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

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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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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) $P > 0.05$.

Table 2
Concentration of total reduced N, NO_3^- and total N in two lines of tobacco plants subjected to N deficit.

Lines/NO_3^- treatment	Total reduced N (mg g^{-1} DW)	NO_3^- (mg g^{-1} DW)	Total N (mg g^{-1} DW)
WT			
Control	30.60 ± 2.22	24.94 ± 1.24	55.61 ± 4.02
7 mM	31.74 ± 1.23	27.52 ± 1.30	59.27 ± 1.29
1 mM	19.65 ± 0.98	3.52 ± 0.18	23.18 ± 1.18
P-value	***	***	***
$LSD_{0.05}$	4.596	3.157	8.760
IPT			
Control	30.41 ± 2.03	29.44 ± 1.09	59.86 ± 2.08
7 mM	29.87 ± 1.53	23.28 ± 1.36	53.16 ± 2.81
1 mM	24.77 ± 0.83	3.71 ± 0.28	28.48 ± 1.16
P-value	*	***	***
$LSD_{0.05}$	4.525	3.079	7.376

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) $P > 0.05$.

(Table 2). A treatment of 7 mM NO_3^- had no effect on the different N-forms with exception of a reduction of 20% in NO_3^- content in the transgenic plants (Table 2).

3.2. Effects of reduced NO_3^- on lipid peroxidation, reactive oxygen species and $NADP^+$, NADPH concentration

MDA concentrations, a measure of lipid peroxidation, together with the concentration of ROS (reactive oxygen species) are good indicators of tissue oxidative stress. In WT plants, N-deficiency caused a significant increase in the $O_2^{\bullet-}$ (Fig. 1A) and H_2O_2 (Fig. 1B) concentrations in the leaves, raising the foliar MDA concentration (Fig. 1C). On the contrary, no increase was seen MDA concentration in the transgenic plants subjected to N deficiency (Fig. 1C). Furthermore, neither the $O_2^{\bullet-}$ (Fig. 1A) nor the H_2O_2 (Fig. 1B) concentrations displayed significant differences with N deficiency, both values remaining close to those from the control treatments (10 mM). Besides, N-deficiency affected the concentrations of $NADP^+$ and NADPH (Table 3). In WT plants $NADP^+$ decreased significantly under N treatments, although no differences were observed in $NADP^+$ concentration in transgenic plants.

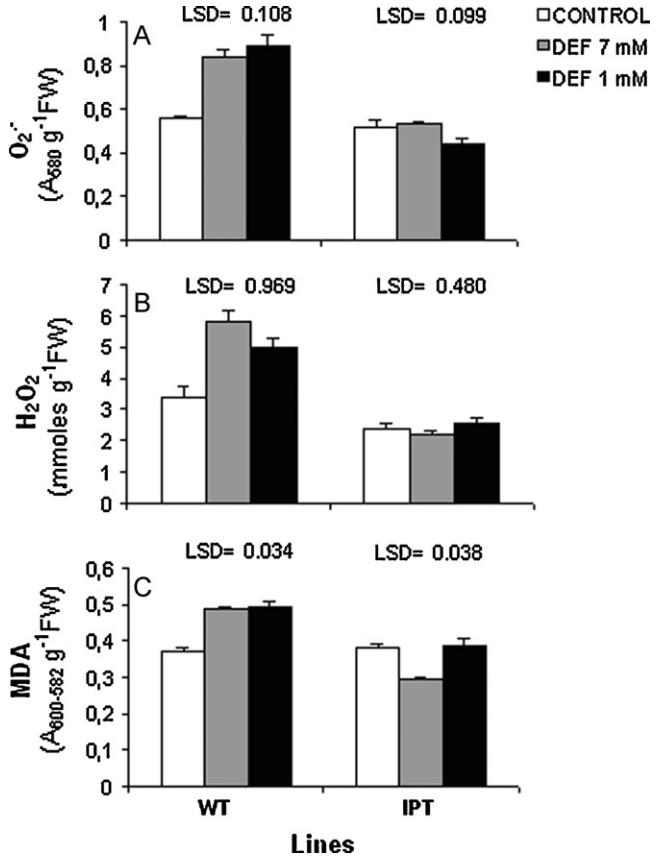

Fig. 1. Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) superoxide ($O_2^{\bullet-}$), (B) hydrogen peroxide (H_2O_2) and (C) malondialdehyde (MDA) concentration in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means ± S.E. ($n = 9$); for each lines.

