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P_{SARK}::*IPT* expression causes protection of photosynthesis in tobacco plants during N deficiency

Maria del Mar Rubio-Wilhelmi [b,*], Maria Reguera [b], Eva Sanchez-Rodriguez [a], Luis Romero [a], Eduardo Blumwald [b], Juan Manuel Ruiz [a]

[a] Department of Plant Physiology, Faculty of Science, University of Granada, 18071 Granada, Spain
[b] Department of Plant Sciences – Mail Stop 5, University of California, One Shields Avenue, Davis, CA 95616, USA

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ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinins (CKs) synthesis, were grown under limited nitrogen (N) conditions. WT tobacco plants under N deficit showed a drastic reduction in photosynthetic rate (*A*) and the maximum carboxylation rate of Rubisco (Vcmax), the maximum rate of the electron transport (Jmax) and the use of triose-P (TPU) parameters. However, the expression an *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter, in tobacco plants under N deficiency leads the maintenance of photosynthesis and leaf biomass related with the maintenance of sm-Rubisco transcripts levels. Limited increase in sugar concentration as well as the maintenance of leaf biomass observed in transgenic plants would indicate CKs may play a role in the sink limitation caused by deficiency of N, enhancing the strength of the sink, such as young leaves, and enhancing foliar biomass under severe N deficiency.

1. Introduction

Nitrogen (N) is a mineral element that plants require in greatest amount and it is often the growth limiting nutrient (Antal et al., 2010). The role of N in agricultural production is intimately connected with photosynthesis because more than half of the total leaf N is allocated to the photosynthesis apparatus (Makino and Osmond, 1991; Lawlor, 2002). Two days of N deprivation led to coordinate repression of the majority of genes assigned to photosynthesis, chlorophyll synthesis, and plastid proteins synthesis (Scheible et al., 2004). Thus, N shortage results in a marked decrease in plant photosynthesis, decreasing in both photosynthetic rate (*A*) and the quantum yield of photosynthesis (Cruz et al., 2003; Boussadia et al., 2010). Limited N supply, causes diminution in the content of chloroplastidic pigments and a reduction in the synthesis of several enzymes involved in the Calvin cycle, particularly in Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) (Terashima and Evans, 1988). Overall, N deficiency leads sink limitation due to decreased in shoot growth, results in accumulation of carbohydrates in matures leaves, higher levels of carbon allocated to the roots and an increase in root-to-shoot biomass ratio (Paul and Foyer, 2001; Hirari et al., 2004; Remans et al., 2006). Therefore N deficiency affects, primary photosynthesis, sugar metabolism and carbohydrate partitioning between source and sink tissues (De Groot et al., 2003; Scheible et al., 2004).

Cytokinins (CKs) are phytohormones involved in numerous important biological processes associated with plant growth and development, including the response of plants to abiotic stress (Haberer and Kieber, 2002; Rivero et al., 2007). Unfavorable environmental conditions have an impact on CKs content in plants and changes in endogenous CKs levels were reported to alter the stress tolerance in plants (Werner et al., 2010; Nishiyama et al., 2011). Thus, CKs, selectively could affect the expression of certain genes, which are important for stress protection (Chernyad'ev, 2009). It has been proposed the protective action of CKs under stress conditions, preserving the structure and function of the photosynthetic machinery inducing the synthesis of proteins of the electron-transport chain, photosynthetic chlorophyll-protein complexes and Rubisco (Chernyad'ev, 2009). Rivero et al. (2010) demostred the prevention of the degradation of the photosynthetic protein complexes, and the maintenance of photosynthesis during water stress in the plants expressing an isopentenyltransferase (*IPT*) gene driven by P_{SARK}, a stress-and maturation-induced promoter. Furthermore it has been suggested that CKs may also be involved in the regulation of assimilate partitioning affecting source-sink relationships (Roitsch and Ehneß, 2000). Peleg et al. (2010) demonstrated that the expression of P_{SARK}::*IPT* in

* Corresponding author. Tel.: +1 5307547322.
E-mail address: mmrubiowilhelmi@ucdavis.edu (M.d.M. Rubio-Wilhelmi).

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rice affected plant hormone homeostasis and altered the source-sink balance showing that CKs could maintain the strength of this sink increasing the amount of starch and sucrose (Suc) during water-deficit. Moreover, it has been demonstrated that CKs supplied at physiological concentration could change the direction of assimilate export from individual leaves, thus completely inverting the source-sink relationship in favor of the shoot.

