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Nitrogen metabolism and gas exchange parameters associated with zinc stress in tobacco expressing an *ipt* gene for cytokinin synthesis

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SUMMARY

Increased endogenous plant cytokinin (CK) content through transformation with an isopentyl transferase (*ipt*) gene has been associated with improved plant stress tolerance. The impact of zinc (tested levels Zn1 = 250, Zn2 = 500, Zn3 = 750 mg kg^{-1} soil) on gas exchange parameters (net photosynthetic rate, transpiration rate, stomatal conductance, intercellular CO_2 concentration) and nitrogen utilization by plants resulted in changes of free amino acid concentrations (glutamic acid, glutamine, asparagine, aspartate, glycine, serine, cystein) and differed for transformed and non-transformed tobacco plants. For pot experiments, tobacco plants (*Nicotiana tabacum* L., cv. Wisconsin 38) transformed with a construct consisting of *SAG*12 promoter fused with the *ipt* gene for cytokinin synthesis (SAG plants) and its wild type (WT plants as a control) were used. Physiological analyses confirmed that SAG 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 amino acids and with lower declines of photosynthetic and transpiration rates. In comparison to WT plants, SAG plants exposed to the highest Zn concentration accumulated lower concentrations of asparagine, which is a major metabolic product during senescence.

Introduction

Zinc is an important component of a large number of plant enzymes. It is associated with carbohydrate metabolism, protein synthesis, and gene expression and regulation (Broadley et al., 2007). Zinc plays critical roles in the defense system of cells against oxidative stress, and thus represents a protective agent against the oxidation of several vital cell components, such as membrane lipid and chlorophyll (Cakmak, 2000). According to Puzina (2004), Zn application to plants shifted the hormonal balance toward a substantial increase in the cytokinin (CK) content and the CK/ABA ratio, as well as a decrease in the IAA/CK ratio. Depending on the plant species and plant part, Zn proportions in the range of 58–91% of total Zn content may be soluble (Brown et al., 1993; Pavlíková et al., 2001). This soluble Zn part is considered to be the physiologically active fraction. A high Zn concentration plays a negative toxic role in plant metabolism. At the organism level, excess Zn inhibits seed germination, plant growth (Mrozek and Funicelli, 1982) and root development (Lingua et al., 2008), and causes leaf chlorosis (Ebbs and Kochian, 1997; Wang et al., 2009). It is likely that trace element stress induces senescence through enhancement of catabolism of key metabolites such as chlorophyll, protein and RNA (Khudsar et al., 2004). At the cellular level, excess Zn can significantly alter mitotic activity (Rout and Das, 2003), affect membrane integrity and permeability (Stoyanova and Doncheva, 2002), and even kill cells (Chang et al., 2005; Wang et al., 2009). At the cellular level, excess Zn can significantly alter gene expression. Many products of these genes are involved in various biological processes (e.g. lignin biosynthesis), among which are many genes encoding proteins that are associated with defense against oxidative stress (Van de Mortel et al., 2006; Wang et al., 2009). Several studies have demonstrated the effects of Zn stress on the activity of many antioxidative enzymes and low-molecular antioxidants in plants

Abbreviations: Asp, aspartate; Asn, asparagine; C_i, intercellular CO_2 concentration; CK, cytokinins; Cys, cysteine; E, transpiration rate; g_s, stomatal conductance; Glu, glutamate; Gln, glutamine; Gly, glycine; *ipt*, isopentyl transferase; P_N, net photosynthetic rate; SAG, tobacco plants transformed with a construct consisting of *SAG*12 promoter fused with *ipt* gene for cytokinin synthesis; Ser, serine; WT, wild type of tobacco plants.

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(Wójcik et al., 2006; Tewari et al., 2008). These data suggest there is cross-talk between Zn-induced differentially expressed genes and antioxidant defensive genes, and represents a complex mechanism developed to cope with Zn toxicity (Van de Mortel et al., 2006).

Zn also causes the decline in protein content and the corresponding increase in the activity of hydrolytic enzymes such as protease due to trace element stress, and this strongly suggests the induction of catabolic activities. A decline in the protein level may be a consequence of a decrease in nitrate reductase activity. Solanki and Dhankhar (2011) reported that when trace element toxicity crosses the threshold limit, the protein level decreases, and this might be due to the breakdown of the protein synthesis mechanism at toxic concentration levels of trace elements or due to reduced incorporation of free amino acids into proteins. According to Atici et al. (2005), high Zn concentrations can affect CK metabolism and decrease CK content in plants, while optimum Zn concentrations increased CK content.