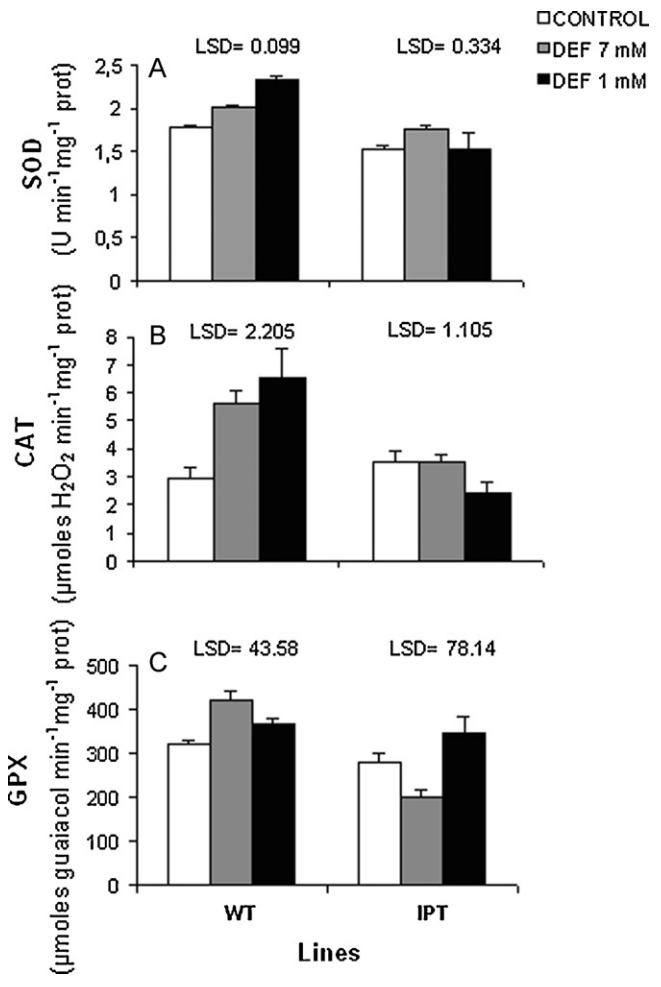

Fig. 2. Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) guaiacol peroxidase (GPX) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means ± S.E. ($n = 9$); for each lines.

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Table 3
Effect of N deficit on $NADP^+$, NADPH and $NADP^+$:NADPH ratio in two lines of tobacco plants.

Lines/NO_3^- treatment	$NADP^+$ (mmol g^{-1} FW)	NADPH (mmol g^{-1} FW)	NADPH/$NADP^+$
WT			
Control	0.554 ± 0.021	0.364 ± 0.017	0.656 ± 0.030
7 mM	0.467 ± 0.017	0.345 ± 0.007	0.739 ± 0.016
1 mM	0.374 ± 0.011	0.412 ± 0.011	1.101 ± 0.030
P-value	***	**	***
$LSD_{0.05}$	0.051	0.037	0.078
IPT			
Control	0.391 ± 0.017	0.333 ± 0.006	0.853 ± 0.017
7 mM	0.338 ± 0.020	0.328 ± 0.030	0.969 ± 0.089
1 mM	0.395 ± 0.027	0.404 ± 0.013	1.024 ± 0.033
P-value	NS	*	NS
$LSD_{0.05}$	0.065	0.056	0.173

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) $P > 0.05$.

NADPH increased with 1 mM treatments in the WT and transgenic plants.

3.3. Effects of N-deficiency on antioxidant activities

During N-deficiency treatments, the activities of some of the foliar antioxidant enzymes increased significantly in the WT plants. SOD, CAT and GPX activities (Fig. 2A–C) increased under both the 1 and 7 mM NO_3^- treatments. While SOD increased with N-deficiency in the WT plants, the activity remained constant in the transgenic plants. A large increase in CAT was observed in the WT plants under low N. Besides, in the transgenic plants, CAT activity remained constant under 7 mM N and was even lower in the 1 mM treatment. GPX activity was higher under 7 mM N (Fig. 2C).

Although no differences were observed in the total AA concentrations in WT plants, there was a significant increase in reduced AA content and a decrease in DHA in the WT plants in the 7 mM and 1 mM treatments (Table 4). As a consequence, the reduced AA to foliar DHA increased significantly during N-deficiency treatments. The transgenic plants showed no differences in total AA concentrations during N-deficiency, although DHA declined in the 1 mM treatment with the concomitant increase in the reduced AA to DHA ratio (Table 4). N-deficiency induced a decrease in total foliar GSH in both WT and transgenic plants (Table 5), and a reduction of GSSG in the WT plants, but not in the transgenic plants.