Besides, the availability of N can alter the levels of CKs. It was proposed that CKs may be root-derived signal which controls uptake and utilization of assimilates and biomass distribution in response to N (Beck, 1999; Sakakibara et al., 2006). Therefore, N deficiency would lower CKs endogenous levels while an application of N would raise CKs levels in the shoot (Argueso et al., 2009). Thus, CKs can act as a signal communicating to the shoot if the N application of the root is adequate, thereby regulating the biomass distribution and nutrient uptake systems (Sakakibara et al., 2006). In addition, previous works indicated that tobacco plants overexpressing P_{SARK}::*IPT* increase the amount of CKs, inhibiting the formation of reactive oxygen species (ROS) and preventing biomass reduction caused by N deficiency (Rivero et al., 2007; Rubio-Wilhelmi et al., 2011). Several evidence linking N deficiency, CKs and photosynthesis, therefore, the aim of the present work was to evaluate the effect of CKs on photosynthetic parameters and sugar metabolism in wild type (WT) and P_{SARK}::*IPT* tobacco plants under N deficiency.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of WT tobacco (Nicotiana tabacum 'SR1') and transgenic line expressing P_{SARK}::*IPT* were sown in soil (Metro-Mix 200; Sun Gro) in a growth chamber (500 μmol photons m^{-2} s^{-1}, 16-h photoperiod, 25 °C) for 15 days until the appearance of the first two true leaves. During this time, no differences in germination time and in plant development between the WT and P_{SARK}::*IPT* lines were observed. After, plants were transferred and transplanted (10-L pots filled with vermiculite) to a greenhouse (UC-Davis, CA, USA), where they were grown for 1 week to allow acclimation of the plants to the new conditions (1000 μmol photons m^{-2} s^{-1}, 16-h photoperiod, 28 °C–30 °C/23 °C–25 °C day/night). During 20 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 $MgCl_2$, 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 40 days after sowing (DAS) and it was maintained for 30 days. 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. All plants were at the reproductive stage when harvested. Middle leaves (positions 7th and 8th) were harvested, frozen immediately in liquid N_2, and kept at −80 °C until used. Leaves, roots and flowers were dried in a forced-air oven at 70 °C for 24 h for dry biomass determination.

2.2. A/CO_i curves

Gas-exchange measurements were conducted in the eighth fully expanded leaf in each genotype (WT, P_{SARK}::*IPT*) with a gas exchange system (LI-6400; Li-Cor). Leaves were first equilibrated at a photon density flux of 1000 mmol m^{-2} s^{-1} for at least 20 min. After this, photosynthesis was induced with 1000 mmol photons m^{-2} s^{-1} and 400 mmol mol^{-1} CO_2 surrounding the leaf (Ca). Leaf temperature was maintained at 25 °C, and the leaf-to-air vapor pressure deficit was kept between 1 and 1.3 kPa. These conditions were kept constant for the determination of *A*. CO_2 response curves were performed at steady state at least 30 min after clamping the leaf. CO_2 response curves, corresponding to eighth fully expanded leaves of 6 different plants, were obtained per each plant genotype (the WT and P_{SARK}::*IPT* lines). *A* and rates of CO_2 assimilation under varying intercellular CO_2 concentrations (Ci) were first measured at 400 mmol mol^{-1} Ca. Then, Ca was increased stepwise up to 1800 mmol mol^{-1} and returned to its original value, followed by a stepwise decrease down to 0 mmol mol^{-1} Ca. *A* and Ci were measured at 12 different Ca values for each curve. From the *A*/Ci curves, the following photosynthetic parameters were calculated according to Long and Bernacchi (2003): the maximum carboxylation rate of Rubisco (Vcmax), the maximum rate of the electron transport (Jmax) that is equivalent to the ribulose-1,5-bisP (RuBP) regeneration rate, as well as use of triose-P (TPU). In order to avoid miscalculation of *A* and Ci due to leakage into the gasket of the gas analyzer, we performed CO_2 response curves using an empty chamber. The values obtained for *A* and Ci in the empty chamber was compared with those of the chamber filled with a tobacco leaf and subtracted from the values obtained with the empty chamber. The relation between *A* and Ci was fitted with the software Photosyn Assistant (Dundee Scientific). The program uses the model proposed by Farquhar et al. (1980), as subsequently modified by von Caemmerer and Farquhar, 1981, Sharkey et al. (1985), Harley and Sharkey (1991), and Harley et al. (1992).

