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Ammonium formation and assimilation in P_{SARK}::*IPT* tobacco transgenic plants under low N

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ABSTRACT

Wild Type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene encoding the enzyme regulating the rate-limiting step in cytokinins (CKs) synthesis, were grown under limited nitrogen (N) conditions. We analyzed nitrogen forms, nitrogen metabolism related-enzymes, amino acids and photorespiration related-enzymes in WT and P_{SARK}::*IPT* tobacco plants. Our results indicate that the WT plants subjected to N deficiency displayed reduced nitrate (NO_3^-) assimilation. However, an increase in the production of ammonium (NH_4^+), by the degradation of proteins and photorespiration led to an increase in the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle in WT plants. In these plants, the amounts of amino acids decreased with N deficiency, although the relative amounts of glutamate and glutamine increased with N deficiency. Although the transgenic plants expressing P_{SARK}::*IPT* and growing under suboptimal N conditions displayed a significant decline in the N forms in the leaf, they maintained the GS/GOGAT cycle at control levels. Our results suggest that, under N deficiency, CKs prevented the generation and assimilation of NH_4^+ by increasing such processes as photorespiration, protein degradation, the GS/GOGAT cycle, and the formation of glutamine.

Introduction

Nitrogen (N) is an essential macronutrient that can frequently act as a limiting factor for growth (Antal et al., 2010). Under normal growth conditions, nitrate (NO_3^-) is the main source of N and its assimilation is essential for normal plant growth and development (Cruz et al., 2004). The reduction of NO_3^- to ammonium (NH_4^+) involves the sequential action of nitrate reductase (NR) (EC.1.6.6.) and nitrite reductase (NiR) (EC.1.7.2.1). The resulting NH_4^+ is then assimilated by glutamine synthetase (GS) (EC.6.3.1.2) and glutamate synthase (GOGAT) (EC.1.4.1.13) to organic forms such as glutamate (Glu) and glutamine (Gln). These amino acids (AAs) are precursors in the synthesis of other AAs, nucleic acids, chlorophylls or hormones (Oliveira et al., 2001). NO_3^- nutrition changes metabolite levels and enzyme activities considerably. Under nitrogen limiting conditions, several authors have described, in various plants such as *Arabidopsis* and barley, a decrease in nitrate reductase activity, while GS activity increased due to greater accumulation of the cytosolic enzyme (Barneix et al., 1984; Lemaître et al., 2008). In addition, compounds such as amino acids, directly related to N metabolism, reduce in concentration in plants subjected to low N (Cruz et al., 2004; Kováčik et al., 2006).

Another process involved in NH_4^+ formation is photorespiration, a process that is a consequence of the oxygenation of ribulose-1,5-biphosphate (RuBP). This reaction is catalyzed by RuBP carboxylase/oxygenase (Rubisco) (EC.4.1.1.39), which generates one molecule of glycerate-3-phosphate and one of glycolate-2-phosphate (2-PG) in the chloroplast. This 2-PG is hydrolyzed by phosphoglycolate phosphatase (EC.3.1.3.18) to glycolate, and converted to glyoxylate in the peroxisome by glyoxylate oxidase (GO) (EC.1.2.3.5). Glyoxylate is transaminated to glycine (Gly) by the reaction catalyzed by glutamate:glyoxylate aminotransferase (GGAT) (EC.2.6.1.4) and is transported to the mitochondria. Subsequently, Gly is transformed into serine (Ser) by the action of the enzymes and hydroxymethyltransferase. This reaction forms NH_4^+, which is integrated in the N-assimilation pathway through GS. Ser formed in the mitochondria is transported to the peroxisome,

Abbreviations: AAs, amino acids; AAT, aspartate aminotransferase; CKs, cytokinins; GDH, glutamate dehydrogenase; GGAT, glutamate:glyoxylate aminotransferase; GO, glyoxylate oxidase; Gln, glutamine; Gly, glycine; Glu, glutamate; GOGAT, glutamate synthase; GS, glutamine synthetase; HR, hydroxypyruvate reductase; IPT, isopentenyltransferase; NH_4^+, ammonium; NO_3^-, nitrate; NR, nitrate reductase; NiR, nitrite reductase; 2-PG, glycolate-2-phosphate; Rubisco, RuBP carboxylase/oxygenase; RuBP, ribulose-1,5-biphosphate; Ser, serine; SGAT, serine:glyoxylate aminotransferase; WT, Wild Type.