With regard to the enzymes involved in the Halliwell–Asada cycle, a decrease in the foliar activities of f APX, DHAR, and MDHAR (Fig. 3A–C) was found in the WT grown under lower N-regimes. However, GR activity was lower under the 7 mM treatment (Fig. 3D). Notably, while an increase of foliar APX activity was seen in the P_{SARK}::IPT plants under the 1 mM dosage of N (Fig. 3A), MDHAR activity remained unchanged (Fig. 3C and D) and an increase in DHAR activity was detected only with the 7 mM (Fig. 3B).

The antioxidant activity in tobacco leaves was studied by the analysis of the FRAP and TEAC tests. Whereas in the WT plants, there was a significant increase in both tests for plants subjected to 1 mM of N deficiency, the transgenic plants did not display significant differences among the N treatment (Table 6).

4. Discussion

N deficiency reduced the RGR as well as the biomass in the leaves of WT tobacco plants (Table 1). Similar results have been reported in *Triticum*, *Morus* or *Matricaria* subjected to N deficiencies (Tewari et al., 2004, 2007; Kovácik and Backor, 2007). These reductions in RGR and biomass have been explained based on the numerous functions of this element within the plant (Maathuis, 2009). Furthermore, the N-deficiency induced decline in biomass and RGR, particularly in the case of severe deficiency (1 mM of N) appeared to be directly related to the diminished foliar concentration of total N, total reduced N and NO_3^- (Table 2). In addition, N deficiency, like other abiotic stresses such as drought or heavy metals (Arora et al., 2002) is capable of altering oxidative metabolism. During this stress conditions, the rate of the Calvin cycle is diminished, which prevents oxidation of NADPH and restoration of $NADP^+$. The insufficient electron acceptor $NADP^+$ pool carried accumulation of ROS (Chaves et al., 2009). In our work, the N deficiency increased NADPH:$NADP^+$ ratio (Table 3), this could explain the accumulation of ROS, $O_2^{\bullet -}$ and H_2O_2, in the WT plants, resulting in greater lipid peroxidation (Fig. 1A–C). It is known that under conditions of N deficiency, an accumulation of ROS, results an increase activity of ROS-detoxifying enzymes (Huang et al., 2004). Our result shown the induction of enzymes involved in detoxification of $O_2^{\bullet -}$ and H_2O_2, such as SOD (Fig. 2A), CAT (Fig. 2B), or GPX (Fig. 2C), in WT plants under the 1 and 7 mM N treatments. These results agree with those of Polesskaya et al. (2004), who reported increased activity of these enzymes in wheat plants subjected to N deficiency. However, the increased CAT and GPX activity in tobacco leaves of the WT plants was not sufficient to detoxify all the H_2O_2 accumulated in the leaf during the N deficient treatments (Fig. 1B). This might be due to the diminished activity in the enzymes of the AA regeneration cycle or the Halliwell–Asada cycle, particularly of APX, the enzyme that detoxify H_2O_2 via the oxidation of reduced AA (Jaleel et al., 2009). High APX endogenous levels have been defined as essential for the maintenance of the antioxidant system that protects against damage due to abiotic stress (Shigeoka et al., 2002). The APX activity in WT plants was reduced with N-deficiency (Fig. 3A), leading to the accumulation of reduced AA (Table 4) which was not used by APX. This, together with the failure to induce enzymes such as MDHAR or GR under N deficiency could be determinant in the accumulation of toxic levels of H_2O_2 in the WT plants, since APX in combination with the rest of the enzymes of the Halliwell–Asada cycle are essential in the detoxification of H_2O_2 in photosynthetic organisms (Asada, 1999).

The non-enzymatic antioxidant levels also play an essential role against the deleterious effects of abiotic stress (Jaleel et al., 2009). Prior studies demonstrated that AA and/or GSH increases could be the result of increased *de novo* synthesis as a response to oxidative stress provoked by the N deficiency (Logan et al., 1999; Kandlbinder et al., 2004). Also, Szalai et al. (2009) postulated that greater GSH synthesis under different types of abiotic stress would prompt stronger tolerance of the plant against stress. The inability of the WT plants to increase the concentrations of AA and GSH during stress correlated with their sensitivity to N-deficiency stress. However, the tests for antioxidant capacity (FRAP and TEAC) would suggest that phenols, tocoferols and/or

