Environmental and Experimental Botany 72 (2011) 167–173

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency

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ARTICLE INFO

Article history:
Received 20 October 2010
Received in revised form 10 March 2011
Accepted 10 March 2011

Keywords:
Cytokinins
Tobacco plants
Nitrogen deficiency
Oxidative stress
Antioxidant system

ABSTRACT

Wild type and transgenic tobacco plants expressing isopentenyltransferase, a gene coding the rate-limiting step in cytokinin synthesis, were grown under limited nitrogen (N) conditions. Our results indicated that the WT plants subjected to N deficiency displayed reduced biomass and relative growth rates, increased levels of oxidative damage and reduced foliar concentrations of the different N forms. However, the transgenic plants expressing P_{SARK}::IPT, in spite of showing a significant decline in all the N forms in the leaf, avoided the alteration of the oxidative metabolism and maintained biomass and the relative growth rates at control levels, under suboptimal N conditions. These results suggest that the increased cytokinin synthesis in the transgenic plants is an effective mechanism to improve N-use efficiency.

1. Introduction

Nitrogen (N) is an essential macronutrient for plants, forming part of nucleic and amino acids, serving its function in protein signalling and regulation. In addition, it is of great importance in the biochemistry of compounds such as enzymes, pigments, secondary metabolites, and polyamines (Maathuis, 2009). The main symptom of N deficiency in plants is leaf senescence provoked by lipid peroxidation and pigment loss as well as protein degradation that leads to the inhibition of photosynthetic capacity (Casano et al., 1994). Thus, during N-deficiency-induced senescence, the rise in reactive oxygen species (ROS), such as superoxide ion (O_2^-) or hydrogen peroxide (H_2O_2) triggers oxidative stress (Grossman and Takahashi, 2001).

Plants can respond to this stress through their antioxidant system, which is composed of non-enzymatic antioxidants such as ascorbate (AA) or glutathione (GSH) and enzymatic antioxidants, including the enzymes superoxide dismutase (SOD) capable of detoxifying the O_2^- and of transforming it into H_2O_2, which later will be eliminated by the action of catalase (CAT), guaiacol peroxidase (GPX) or enzymes belonging to the AA-GSH or Halliwell–Asada cycle, such as ascorbate peroxidase (APX), which reduces H_2O_2 by AA oxidation (Jaleel et al., 2009). The induction of the plant antioxidant system during moderate N-deficiency has been reported (Polesskaya et al., 2004; Tewari et al., 2007). Polesskaya et al. (2004) showed greater activity of SOD, APX, or CAT in wheat plants exposed to N deficiency. Also, Tewari et al. (2007) demonstrated a higher H_2O_2 concentration in leaves of Mulberry subjected to N deficiency, which prompted a rise in lipid peroxidation, in the concentration of antioxidant compounds such as AA or GSH, and in the activity of enzymes in charge of detoxification.

Cytokinins (CKs) are phytohormones that control the plant developmental programme. CKs can also regulate plant responses against abiotic stress (Haberer and Kieber, 2002; Rivero et al., 2007). In addition, a relationship between CKs and macronutrient acquisition has been postulated (Franco-Zorrilla et al., 2002; Brenner et al., 2005). Recent studies indicated that CKs act as long-distance messengers signalling the N status of the plant (Forde, 2002; Takei et al., 2002). Therefore, CKs can act as a signal communicating to the shoot if the N application of the root is adequate, thereby regulating the nutrient uptake systems (Sakakibara et al., 2006). Gan and Amasino (1995) showed that leaf senescence could be delayed in transgenic plants overexpressing isopentenyltransferase (IPT), an enzyme that catalyses the limiting step in CK synthesis. Therefore, the aim of the present work was to evaluate the effect of N deficiency on oxidative metabolism in WT and transgenic tobacco plants expressing P_{SARK}::IPT.

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doi:10.1016/j.envexpbot.2011.03.005

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of WT (*Nicotiana tabaccum* cv.SR1, Wild Type) and transgenic plants expressing P_{SARK}::IPT were germinated and grown in soil for 30 days (d) in a tray with wells (each well 3 cm × 3 cm × 10 cm). During this time, no differences in germination or plant development between WT and the transgenic plants were observed. Afterwards, the seedlings were transferred to a growth chamber under controlled conditions with relative humidity of 50 ± 10%, at 28 °C/20 °C (day/night), and a 16 h/8 h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 μmol m^{-2} s^{-1} (measured with an SB quantum 190 sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions, plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. During 30 d, the plants were grown in a complete nutrient solution containing: 10 mM $NaNO_3$, 2 mM NaH_2PO_4, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM Cl_2Mg, 2 mM Na_2SO_4, 2 μM $MnCl_2$, 0.75 μM $ZnCl_2$, 0.25 μM $CuCl_2$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 5 μM Fe-EDDHA, and 50 μM H_3BO_3, pH 5.8. The nutrient solution was renewed every 3 d and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 60 DAS and were maintained for 30 d. The treatments were 10 mM (Control), 7 mM and 1 mM $NaNO_3$. The experimental design was a randomized complete block with six treatments, arranged in individual pots with six plants per treatment, and three replicates. The experiment was repeated three times under the same conditions ($n = 9$).