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where it is transformed by serine:glyoxylate aminotransferase (SGAT) (EC.2.6.1.45) into hydroxypyruvate, which in turn is reduced to glycerate by hydroxypyruvate reductase (HR) (EC.1.1.1.81). Finally, the glycerate enters the chloroplast, where it is phosphorylated by glycerate kinase, giving rise to a molecule of 3-PGA, which enters the Calvin-Benson cycle (Wingler et al., 2000). Although photorespiration involves a reduction of photosynthesis and CO_2 assimilation, photorespiration allows the supply of RuBP to the Calvin-Benson cycle (Osmond, 1981; Wu et al., 1991) and also can generate Gly and Ser (Madore and Grodzinski, 1984). Moreover, photorespiration plays a role in N assimilation, since the production of NH_4^+ during photorespiration can be significant, in particular during NO_3^- reduction (Key et al., 1978). The reassimilation of NH_4^+ caused during photorespiration by the enzymes GS and GOGAT has been defined as essential in the maintenance of the N status in the plant (Wingler et al., 2000).

The N status of the whole plant can regulate the plant N assimilation (Dluzniewka et al., 2006). NO_3^- can also induce the accumulation of mRNA and enzymes such as NR, GS, and GOGAT (Fan et al., 2002; Redinbaugh and Campbell, 1993). In addition to external factors such as temperature or atmospheric CO_2, the signaling of the internal status of N is essential to coordinate the absorption and assimilation of this element (Imsande and Touraine, 1994). It is known that, in addition to reduced N compounds such as AAs, cytokinins (CKs) are involved in N-dependent signaling. In response to NO_3^-, CKs accumulate in the plant tissues and induce the response of protein and enzyme regulators involved in assimilation (Takei et al., 2002; Sheen, 2002). The rate-limiting step of cytokinin biosynthesis, the transfer of an isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to the N^6 position of ATP/ADP, is catalyzed by the enzyme isopentenyltransferase (IPT) (Argueso et al., 2009). In *Arabidopsis*, IPT is encoded by seven genes that are differentially expressed in various tissues. Among these seven genes, *At*IPT3 is NO_3^- inducible (Kiba et al., 2011).

Here, we aim to characterize the effects of N deficiency on N-assimilation pathways and on photorespiration of Wild Type (WT) tobacco plants and transgenic tobacco expressing P_{SARK}*::IPT* (Rivero et al., 2007, 2009; Rubio-Wilhelmi et al., 2011).

Materials and methods

Plant material, growth conditions and plant growth

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 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. Over a period of 30 days, 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 days and the soil was rinsed with distilled water to avoid nutrient accumulation. The N treatments began 60 day after sowing (DAS) and were maintained for 30 day. 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 ($n = 9$).

Plant sampling

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 use. A part of the plant material was used for the determination of fresh weight (FW), nitrate reductase (NR) (EC.1.6.6.1), nitrite reductase (NiR) (EC.1.7.2.1), glutamine synthetase (GS) (EC. 6.3.1.2), glutamate synthase (GOGAT) (EC.1.4.1.13), aspartate aminotransferase (AAT) (EC. 2.6.1.1), glyoxylate oxidase (GO) (EC.1.2.3.5), glutamate:glyoxylate aminotransferase (GGAT) (EC.2.6.1.4), hydroxypyruvate reductase (HR) (EC.1.1.1.81) and glutamate dehydrogenase (GDH) (EC.1.4.1.2) enzymatic activities. The rest of the plant material was lyophilised and used to determine amino acids (AAs), nitrate (NO_3^-) and ammonium (NH_4^+).

Analysis of N forms and free AAs concentration

NO_3^- was measured by spectrophotometry following Cataldo et al. (1975), and NH_4^+ was determined as described by Krom (1980).