2.3. qPCR

cDNAs were obtained from two independent RNAs corresponding to the same sample using the SuperScript VILO synthesis kit (Invitrogen). This procedure was done with tissues from WT plants and P_{SARK}::*IPT* plants growing under control conditions (10 Mm) and N deficient conditions (7 and 1 Mm) so that every sample was represented by two independent cDNAs.

From each cDNA, three replicates were placed on a 96-well plate, so that every sample was represented by six replicates. For all targets analyzed, the primers were designed using ABI Primer Express software. For IPT expression, the primers used were IPT-forward (5′-CCAAGGCCAGAGTTAAGCAG-3′) and IPT-reverse (5′-TTTGCGTCAAGCTGCAATAG-3′). For Rubisco small subunit (smRubisco), the primers used were smRubisco-forward (5′-AGTGCGGCAACGGTAATATC-3′) and smRubisco-reverse (5′-TCAACAAAGTCCGGAGAACC-3′). An internal control Elongation factor 1 α (EF-1α) which expression did not change over the amplification in the different samples, were processed in parallel. The primers used were as follows: EF-1α-forward (5′-TGAGATGCACCACGAAGCTC-3′) EF-1α-reverse (5′-CCAACATTGTCACCAGGAAGTG-3′). The amplification was performed in a total reaction volume of 20 mL. Reactions included 2 mL of template, 10 mL of Fast SYBR Green Master Mix, 0.9 mL of reverse primer, 0.9 mL of forward primer, and sterile molecular biology-grade water to a total volume of 20 mL. All PCRs were performed with the exact reaction cycling conditions as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt curve for every target analyzed was included with the following conditions: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Amplification and data analysis were carried out on an ABI StepOne Plus real-time PCR system (Applied Biosystems) taking as internal control EF-1α and as a sample control WT plants growing under control conditions (10 mM). All template and primer concentrations were optimized for the reactions.

2.4. Sugar determination

Leaves glucose-6P (Glu-6P), Suc, glucose (Glu), mannose (Man), fructose (Fru) were quantified as described by Smith and Zeeman (2006) with some modifications. Middle leaves were sampled from WT and P_{SARK}::*IPT* tobacco plants and immediately frozen in liquid-N. Then 0.25 g was incubated in ethanol (80%) for 3 min at 95 °C and centrifuged 3000 × *g* 10 min, keeping supernatant (soluble carbohydrate fraction). For sugars quantification, the soluble carbohydrate fraction dissolved in ethanol (80%) was evaporated using a rotary evaporator. The samples were dissolved in ddH_2O and subsequently analyzed by high performance liquid chromatography (HPLC) using Aminex HPX-87C column.

2.5. Enzymes assay

Enzymes were extracted from 0.1 g of leaves by homogenizing in 50 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.005% Triton X-100, 1% insoluble PVPP, and 5 mM DTT. The homogenate was centrifuged at 20 000 × *g* for 20 min. Supernatant was used to measure Suc-P synthase (SPS, EC 2.4.1.14; SPS), Suc synthase (SS, EC 2. 4. 1. 13), vacuolar and cytosolic invertases (EC 3.2.1.26) activities. Resuspended pellet was used for measured of cell wall invertase (EC 3.2.1.26).

SS and SPS assays were performed in a manner similar to that previously described (Hubbard et al., 1989; Miron and Schaffer, 1991; Klann et al., 1992). SS and SPS reactions and controls contained 40 μL of extract in a total volume of 70 μL. SS assays contained 50 mM Hepes/NaOH (pH 7.5), 15 mM $MgCl_2$, 25 mM Fru, and 25 mM UDP-Glu. SPS assays contained 50 mM Hepes/NaOH (pH 7.5), 15 mM $MgCl_2$, 25 mM Fru-6-P, 25 mM Glu-6-P, and 25 mM UDP-Glu. Controls were boiled for 10 min immediately after the addition of the enzyme extract. The reactions were incubated for 30 min at 37 °C and then stopped by transfer to a boiling water bath. Reactions and controls were stored at −20 °C until the Suc was assayed using the anthrone assay (Van Handel, 1968). To the 70 μL sample, 70 μL of 30% KOH was added, boiled for 10 min, and cooled. Anthrone reagent was then added, and the tubes incubated for 20 min at 40 °C. Absorbance was measured at 650 or 595 nm and compared to Suc standards.