2.2. Sampling and determination of the relative growth rate (RGR)

All plants were at the late vegetative stage when harvested. Middle leaves (positions 7th and 8th) were harvested, frozen immediately in liquid N_2, and kept at −80 °C until used. To determine the RGR, leaves from three plants per line were sampled at 60 DAS, before starting the N treatment. The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry biomass (DB) was recorded. The remaining plants were sampled at 90 DAS. The relative growth rate was calculated from the increase in leaf DW at the beginning and at the end of N-treatment, using the equation $RGR = (\ln DW_f - \ln DW_i)/(T_f - T_i)$ where T is the time and the subscripts denote the final and initial sampling (i.e., d 0 and 30, respectively, after nitrogen treatment) (Cervilla et al., 2007).

2.3. Analytical methods

Total reduced N concentration was analysed as described by Baethgen and Alley (1989). NO_3^- was measured by spectrophotometry following Cataldo et al. (1975), and NH_4^+ was determined as described by Krom (1980). Malondialdehyde (MDA) concentration in leaves was determined as described before (Sanchez-Rodriguez et al., 2010).

H_2O_2 content of leaf samples was determined as described by Mukherje and Choudhuri (1983) and the detection of $O_2^{\bullet -}$ was based on its ability to reduce nitro blue tetrazolium (NBT) as described by Kubis (2008). The extraction and quantification of total and reduced ascorbic acid and dehydroascorbate (DHA) was performed according to Okamura (1980) with the modifications by Law et al. (1992). GSH was measured by the recycling assay initially described by Tietze (1969) and modified by Noctor and Foyer (1998).

Pyridine nucleotides were extracted from liquid N-frozen leaves material in 1 mL of 100 mM NaOH for NADPH or 5% TCA for $NADP^+$. Nucleotides were quantified by the enzyme-cycling method (Matsumura and Miyachi, 1980) with some modification (Gibon and Larher, 1997).

SOD (EC 1.15.1.1) activity was assayed according to the methods of Giannopolitis and Ries (1977) and Beyer and Fridovitch (1987), with some modifications (Yu et al., 1998). CAT (1.11.1.6) activity was determined according to Nakano and Asada (1981). GPX (EC 1.11.1.7) activity was determined following a modified version of Cara et al. (2002) using 100 mM K-phosphate buffer (pH 7) for extraction.

APX (EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.1) were assayed following Rao et al. (1996). Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) was measured at 265 nm for 3 min following the change in absorbance resulting from the formation of AA (Nakano and Asada, 1981). The free radical scavenging capacity of extracts was determined as described by Re et al. (1999). The Ferric Reducing Ability of Plasma (FRAP) assay was made with 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 20 mM $FeCl_3$ in 0.25 M CH_3COONa, pH 3.6 (FRAP reagent). An aliquot of 100 mL of extract (1 g per 10 mL in methanol) was added to 2 mL of FRAP reagent and mixed thoroughly. After the mixture was left at room temperature (20 °C) for 5 min, absorbance at 593 was measured. Calibration was against a standard curve (25–1600 mM Fe^{3+}) using freshly prepared ammonium ferrous sulphate (Benzie and Strain, 1996). The Trolox Equivalent Antioxidant Capacity (TEAC) value of an extract represents the concentration of Trolox solution that has the same antioxidant capacity as the extract. TEAC was expressed as mg Trolox g DW^{-1}.

The protein concentration of the extracts was determined according to the method of Bradford (1976), using BSA as the standard.

2.4. Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Fisher's least-significant differences test (LSD).

3. Results

3.1. Effects of reduced NO_3^- on plant biomass and RGR

Nitrogen deficiency resulted in reduced foliar biomass and RGR in the WT plants (Table 1). The application of 7 mM and 1 mM NO_3^- resulted in reductions of 20–33% and 21–33% in foliar biomass and RGR, respectively. Besides, neither biomass nor growth rates of the transgenic plants were affected by the reduction in NO_3^- (Table 1). N-deficiency affected the concentrations of the different N-forms in both WT and transgenic plants. Both lines showed the minimum concentrations of total reduced N, NO_3^- and total N under the 1 mM NO_3^- treatment (Table 2). The reduction in total N was more pronounced in the WT plants (36%) than in the P_{SARK}::IPT plants (19%)

Table 1
Foliar biomass and foliar RGR in two lines of Tobacco plants subjected to N deficit.

NO_3^-	Foliar biomass (g DW)		Foliar RGR (g day^{-1})	
	WT	IPT	WT	IPT
Control	6.77 ± 0.36	9.15 ± 0.23	0.042 ± 0.002	0.048 ± 0.001
7 mM	5.41 ± 0.01	7.88 ± 0.07	0.033 ± 0.001	0.044 ± 0.001
1 mM	4.53 ± 0.08	8.22 ± 0.62	0.028 ± 0.001	0.045 ± 0.001
P-value	**	NS	***	NS
$LSD_{0.05}$	0.745	1.338	0.004	0.004

Values are means ± S.E. ($n = 9$) and differences between means were compared using LSD ($P = 0.05$). Levels of significance are represented by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and NS (not significant) $P > 0.05$.