AAs were measured by high performance liquid chromatography (HPLC, Agilent 1100). Fresh leaf tissue was homogenized in methanol containing DL-3-aminobutyric acid as an internal standard and samples were agitated. Subsequently, 200 μL of chloroform were added and samples were agitated again for 5 min. Finally, 400 μL of ultra-pure water were added and samples were then vortexed and centrifuged at 13,000 × *g* for 5 min. The upper phase was transferred to a microtube and dried under vacuum overnight. Dry residues were resuspended in an appropriate volume of ultra-pure water and 10 μL of the resulting extract were sampled for amino acid derivatization according to the AccQ Tag Ultra Derivitization Kit protocol (Waters Corp., Milford, USA).

Enzyme extractions and assays

Leaves were homogenized in 50 mM buffer KH_2PO_4 (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 30,000 × *g* for 20 min. Supernatant was used to measure enzyme activity of NR, NiR, GOGAT and GDH (Groat and Vance, 1981; Kaiser and Lewis, 1984; Lillo, 1984; Singh and Srivastava, 1986). The NR assay followed the methodology of Kaiser and Lewis (1984). The NO_2^- formed was determined colorimetrically at 540 nm after azocoupling with sulphanilamide and naphthylethylenediamine dihydroclhoride according to the method of Hageman and Hucklesby (1971). NiR activity was measured by the disappearance of NO_2^- from the reaction medium (Lillo, 1984). After incubation at 30 °C for 30 min, the NO_2^- content was determined colorimetrically. GOGAT and GDH activities were assayed spectrophotometrically at 30 °C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). GS and AAT was determined by an adaptation of the hydroxamate synthetase assay by Kaiser and Lewis (1984) and according to Gonzalez et al. (1995) respectively. Leaves were homogenized in 50 mM of maleic acid-KOH buffer (pH 6.8), containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol and 20% (v/v) ethyleneglycol. The homogenate was centrifuged at 30,000 × *g* for 20 min. For GS, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove et al.,

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

NO_3^-	Foliar biomass (g DW)	
	WT	IPT
Control	6.77 ± 0.36 a	9.15 ± 0.23
7 mM	5.41 ± 0.01 b	7.88 ± 0.07
1 mM	4.53 ± 0.08 c	8.22 ± 0.62
P-Value	**	NS
$LSD_{0.05}$	0.745	1.338

Values are means ± SE ($n = 9$) and differences between means were compared using LSD ($P = 0.05$). Means followed by the same letter in the same column do not differ significantly.
* $P < 0.05$.
*** $P < 0.001$.
** $P < 0.01$.
NS (not significant).

Table 2
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on nitrate reductase (NR) and nitrite reductase (NiR) activities in two tobacco lines: 'WT' and 'IPT'.

N treatment	NR (μmol min^{-1} mg^{-1} prot)		NiR (mmol min^{-1} mg^{-1} prot)	
	WT	IPT	WT	IPT
Control	90.40 ± 9.53 a	34.36 ± 9.43	0.90 ± 0.02 b	1.39 ± 0.12 a
7 mM	33.67 ± 6.64 b	47.17 ± 8.03	1.02 ± 0.06 ab	1.28 ± 0.10 ab
1 mM	7.09 ± 0.77 c	32.75 ± 19.9	1.08 ± 0.03 a	1.05 ± 0.02 b
P-Value	***	NS	*	*
$LSD_{0.05}$	19.62	39.56	0.13	0.27

Values are means ± SE ($n = 9$) and differences between means were compared using LSD ($P = 0.05$). Means followed by the same letter in the same column do not differ significantly.
** $P < 0.01$.
* $P < 0.05$.
*** $P < 0.001$.
NS (not significant).

1979). The supernatant was used to measure AAT activity spectrophotometrically at 340 nm according to Gonzalez et al. (1995). For the determination of GO activity, fresh leaf tissue was homogenized with PVPP and 1 mL of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at 30,000 × *g* for 20 min. GO activity was assayed as described by Feierabend and Beevers (1972). For determination of GGAT and HR activities, leaves were homogenized in 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000 × *g* for 10 min. The supernatant was used to measure GGAT activity by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH (Igarashi et al., 2006). HR activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder and Rej, 1983). The protein concentration of the extracts was determined according to the method of Bradford (1976), using BSA as the standard.