Vacuolar, cytosolic and cell wall invertases assays contained either 100 μL of extract in a total volume of 300 μL. The final assay contained 500 mM sodium acetate/NaOH pH 4.5 for vacuolar and cell wall invertase or pH 7.5 for cytosolic invertase and 120 mM Suc. Both reactions and controls were neutralized with the addition of 30 μL of 2.5 M Tris base before boiling to prevent acid hydrolysis of the Suc. Controls were boiled immediately, and the reactions were incubated at 37 °C for 30 min before boiling. Reaction and controls were stored at −20 °C until the reducing sugar assay was performed. Reducing sugars in the invertases assays were measured using the Somogyi modification of Nelson's reducing sugar assay (Nelson, 1944; Somogyi, 1951).

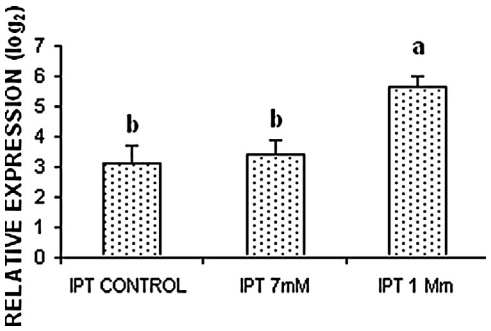

Fig. 1. Relative expression *IPT* gene driven by P_{SARK}, a stress- and maturation-induced promoter levels during control (10 mM) and under N deficient (7 and 1 mM) in P_{SARK}::*IPT* transgenic plants. Bars represent means + SE ($n = 9$); for each lines.

3. Results

3.1. Effects of N deficiency on plant biomass

Table 1 shows foliar, root and flower biomass in WT and P_{SARK}::*IPT* tobacco plants under N deficiency. The application of 1 mM of NO_3^- resulted in a significant decrease in foliar biomass in WT plants. However, transgenic line did not show significant differences in foliar biomass under any of the N treatments (Table 1). Besides, root biomass in WT plants was not affected by the N deficiency; on the contrary, P_{SARK}::*IPT* plants shown significant decreased in this parameter under 1 mM treatment (Table 1). Finally, in WT plants, 7 mM dosage of N produced a significant increase in flower biomass compared to control conditions (10 mM); while transgenic plant shown a reduction in flower biomass under N severe deficiency (1 mM), however decrease in flower biomass was not significant in transgenic plants (Table 1).

3.2. Effects of N deficiency on IPT and sm-Rubisco expression analysis

Relative expression *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter levels during control and under N deficient in P_{SARK}::*IPT* transgenic plants is shown in Fig. 1. When the plants grown under 7 mM N treatment did not show differences in P_{SARK}::*IPT* expression levels, however severe N deficiency (1 mM) increased P_{SARK}::*IPT* expression levels compared to control (10 mM). On the other hand, sm-Rubisco relative expression levels in both lines of tobacco plants are shown in Fig. 2. WT tobacco plants registered a significant decrease in sm-Rubisco relative expression levels under N deficiency (Fig. 2). The lowest expression levels were shown under 1 mM N treatment (Fig. 2). However, in transgenic plants, sm-Rubisco relative expression levels remained unchanged under different N dosages (7 and 1 mM) compared to control conditions (10 mM; Fig. 2).

Table 1
Foliar, root and flower biomass in WT and transgenic tobacco plants under control (10 mM) and N deficiency (7 and 1 mM).

NO_3^- treat.	Foliar biomass (g DW)		Root biomass (g DW)		Flower biomass (g DW)	
	WT	IPT	WT	IPT	WT	IPT
Control	9.89 ± 0.53a	12.66 ± 0.89	8.44 ± 1.15	5.77 ± 0.23a	6.83 ± 0.58b	7.31 ± 0.80
7 mM	9.80 ± 0.29a	12.03 ± 0.67	8.24 ± 1.37	6.70 ± 0.90a	9.14 ± 0.27a	7.16 ± 0.36
1 mM	6.61 ± 0.34b	11.15 ± 1.01	7.42 ± 0.20	4.02 ± 0.50b	5.92 ± 0.41b	5.57 ± 0.66
P-value	***	NS	NS	*	**	NS
$LSD_{0.05}$	1.214	2.631	3.597	1.009	1.338	1.916

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. Levels of significance are represented by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and NS (not significant) $P > 0.05$.