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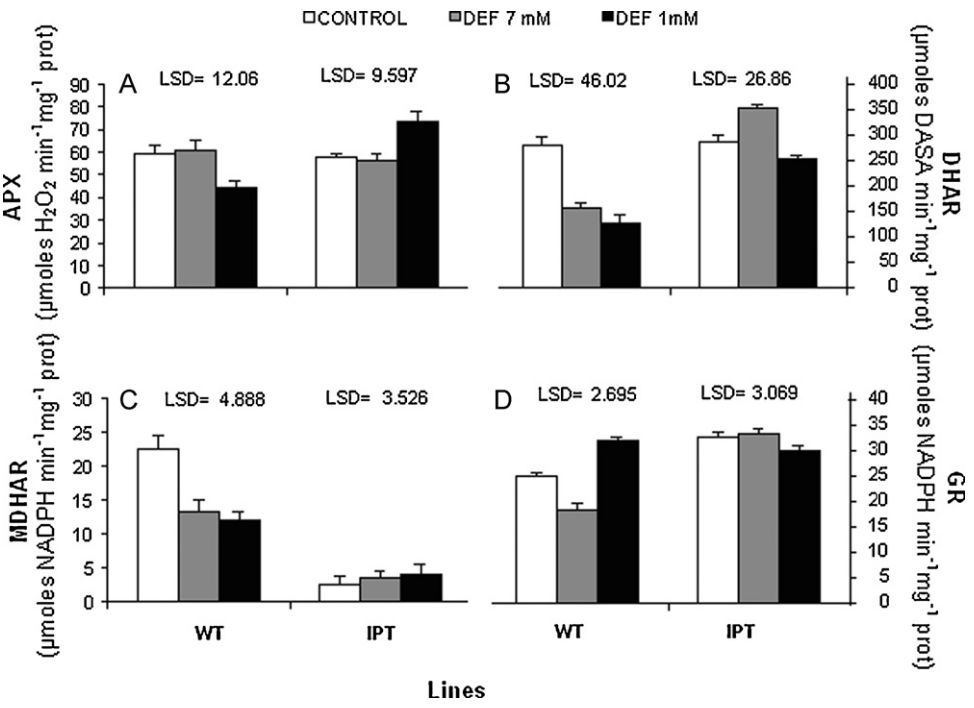

Fig. 3. Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on (A) ascorbate peroxidase (APX), (B) dehydroascorbate reductase (DHAR), (C) monodehydroascorbate reductase (MDHAR) and glutation reductase (GR) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means ± S.E. ($n = 9$); for each lines.

Table 4
Concentration of reduced AA, DHA, total AA and the ratio of reduced AA/DHA in the leaves of two lines of tobacco plants subjected to 3 treatment of N deficit.

Lines/NO_3^- treatment	AA red (mmol g^{-1} FW)	DHA (mmol g^{-1} FW)	Total AA (mmol g^{-1} FW)	AA red/DHA (mmol g^{-1} FW)
WT				
Control	5.99 ± 0.06	2.69 ± 0.20	8.69 ± 0.26	2.34 ± 0.19
7 mM	7.14 ± 0.20	1.48 ± 0.06	8.62 ± 0.15	4.93 ± 0.33
1 mM	7.11 ± 0.06	2.07 ± 0.10	9.19 ± 0.15	3.50 ± 0.19
P-value	***	***	NS	***
$LSD_{0.05}$	0.372	0.409	0.575	0.731
IPT				
Control	4.93 ± 0.04	3.41 ± 0.17	8.34 ± 0.21	1.47 ± 0.06
7 mM	5.06 ± 0.08	3.59 ± 0.17	8.66 ± 0.20	1.43 ± 0.06
1 mM	4.99 ± 0.05	2.75 ± 0.22	7.75 ± 0,21	1.91 ± 0.17
P-value	NS	*	*	**
$LSD_{0.05}$	0.185	0.553	0.615	0.327

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) $P > 0.05$.

carotenoids could increased in the WT plants during N-deficiency (Table 6). Similar results have been reported during the effects of stress on lettuce and tomato (Cervilla et al., 2007; Blasco et al., 2008).

Nitrogenous nutrition has been associated with the synthesis of CKs, since CKs are believed to act as N-sensing because of their role in the regulation of genes involved in the uptake of nutrients (Sakakibara et al., 2006; Rubio et al., 2009). It has been postu-

Table 5
Concentration of reduced GSH, GSSG, total GSH and the relationship of reduced GSH/GSSG in the leaves of two lines of tobacco plants subjected to 3 treatment of N deficit.