Statistical analysis

The data were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P > 0.05$).

Results

Effects of N deficiency on foliar biomass and foliar NO_3^- and NH_4^+ concentrations

Nitrogen deficiency resulted in reduced foliar biomass in the WT plants (Table 1). The application of 7 mM and 1 mM NO_3^- resulted in reductions of 20–33% in foliar biomass. Besides, foliar biomass of the transgenic plants was not affected by the reduction in NO_3^- (Table 1). The internal NO_3^- concentrations was severely reduced in both WT and P_{SARK}*::IPT* plants growing at low N-concentrations (i.e. 1 mM) (Fig. 1A). A significant reduction in leaf NH_4^+ concentrations was also observed in plants growing under low N, although the decrease was less pronounced in the P_{SARK}*::IPT* plants (Fig. 1B).

Effects of N deficiency on NH_4^+ formation and assimilation

We measured the activities of enzymes comprising different biochemical pathways associated with the formation of NH_4^+ in plants. The NR activity of WT plants declined with the N deficiency treatments, while a slight increase was seen in NiR activity (Table 2). Under control conditions, the transgenic plants displayed lower NR activity levels compared to WT plants, but the activity remained constant (or increased at 7 mM N) when grown at N-deficient conditions (Table 2). In contrast to WT plants, the transgenic plants displayed a reduction in NiR activity under N deficiency.

We tested the activity of enzymes involved in the photorespiration-mediated NH_4^+ production and NH_4^+ assimilation. GO and GGAT activities increased and HR activity decreased in WT plants grown under N deficiency (Table 3). On the other hand, GO and HR decrease in P_{SARK}*::IPT* plants grown under 1 mM N, GGAT activity decreased under both 7 and 1 mM N treatments. GS, GOGAT, GDH, and ATT increased their activity in the WT plants grown under N deficiency, showing the highest values at 1 mM of N (Fig. 2). The transgenic plants did not display differences in their GS and ATT activities (Fig. 2A and D), while in the P_{SARK}*::IPT* plants grown in the presence of 7 mM N, GOGAT and GDH displayed their highest and lowest activity, respectively (Fig. 2B and C), although these values did not differ from those obtained with plants grown under control N conditions.

Effects of N deficiency on free amino acid contents

The concentration of total free AAs decreased drastically with the N deficiency treatments in both WT and transgenic lines (Table 4). In spite of this decrease in AA contents, the relative content of Glu and Gln increased in WT plants (Table 4). In the transgenic plants, the relative content of Gln remained constant with N deficiency, while the relative content of Glu and alanine (Ala) increased. The relative contents of other AAs such as Gly, arginine (Arg), valine (Va), lysine (Lis), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe) slightly increased with N deficiency in both plant lines (Table 4). A sharp decrease in the relative content of proline (Pro) was observed under the N deficiency treatments in both tobacco plant lines (Table 4).

Discussion

N deficiency reduced the foliar biomass in the leaves of WT tobacco plants (Table 1). Similar results have been reported in plants as different as *Triticum*, *Morus* or *Matricaria* subjected to N deficiencies (Tewari et al., 2004, 2007; Kováčik and Backor, 2007). This reduction in foliar biomass has been explained based on the numerous functions of this element within the plant (Maathuis, 2009). Furthermore, the N deficiency-induced decline in biomass, particularly in the case of severe deficiency (1 mM of N), appeared to be directly related to the diminished foliar concentration of NO_3^- and NH_4^+ (Fig. 1). In spite of the decrease in NO_3^- and NH_4^+ (Fig. 1), the transgenic plants expressing P_{SARK}*::IPT* maintained active foliar biomass under severe N deficiency (Table 1). These results suggest that the higher CK content of the transgenic P_{SARK}*::IPT* plants resulted in the ability of the transgenic plants to