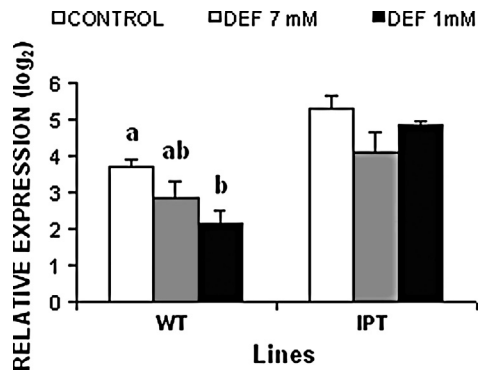

Fig. 2. Relative expression sm-Rubisco subunit levels during control (10 mM) and under N deficient (7 and 1 mM) in WT and transgenic plants. Bars represent means + SE ($n = 9$); for each lines.

3.3. Effects of N deficiency on A/C_i curves, Vcmax, Jmax and TPU

We measured rates of CO_2 assimilation under varying Ci and produced *A*/Ci curves (Fig. 3A and B). *A*/Ci curves showed a decrease in both lines (WT and P_{SARK}*::IPT*) of tobacco plants under 1 mM dosage of N (Fig. 3A and B). The reduction in A/Ci curve was more pronounced in WT plants than in transgenic plants (Fig. 3A). Neither of tobacco lines was affected by 7 mM of N treatment (Fig. 3A and B).

From the curves, one can calculate biochemical factors such as the Vcmax, Jmax and TPU (Fig. 4A–C). The application of 1 mM N resulted in WT plants, in reductions of 72, 70 and 66% respectively compared to control (10 mM; Fig. 4A–C). Vcmax, Jmax and TPU did not show significant differences under 7 mM N treatment and control condition (10 mM) in WT plants (Fig. 4A–C). In transgenic plants, P_{SARK}*::IPT*, a decrease in Vcmax and Jmax were observed under severe N deficiency (1 mM; 48 and 45% respectively) (Fig. 4A–C). However, TPU showed significant differences under both N rates (1 and 7 mM) and control conditions, resulted in reduction of 21% for 1 mM N dosage (Fig. 4A–C).

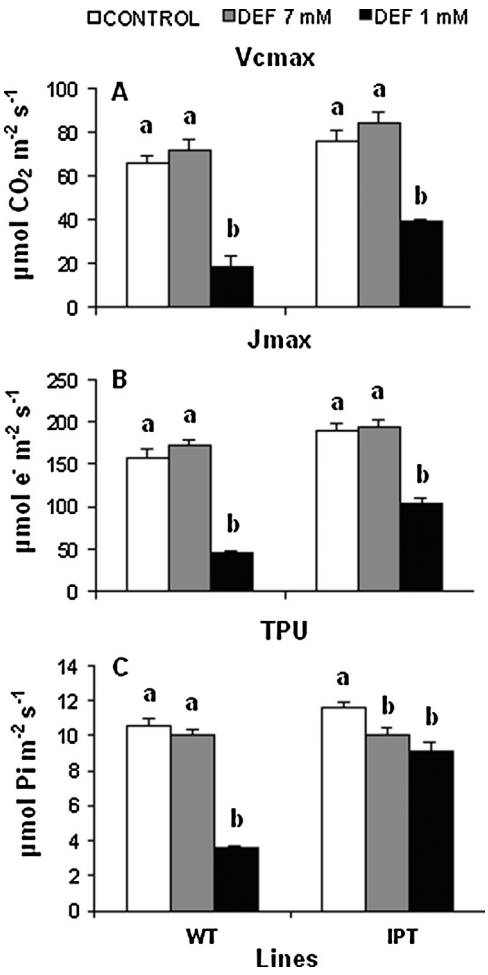

Fig. 4. Vcmax (A), Jmax (B), TPU (C) in WT and transgenic tobacco plants under control (10 mM) and N deficiency (7 and 1 mM). Bars represent means + SE ($n = 9$); for each lines.

