the concentrations in soil. The increases of 154 percent for WT Zn3 and 123 percent for SAG Zn3 were determined (all data are not shown here).

Amino acids were differentially regulated between two tested plant lines. Total content of free amino acids in all treatments of WT plants was lower compared to SAG plants. All tested amino acids displayed significant linear relation with Zn content in above ground biomass of WT plants (R²=0.70–0.95). For SAG plants calculated correlation was less significant (R²=0.36–0.87) (Tables 2 and 3).

Levels of free proline (Pro) increased in response to Zn stress in both plant lines, however, SAG control plants had significantly greater content of Pro than WT plants (by 43 percent). Compared to the untreated control, concentration of free Pro in WT plants under Zn3 treatment was enhanced up to 1.85-fold (Table 1). Increase of this amino acid in SAG plants was only 1.35-fold. The linear correlations between Pro and Zn content in WT plants (R²=0.70) and in SAG plants (R²=0.56) were calculated. This amino acid (Tables 2 and 3) shows significant linear relation with all amino acids discussed here (R²=0.55–0.99). The significant relation between biomass yield and Pro content was confirmed only for WT (Table 2).

Ala and GABA contents were declined in WT and SAG plants but the changes in SAG plants are less significant. Zn3 treatment caused a decrease of Ala (15 percent WT plants; ten percent SAG plants) and GABA (99 percent WT plants; 12 percent SAG plants) contents. The relationship between contents of GABA and Ala in plant biomass was confirmed by our results using linear correlation and significant relationship was calculated for both WT and SAG plants (R²=0.93 and 0.82, respectively).

The importance of the branched-chain amino acids – valine (Val), leucine (Leu), and isoleucine (Ile) – as building blocks of proteins in plants is obvious. The contents of Val and Ile were

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