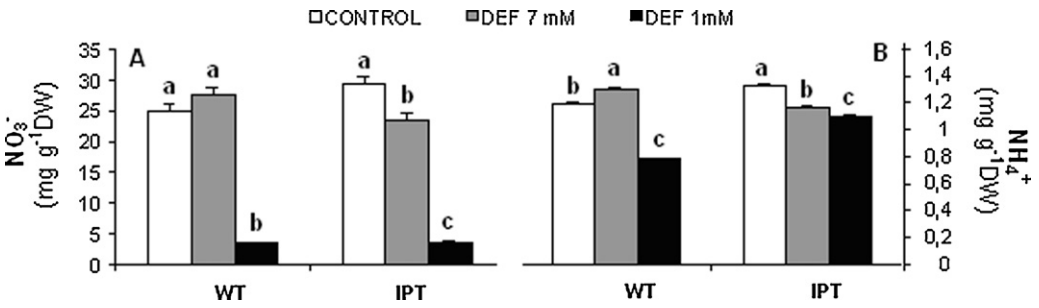

Fig. 1. Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) foliar NO_3^- concentration, (B) foliar NH_4^+ concentration of two tobacco lines: 'WT' and 'IPT'. Bars represent means + SE ($n = 9$); for each lines.

Table 3
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on glyoxylate reductase (GO), glutamate:glyoxylate reductase (GGAT) and hidroxypyruvate reductase (HR) activities in two tobacco lines: 'WT' and 'IPT'.

N treatment	GO (ΔA_{324} h^{-1} mg^{-1} prot)	GGAT (ΔA_{340} h^{-1} mg^{-1} prot)	HR (ΔA_{340} h^{-1} mg^{-1} prot)
WT			
Control	0.19 ± 0.03 b	1.06 ± 0.09 b	6.56 ± 0.29 a
7 mM	0.25 ± 0.03 b	1.50 ± 0.08 a	4.25 ± 0.31 c
1 mM	0.45 ± 0.05 a	1.51 ± 0.14 a	5.34 ± 0.41 b
P-Value	***	**	***
$LSD_{0.05}$	0.12	0.31	1.00
IPT			
Control	0.63 ± 0.04 a	1.17 ± 0.12 a	6.61 ± 0.27 a
7 mM	0.66 ± 0.07 a	0.92 ± 0.05 ab	6.40 ± 0.14 a
1 mM	0.32 ± 0.02 b	0.83 ± 0.11 b	4.62 ± 0.10 b
P-Value	***	*	***
$LSD_{0.05}$	0.14	0.29	0.55

Values are means ± SE ($n = 9$) and differences between means were compared using LSD ($P = 0.05$). Means followed by the same letter in the same column do not differ significantly.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
NS (not significant).

grow under low N. It has been shown that, in plants growing under nutrient deficiency, the decrease in growth was correlated with a decrease in CK content (Kuiper, 1988), and that NO_3^- deficiency promoted lower CK levels, while NO_3^- applications resulted in increased shoot CKs and the activation of genes associated with nutrient uptake and homeostasis (Argueso et al., 2009). NR and NiR are critical steps in NO_3^- assimilation (Lea and Azevedo, 2006). The decrease in NR activity seen in the WT plants is supported by the reported NO_3^- induced NR activity (Cruz et al., 2004). It has been shown that high endogenous CK levels reduced the inducibility of NR by NO_3^- (Lexa and Genkov, 2002). This observation is in agreement with our results showing that the P_{SARK}::*IPT*