3.4. Effects of N deficiency on sugars metabolism

Table 3 shows Glu-6P, Suc, Glu, Man and Fru concentrations in WT and P_{SARK}*::IPT* tobacco plants under N deficiency. In WT plants, severe N deficiency (1 mM) affected the concentration of Glu-6P, Glu, Man y Fru, increasing 45, 89, 194 y 51% respectively compared to control conditions (10 mM; Table 3). When the WT plants grown under 7 mM N treatment, only Suc concentration showed a significant decrease respect to the control conditions (10 mM; Table 2). However, in P_{SARK}*::IPT* plants under severe N deficiency dosage (1 mM), only Glu and Man concentrations presented an increase compared to control conditions (10 mM; 50 years 57% respectively).

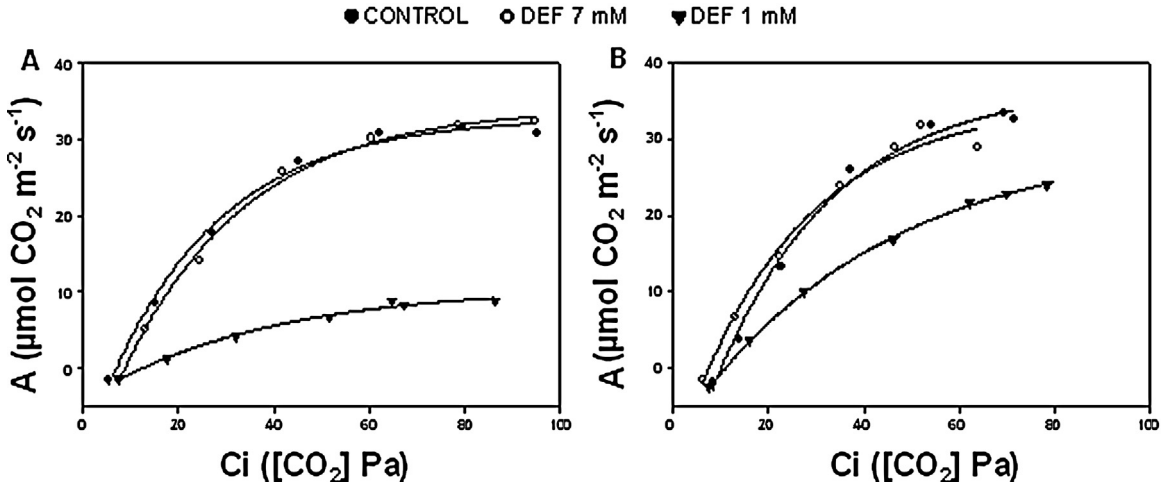

Fig. 3. CO_2 assimilation rates at different intercellular CO_2 concentrations (A/Ci curves) of WT and transgenic tobacco plants under control (10 mM) and N deficiency (7 and 1 mM).

Table 2
Glu-6P, Suc, Glu, Man, Fru concentrations in WT and transgenic tobacco plants under control (10 mM) and N deficiency (7 and 1 mM).

Lines/NO_3^- treatment	Glucose 6P (mg g^{-1} DW)	Sucrose (mg g^{-1} DW)	Glucose (mg g^{-1} DW)	Mannose (μg g^{-1} DW)	Fructose (mg g^{-1} DW)
WT					
Control	56.20 ± 3.45 b	17.24 ± 0.25 a	11.20 ± 0.47 b	1.22 ± 0.29 b	8.39 ± 0.52 b
7 mM	78.98 ± 3.50 a	14.97 ± 0.63 b	9.15 ± 0.79 b	0.81 ± 0.25 b	9.32 ± 0.71 b
1 mM	81.01 ± 5.78 a	18.55 ± 0.36 a	21.22 ± 1.21 a	3.59 ± 0.45 a	12.67 ± 0.43 a
P-value	**	**	***	***	**
$LSD_{0.05}$	14.027	1.429	2.818	1.105	1.848
IPT					
Control	71.31 ± 6.12 a	17.03 ± 0.58 a	9.81 ± 0.30 b	1.22 ± 0.09 b	8.24 ± 0.67
7 mM	62.90 ± 0.59 ab	13.20 ± 1.15 b	12.02 ± 0.92 b	1.35 ± 0.16 b	9.25 ± 1.14
1 mM	52.19 ± 1.12 b	14.63 ± 0.24 ab	14.67 ± 0.87a	1.92 ± 0.31 a	9.49 ± 0.04
P-value	*	*	**	***	NS
$LSD_{0.05}$	11.559	2.432	2.421	0.263	2.459

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. Levels of significance are represented by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and NS (not significant) $P > 0.05$.