CK levels have been found to change significantly in plants under a variety of stress conditions. The role of CK in plant during stress is relatively inconsistent (Ha et al., 2012). Their endogenous level decreases in response to various stress conditions (Hare et al., 1997). Alvarez et al. (2008) found that isoprene-type CK declined, but benzylaminopurine was concurrently elevated in drought-stressed maize. The effect of CK on the plant stress response was investigated mainly by exogenous CK application and in plants over-expressing the isopentyl transferase (*ipt*) gene under different promoters. A positive role of CK application in abiotic stresses has been shown in many studies. For example, CK alleviated drought (Hu et al., 2013), heat (Barciszewski et al., 2000; Wang et al., 2012) and salinity (Barciszewski et al., 2000). CKs reverse ABA-induced stomatal closure (Pospíšilová, 2003), thus promoting stomatal reopening following drought, leading to enhanced stomatal conductance (g_s) (Hu et al., 2013). Plants with the introduced *SAG*12:*ipt* gene construct, which increases CK biosynthesis in response to senescence initiation. Thus, these plants have a longer life span and show better tolerance against abiotic stresses compared to non-transformed plants (Merewitz et al., 2010; Procházková et al., 2012; Pavlíková et al., 2014). Their resistance has been associated with the maintenance of greater antioxidant enzyme activities (Merewitz et al., 2011a,b). According to Thomas et al. (2005), enhanced CK production in transgenic tobacco also led to lower lipid peroxidation compared to controls under non-stressed and copper-stressed conditions.

The objectives of the study were to evaluate metabolite changes differentially exhibited between *ipt* and non-transgenic tobacco plants under zinc stress conditions in order to identify potential tolerance mechanisms related to the maintenance of CK content under trace element stress in tobacco. We hypothesized that the impact of Zn on photosynthesis and nitrogen utilization by plants can result in changes of free amino acid concentrations, and that these changes can differ in transformed and non-transformed plants.

Material and methods

Plant material and cultivation conditions

For the pot experiment, tobacco plants (*Nicotiana tabacum* L., cv. Wisconsin 38) transformed with a construct consisting of the *SAG*12 promoter fused with *ipt* gene for CK synthesis (SAG) were planted. The seeds were a gift from Prof. R. Amasino at the University of Wisconsin, USA. As the control, its wild type (WT plants) was used. After 30 days of *in vitro* pre-cultivation, plants (three plants per pot) were cultivated for 90 days in pots. The pots were filled with the soil from the non-polluted site Prague-Suchdol (Chernozem – pH = 7.2, CEC = 258 $mol_{(+)}$/kg, $C_{org.}$ = 1.8%, Zn_T = 106.0 mg kg^{-1}).

For cultivation of tobacco plants, 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 ammonium nitrate and potassium hydrogen phosphate for control treatment, and with the same amount of nutrients plus Zn (applied in $Zn(CH_3COO)_2{\cdot}2H_2O$) for treated variants. Three concentrations of Zn (Zn1 = 250, Zn2 = 500, Zn3 = 750 mg kg^{-1} soil) were applied. Plants were grown in a greenhouse under natural light and temperature conditions. The water regime was controlled and the soil moisture was kept at 60% MWHC (maximum water-holding capacity). Each treatment was performed in five replications.

Analyses

Determination of gas-exchange parameters

The net photosynthetic rate (P_N), transpiration rate (E), g_s, and intercellular CO_2 concentration (C_i) were measured in the leaves in situ using the portable gas-exchange system LCpro+ (ADC BioScientific Ltd., Hoddesdon, Great Britain) from 10:00 to 11:30 Central European summer time. The irradiance was 565 μmol m^{-2} s^{-1} photosynthetically active radiation, the temperature in the measurement chamber was 26.5–27.9 °C, the CO_2 concentration was 550 ± 50 cm^3 m^{-3}, the air flow rate was 205 ± 30 μmol s^{-1} and the duration of the measurement of each sample was 15 min after the establishment of steady-state conditions inside the measurement chamber (Holá et al., 2010).

Analysis of free amino acids in plant biomass

The amino acids from methanol + H_2O extracts were determined using the EZ-fast amino acid analysis procedure (Phenomenex, USA). Samples were analyzed for amino acid contents by GC–MS using a 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 using constant carrier gas (He) flow (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. 1.5–2 μl sample was 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 was 70 eV, scan m/z range 45–450 and sampling rate was 3.5 scan s^{-1} (Neuberg et al., 2010).

The complex of free amino acids was determined, and glutamic acid, glutamine (Gln), asparagine (Asn), aspartate (Asp), glycine (Gly), serine (Ser), and cystein were chosen for evaluation. These amino acids play an important role in the synthesis of active forms of phytohormones or in photorespiration. The concentrations of the free Gln, tryptophan and cysteine (Cys) were below the detection limits of GC.

Analyses of zinc in plant biomass

Plant samples were decomposed 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 decomposed in a mixture of oxidizing gases (O_2 + O_3 + 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% HNO_3 (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until 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).

Analyses of total nitrogen in plant biomass

The dried above-ground biomass was used for determination of total N. For determination of total N content, the plant material was decomposed by a liquid ashing procedure in H_2SO_4 solution (1:20,