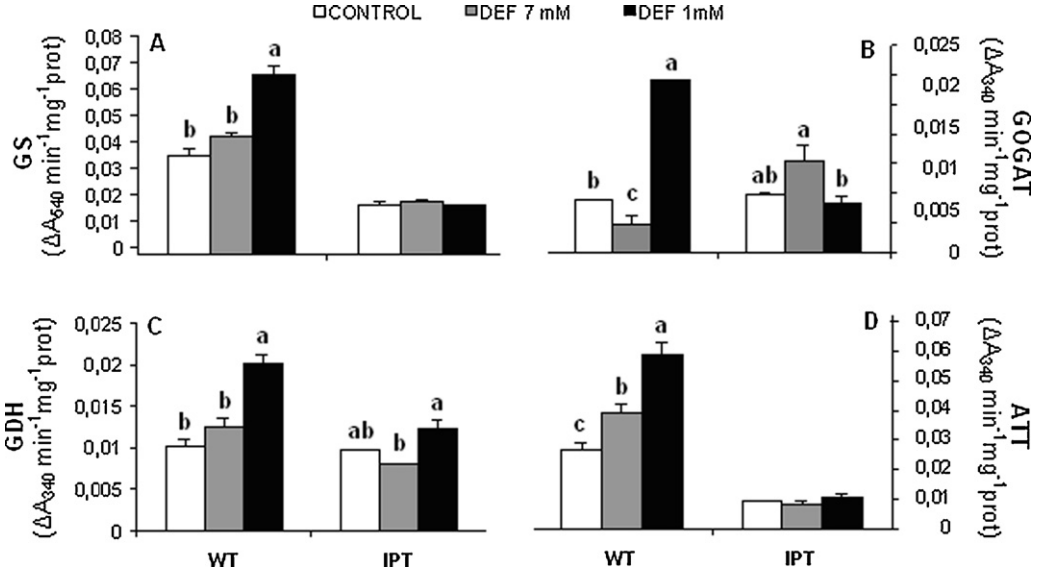

Fig. 2. Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) glutamine synthetase (GS), (B) glutamate synthase (GOGAT), (C) glutamate dehydrogenase (GDH) and (D) aspartate aminetransferase (AAT) activity in leaves of two tobacco lines: 'WT' and 'IPT'. Bars represent means + SE ($n = 9$); for each line.

Table 4
Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on foliar amino acids concentration in two tobacco lines: 'WT' and 'IPT'.

N treatment	Amino acids concentration (nmol g^{-1} FW) and proportion (%)					
	WT			IPT		
	Control	Def 7 mM	Def 1 mM	Control	Def 7 mM	Def 1 mM
Aspartate	4657.33 (4.8)	3415.05 (4.8)	1791.46 (3.5)	4221.68 (4.4)	4020.91 (6.2)	1700.02 (3.8)
Serine	7993.42 (8.3)	7456.07 (10.5)	4037.21 (7.9)	10786.62 (11.3)	7104.29 (11.0)	6127.70 (13.7)
Glutamate	13899.41 (14.5)	5940.17 (8.4)	9398.65 (18.5)	9379.66 (9.8)	8452.01 (13.1)	7934.68 (17.1)
Glycine	2599.64 (2.7)	2428.42 (3.4)	1994.56 (3.9)	2186.21 (2.3)	1309.97 (2.0)	2596.99 (5.8)
Histidine	5716.29 (5.9)	4987.94 (7.0)	1456.72 (2.8)	7133.14 (7.5)	3197.57 (5.0)	1465.07 (3.3)
Asparagine	1531.69 (1.59)	1119.34 (1.5)	721.02 (1.4)	1678.91 (1.7)	1420.01 (2.2)	867.09 (1.94)
Glutamine	17658.01 (18.4)	15645.83 (22.1)	15137.40 (29.9)	18742.72 (19.7)	13486.03 (20.9)	8860.63 (19.88)
Arginine	437.53 (0.4)	457.49 (0.6)	389.38 (0.7)	767.65 (0.8)	450.05 (0.7)	433.99 (0.97)
Threonine	3046.95 (3.1)	2306.17 (3.2)	1661.17 (3.2)	4765.50 (5.0)	3659.12 (5.7)	2662.25 (5.9)
Alanine	5427.07 (5.6)	5371.04 (7.6)	1714.20 (3.4)	3930.18 (4.1)	3158.13 (4.9)	3638.80 (8.2)
Proline	27557.23 (28.7)	16384.47 (23.2)	7129.48 (14.1)	25976.87 (27.3)	14048.94 (21.8)	3018.55 (6.7)
Cysteine	nd	nd	nd	nd	nd	nd
Thyroxine	339.46 (0.4)	752.73 (1.1)	210.42 (0.4)	778.77 (0.8)	303.41 (0.4)	735.52 (1.6)
Valine	658.19 (0.7)	1137.06 (1.6)	847.47 (1.7)	1079.13 (1.1)	908.47 (1.4)	1109.99 (2.5)
Methionine	756.60 (0.8)	nd	nd	nd	nd	nd
Lysine	1115.60 (1.2)	744.85 (1.1)	968.23 (1.9)	285.78 (0.3)	279.19 (0.4)	780.57 (1.7)
IsoLeucine	666.59 (0.7)	522.98 (0.7)	647.35 (1.3)	858.16 (0.9)	441.28 (0.7)	781.93 (1.7)
Leucine	930.48 (1.0)	925.64 (1.3)	742.78 (1.5)	917.17 (1.0)	846.69 (1.3)	923.42 (2.0)
Phenylalanine	987.11 (1.0)	996.82 (1.4)	1019.45 (2.0)	1357.33 (1.4)	1184.54 (1.8)	923.39 (2.0)
Total	95970.09 (100)	70592.13 (100)	50688.02 (100)	94845.55 (100)	64270.68 (100)	44560.67 (100)