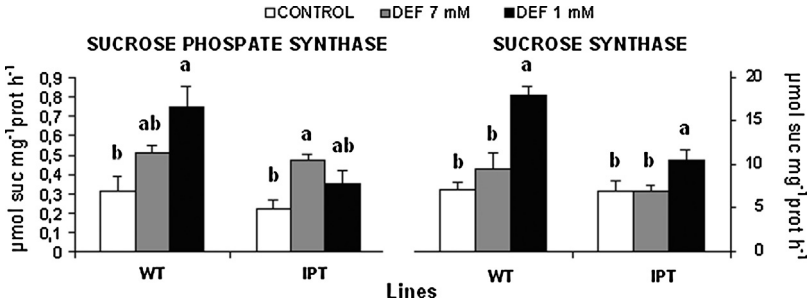

Fig. 5. SPS and SS activity in WT and transgenic tobacco plants under control (10 mM) and N deficiency (7 and 1 mM). Bars represent means + SE ($n = 9$); for each lines.

Table 3
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on invertases activity in WT and transgenic tobacco plants.

Lines/NO_3^- treatment	Cell wall invertase (μmol Glu mg^{-1} prot h^{-1})	Vacuolar invertase (μmol Glu mg^{-1} prot h^{-1})	Cytosolic invertase (μmol Glu mg^{-1} prot h^{-1})
WT			
Control	1.65 ± 0.08 b	4.33 ± 0.37 b	1.09 ± 0.11 b
7 mM	1.54 ± 0.30 b	3.89 ± 0.37 b	1.01 ± 0.09 b
1 mM	2.88 ± 0.30 a	6.26 ± 0.70 a	1.58 ± 0.13 a
P-value	**	*	**
$LSD_{0.05}$	0.776	1.527	0.356
IPT			
Control	1.88 ± 0.16 ab	3.35 ± 0.31	1.03 ± 0.13
7 mM	1.53 ± 0.07 b	3.25 ± 0.11	1.17 ± 0.14
1 mM	2.13 ± 0.17 a	4.08 ± 0.28	1.13 ± 0.11
P-value	*	NS	NS
$LSD_{0.05}$	0.432	0.595	0.391

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. Levels of significance are represented by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and NS (not significant) $P > 0.05$.

Fru concentration was not affected by the reduction in N. On the contrary, Glu-6P, Suc concentrations showed a reduction under severe N deficiency treatment in transgenic tobacco plants (1 mM; Table 2).

The enzymes involved in Suc synthesis are shown in Fig. 5. WT tobacco plants presented an increase in SPS and SS activities under both N deficiency treatments (7 and 1 mM). Activities were higher under 1 mM dosage (Fig. 5). In P_{SARK}*::IPT* plants, 7 mM of N resulted in increase of SPS, while SS activity showed a significant increase when plant grown under severe N deficiency (1 mM; Fig. 5). Finally, in WT tobacco plants, the enzymes involved in Suc degradation, cell wall, vacuolar and cytosolic invertases, followed a similar trend, with higher activities under the severe N deficiency treatment (1 mM; Table 3), while in P_{SARK}*::IPT* plants, vacuolar and cytosolic invertases activities remain unchanged under N deficiency, only cell wall invertase activity showed significant decrease under 7 mM treatment (Table 3).

4. Discussion

The effect of low N availability on partitioning plant biomass has already been widely studied (Nguyen et al., 2003; Scheible et al., 2004). N content was negatively correlated with the proportion of C allocated to the root (De Groot et al., 2003). This observation is in agreement with our results in WT plants under severe N deficiency showing that the reduction in foliar biomass as well as the root biomass maintenance is related with low N availability (Table 1). On the contrary, P_{SARK}*::IPT* plants under severe N deficiency (1 mM) reduced root biomass and maintained leaf biomass. Our results suggest that in transgenic plants the translocation of sugars (carbon compounds) to the root is disrupted despite N deficiency. This response could be related to increased *IPT* gene expression levels which demonstrate the response of promoter P_{SARK} to severe N deficiency (1 mM; Fig. 1). Therefore, CKs may play a role in plant biomass partitioning under low availability of N. In this sense, it has

been indicated that CKs supplied at physiological concentrations to *Urticadioica* plants could change the direction of photoassimilates, thus completely inverting the source-sink relationship in favor of the shoot (Beck, 1999).