Lines/NO_3^- treatment	GSH red (mmol g^{-1} FW)	GSSG (mmol g^{-1} FW)	GSH total (mmol g^{-1} FW)	GSH red/GSSG (mmol g^{-1} FW)
WT				
Control	0.16 ± 0.03	0.28 ± 0.01	0.44 ± 0.03	0.60 ± 0.11
7 mM	0.13 ± 0.04	0.12 ± 0.00	0.25 ± 0.02	1.06 ± 0.12
1 mM	0.15 ± 0.02	0.18 ± 0.01	0.33 ± 0.04	0.86 ± 0.29
P-value	NS	***	**	NS
$LSD_{0.05}$	0.101	0.036	0.107	0.569
IPT				
Control	0.17 ± 0.03	0.15 ± 0.00	0.32 ± 0.04	1.11 ± 0.25
7 mM	0.10 ± 0.02	0.21 ± 0.00	0.31 ± 0.09	0.53 ± 0.12
1 mM	0.09 ± 0.00	0.15 ± 0.00	0.23 ± 0.00	0.66 ± 0.06
P-value	*	**	*	*
$LSD_{0.05}$	0.075	0.037	0.077	0.483

Values are means ± SE ($n = 9$) 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) $P > 0.05$.

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Table 6
Effect of N deficit on antioxidant test FRAP and TEAC in two lines of tobacco plants.

Lines/NO_3^- treatment	FRAP (mg g^{-1} FW)	TEAC (mg g^{-1} FW)
WT		
Control	2.84 ± 0.03	1.10 ± 0.02
7 mM	2.36 ± 0.05	1.09 ± 0.02
1 mM	4.28 ± 0.06	1.42 ± 0.03
P-value	***	***
$LSD_{0.05}$	0.381	0.076
IPT		
Control	1.72 ± 0.06	0.97 ± 0.01
7 mM	2.18 ± 0.10	1.00 ± 0.02
1 mM	2.17 ± 0.33	1.00 ± 0.05
P-value	NS	NS
$LSD_{0.05}$	0.597	0.109

Values are means ± SE ($n = 9$) 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) $P > 0.05$.

lated that CKs play a role in the NO_3^--regulated plant growth response (Samuelson et al., 1992), and that the amounts of available NO_3^- regulates plant CK levels in plants, thus, NO_3^- deficiency would lower CKs endogenous levels while an application of NO_3^- would raise CK levels in the shoot, thereby activating genes associated with nutrient uptake and homeostasis (Argueso et al., 2009). Kuiper (1988) argued for the importance of CKs in growth regulation, demonstrating that the decrease in CKs contents in *Plantago mayor* plants subjected to nutrient deficiencies was due to diminished endogenous cytokinins and not a decrease in the nutrient concentration in plant tissues. The expression of *IPT* under the control of the maturation- and stress-induced *SARK* promoter induced CKs synthesis during stress and drought tolerance in tobacco plants (Rivero et al., 2007, 2009). The higher CK content of the transgenic P_{SARK}::*IPT* plants used in this study would explain the ability of the transgenic plants to maintain plant biomass and RGR during N-deficit (Table 1), despite the decrease in foliar concentrations of total N, total reduced N, and NO_3^- seen when plants were grown under 1 mM treatment (Table 2). The P_{SARK}::*IPT* plants displayed low H_2O_2 content during a pronounced drought stress (Rivero et al., 2007). A decrease in the concentration of radical hydroxyl and MDA formation was also observed in wheat plants exposed to N-deficiency and treated with exogenous CKs (Stoparic and Maksimovic, 2008). These results, together with the results presented here, showing the lack of induction of lipid peroxidation in the transgenic plants during N-deficiency, support the notion of a role of CKs in the plant response to N-deficiency.

The absence of oxidative stress in the transgenic plants under N-deficient conditions was also indicated by the unchanged total AA, reduced AA and in other antioxidant compounds as shown by the FRAP and TEAC tests. The decrease in the concentrations of total GSH, reduced GSH, and the reduced-GSH:GSSG ratio under these conditions would correlate with the sharp decrease in the N forms under N-limited conditions and the need of N for GSH synthesis. Nonetheless, the normal growth displayed by the transgenic plants indicated that the decrease in GSH content was not harmful for the plants.

5. Conclusion

Our results showed that the tobacco WT plants displayed reduced biomass during N-limited conditions that produced a decrease in all of the different N forms and the generation of ROS that induced a damaging oxidative stress. In contrast, under N-deficiency transgenic P_{SARK}::IPT did not produce high ROS concentrations and maintained biomass and RGR values similar to the control plants. Our results suggest that the expression of P_{SARK}::*IPT* in plants could improve N use efficiency, favouring a reduction in the application of nitrogenous fertilizers, without losses in crop productivity.

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