plants displayed a low response of NR activity to N deficiency (Table 2).

NH_4^+ can be formed not only by NO_3^- assimilation, but also by diverse metabolic pathways such as photorespiration or protein degradation (Temple et al., 1998; Tercé-Laforgue et al., 2004). The increase in the enzymes GO and GGAT (Table 3) seen in WT plants during N deficiency could lead to greater NH_4^+ formation and an increase in N assimilation (Wingler et al., 2000). In addition, the increased GDH activity observed in the deficient WT plants (Fig. 2C) would result in increased protein degradation, especially in senescent parts of the plant. Thus, increased photorespiration and GDH activity in WT plants would increase NH_4^+ formation. These functions are carried out by the GS/GOGAT cycle, which increased in the WT plants grown under severe N deficiency (1 mM) (Fig. 2A and B). This strategy would also include the elimination of excess toxic NH_4^+, and contribution to the maintenance of N status during N deficiency.

The transgenic plants did not display the activation of enzymes associated with photorespiration under N deficiency (Table 3). Consequently, the formation of NH_4^+ by this pathway did not appear to be affected. The relatively low amounts of NH_4^+ in the P_{SARK}*::IPT* plants were paralleled by the maintenance in GDH activity and the low response of the GS/GOGAT cycle in the transgenic plants.

N deficiency lowered the amounts of AAs in both WT and transgenic plants (Table 4). A similar decline in AAs under N deficiency conditions has been reported in tobacco plants as well as other species such as *Cassava* or *Matricaria* (Tercé-Laforgue et al., 2004; Cruz et al., 2004; Kováčik et al., 2006). Despite the general decrease in AAs, there was a relative increase in Glu and Gln during N deficiency. However, the relative amounts of Pro decreased (Table 4). The degradation of proteins by GDH and the decline in Pro could have favored the synthesis of Glu and Gln. In addition to their role as donors of the amino group for the synthesis of other AAs or compounds such as ureides or nucleic acids, Glu and Gln constitute the main form of N transported by the phloem towards the young parts in growth, a vital process for plant growth under N deficiency (Lea and Azevedo, 2006). In the transgenic plants, the relative amounts of Gln remained constant with N deficiency, while there was a relative increase in Glu and Ala. It has been suggested that CKs may support the AA-mediated communication of N demand from the shoot to the roots and participate in the regulation of N assimilation (Collier et al., 2003; Gessler et al., 2004). In our work, the transgenic plants showed similar quantities of total AAs as in WT plants, reducing their amount in similar proportions under N deficiency (Table 4). These results are in agreement with Dluzniewka et al. (2006), who showed that the application of external CKs did not raise the levels of AAs in *Poplar*.

Conclusions

Nitrogen often limits plant growth and development, and in response to N deficiency, plants have developed a number of physiological as well as morphological strategies to adjust and maintain their growth and development. In this work, we demonstrate that, in transgenic tobacco expressing P_{SARK}*::IPT* plants, CKs prevented the generation and assimilation of NH_4^+ by stimulating such processes as photorespiration, protein degradation, the GS/GOGAT cycle, and the formation of AAs such as Gln, responses that were seen in the WT plants grown under N deficiency. In spite of the N-deficient conditions, the transgenic tobacco P_{SARK}*::IPT* plants, maintained their foliar growth constant, suggesting a role of the CKs in the efficient use of the N available under these conditions.

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