It has been demonstrated the protective action of CKs under stress conditions preserved the structure and function of the photosynthetic machinery (Chernyad'ev, 2009), thus CKs could induce the synthesis of proteins of the electron-transport chain, photosynthetic chlorophyll-protein complexes and Rubisco (Chernyad'ev, 2009). Several authors show *A* maintenance under water stress in plants which expressing *IPT* gene driven by different promoters (Rivero et al., 2009; Merewitz et al., 2011). In WT plants, severe N deficiency (1 mM) provoked drastic decrease in *A* (Fig. 3A) linked to drastic decrease in Vcmax, Jmax and TPU parameters (72, 70 and 66% respectively; Figs. 4A–C). Furthermore, in P_{SARK}*::IPT* plants under 1 mM N dosage, reduction of *A* (Fig. 3B) and Vcmax; Jmax and TPU parameters (48, 45 and 21% respectively; Figs. 4A–C) were less pronounced than in WT tobacco plants. It suggests that CKs could protect biochemical processes such as carboxylation rate of Rubisco which may enhance *A*. In P_{SARK}*::IPT* tobacco plants under severe N deficiency, the maintenance of Jmax and TPU appears to supports a role of CKs in the protection of the electron transport, leading to the regeneration of RUBP and the capacity of the chloroplast reactions to use triose-P under low availability of N.

Sm-Rubisco are encaged by a family of nuclear genes. It has been proposed that this subunit may be involved in the regulation of photosynthetic ratios and thus the carboxylase/oxygenase Rubisco activity (Khrebtukova and Spreitzer, 1996). Our results show a decrease in sm-Rubisco transcripts in WT plants under N deficiency according to a decrease Vcmax. However, P_{SARK}*::IPT* plants preserved sm-Rubisco transcripts levels under N deficiency compared to control conditions (10 mM). It suggests the CKs could be involved transcriptional regulation of this gene under N deficiency regulating photosynthetic ratios and carboxylase/oxygenase Rubisco activity.

Regarding the enzymes involved in sugars metabolism, an increase in both synthesis and degradation of Suc were observed in the WT plants subjected to severe N deficiency (1 mM, Table 3 and Fig. 5). P_{SARK}*::IPT* tobacco plants showed a similar trend although both synthesis and degradation were increased less than in WT plants (Table 3 and Fig. 5). It has been demonstrated that N deficiency leads a sugar accumulation due to less carbon is consumed and exported from source leaves for N assimilation and growth (Paul and Driscoll, 1997; Scheible et al., 2004). The degradation of Suc by invertases, observed especially in WT plants (Table 3) could be a carbon source for the formation of carbon skeletons. Thus, carbon skeletons may be aminated forming new amino acids, particularly affected during the N deficiency (Sánchez et al., 2004). In fact previous work by our group shown that WT plants under N deficiency increased processes such as protein degradation or photorespiration which lead an ammonia formation. This ammonia is reassimilated by the cycle GS/GOGAT. Thus carbon skeletons, from the Suc degradation could enhance N remobilization process, which are critical under low availability of N (Rubio-Wilhelmi et al., 2012). Besides, general increase in other sugars concentration in mature leaves (Table 2) and a reduction in leaf biomass (Table 1) found in WT plants subjected to severe N deficiency (1 mM) could be related with sink limitation within the whole plant due to decreased the growth under N deficiency (Paul and Foyer, 2001). In transgenic plants, limited increase in sugar concentration as well as the maintenance of leaf biomass observed would indicate that CKs may play a role in the sink limitation caused by deficiency of N. Overall, our results could be agree with several authors that indicated CKs may be involved in the maintenance of sink strength in plants subjected several stress conditions such as drought or salinity (Peleg et al., 2010; Pérez-Alfocea et al., 2010).

In conclusion, our work indicated that expression an *IPT* gene driven by P_{SARK}, a stress-and maturation-induced promoter, leads the maintenance of photosynthesis as well as VCmax, Jmax and TPU parameters and leaf biomass under N deficiency. Therefore, our results suggest that CKs could protect biochemical processes such as carboxylation rate of Rubisco, enhancing sm-Rubisco transcripts levels under low availability of N. Also, CKs could be involved in the protection of the electron transport, leading to the regeneration of RUBP and the capacity of the chloroplast reactions to use triose-P under N deficiency.

Regarding sink-source relationships, CKs would act maintain the strength of the sink, such as young leaves, enhancing foliar biomass under severe N deficiency (1 mM).

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