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Cytokinin-Dependent Improvement in Transgenic P_{SARK}::IPT Tobacco under Nitrogen Deficiency

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ABSTRACT: Wild-type (WT) and transgenic tobacco plants overexpressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinin (CKs) synthesis, were grown under limited nitrogen (N) conditions to evaluate the role of CKs in NUE (N-use efficiency) and in different parameters that determine the quality of tobacco leaves. The results indicate that WT tobacco plants submitted to N deficiency show a decline in the leaf/root ratio, associated with a decrease in the NUE and in tobacco-leaf quality, defined by an increase in the quantity of nicotine. On the contrary, the transgenic plants submitted to N deficiency maintained the leaf/root ratio, presenting a higher NUE and greater quality of tobacco leaves than the WT plants, as the latter showed reduced nicotine and an increase in reducing sugars under severe N-deficiency conditions. Therefore, the overexpression of CKs under N deficiency could be a useful tool to improve tobacco cultivation, given that it could reduce N-fertilizer application and thereby provide economic savings and environmental benefits, maintaining yield and improving tobacco leaf quality.

KEYWORDS: nitrogen deficiency, cytokinins (CKs), tobacco plants, N-use efficiency (NUE), nicotine, reduced sugars

INTRODUCTION

Nitrogen (N) availability is considered one of the main limiting factors in crop production.[1] This is particularly true for tobacco plants, in which the decline in foliar biomass is directly correlated to N deficiency,[2] because tobacco cultivation requires high quantities of nitrate (NO_3^-) for maximum yield.[3] Only 30−40% of the N applied to the soil is used by the plant, so greater N-use efficiency (NUE) could improve crop yield and quality, reducing economic costs as well as decreasing environmental degradation caused by N fertilizer application.[4] Therefore, the selection of cultivars with high NUE becomes critical, especially in crops such as tobacco, in which N is essential to reach harvest. Thus, crops that have high NUE offer greater yield under conditions of limited N supply or require lower N quantities to reach the same yield as crops with lower NUE.[3,5]

NUE is defined as biomass production per unit of N available in the soil.[6] This can be divided into two fundamental processes: (i) the ability of the plant to take up N from the soil and (ii) the efficient use of the N taken up, that is, the capacity of the plant to transfer and utilize this element in plant organs.[3] Genetic variability in NUE has been demonstrated in several species such as rice, alfalfa, and maize.[7,8] Improved NUE together with the evaluation of external factors such as agricultural practices or soil types has today become essential for maintaining agricultural output, particularly when N is limited in the environment.[4,9]

The quality of tobacco is a complex combination of visual, physical, and chemical characteristics that are strongly influenced by N fertilization.[10] The NO_3^- levels in tobacco leaves have a marked effect on their chemical composition, as it stimulates the formation of compounds that are harmful for human health, such as nitric oxide, volatile carcinogenic compounds, or specific nitrosamines that are directly related to the quantity of this anion in the leaf.[3] Furthermore, alterations in the availability of NO_3^- in tobacco plants could provoke changes in the leaf nicotine content, and a close relationship has been reported between N metabolism and alkaloid synthesis.[11] In addition, another parameter used to evaluate tobacco quality are sugars, which are closely related to the quantity of NO_3^-, given that an increase in the quantity of NO_3^- in tobacco leaves lowers the sugar quantity and thus quality.[12]

Cytokinins (CKs) are phytohormones that control the plant developmental program. In addition, a relationship between CKs and macronutrient acquisition has been postulated.[13] Recent studies have indicated that CKs act as long-distance messengers, signaling the N status of the plant[14] and thus regulating the nutrient uptake systems.[15] Previous work indicated that plants overexpressing isopentenyltransferase (IPT), an enzyme that catalyzes the limiting step in CK synthesis, do not display biomass reduction caused by N deficiency,[16] and a CK-dependent NUE was suggested. Here, we aimed to examine the response of NUE and leaf quality of P_{SARK}::IPT tobacco plants submitted to N deficiency.

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 as described before.[16] Growth conditions and N treatments were as described elsewhere.[16] The N treatments began 60 days after sowing (DAS) and were maintained for 30 days. The treatments were 10 mM (control) and 7 and 1 mM $NaNO_3$. The experimental design was a randomized

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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 Analysis. All plants were at the late vegetative stage when harvested. Middle leaves (positions 7 and 8) were harvested, frozen immediately in liquid N_2, and kept at −80 °C until used. Plant material was lyophilized and used to determine N forms, nicotine, and soluble sugar and starch. To determine the leaves/roots ratio, leaves and roots from three plants per line were sampled at 90 DAS. The leaves and roots were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded.

NUE Parameters. NO_3^- was analyzed from an aqueous extraction of 0.2 g of dried leaf and root material in 10 mL of Millipore-filtered water.[17] Reduced N concentrations were analyzed after digestion of 0.2 g of dry and milled leaf and root material with H_2SO_4 (5 mL at 98%) and H_2O_2 (30%) (Sigma-Aldrich, Madrid, Spain).[18] Total nitrogen content (TNC) represents the sum of reduced N and NO_3^- and was expressed as milligrams gram DW. Total nitrogen accumulation (TNA) was calculated as TNC divided by leaf tissue DW values,[19] the result being expressed as milligrams of N. Nitrogen utilization efficiency (NUtE) was calculated as leaf tissue DW divided by TNC.[20] The results were expressed as grams DW per milligram N. Nitrogen uptake efficiency (NUpE) was calculated as TNA divided by root tissue DW values,[21] the results being expressed as milligrams N per gram DW root. Over the period under study, determinations of N uptake fluxes were calculated from the relative growth rate (RGR), fresh weight (FW), nutrient total concentrations, and NO_3^- concentration contents of leaves and roots.[22]

Nicotine Analysis. Half a leaf without midvein (100 mg) was harvested and frozen in liquid N_2. Leaf material was ground frozen in a 2 mL microcentrifuge tube. The samples were extracted by shaking the tubes vigorously for 2 h with 1.5 mL of 40% aqueous MeOH, containing 0.5% acetic acid. The resulting extract was centrifuged (13000 rpm, 12 min) and filtered through a 0.45 μm PVDF membrane. Chromatographic analyses were carried out on a Phenomenex reverse-phase column (250 × 4.6 mm, Luna 5 μm C18 (2) 100A).[23] Nicotine standard was from Sigma-Aldrich. The mobile phase consisted of two solvents: (A) H_3PO_4 in water (pH 2.2) and (B) acetonitrile; HPLC gradient: 0−6 min, 12% B; 6−10 min, 18% B; reaching 58% B in 30 min. The flow rate was 1 mL/min, the injection volume was 20 μL, and the column oven was set at 24 °C. The eluent was monitored at 210, 254, 320, and 365 nm. The HPLC-UV analyses were carried out with an Agilent HPLC 1100 series.

Reduced Sugar and Starch Analysis. Frozen leaves were homogenized for sugar analysis using mortar in 5 mL of ethanol at 96%. After centrifugation, the insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 5500 rpm for 10 min and the supernatant stored at 4 °C for the determination of reduced sugars such as sucrose, glucose, and fructose,[24] whereas the residue remaining from the centrifugation was dried for 48 h at 40 °C and used in the analysis of starch concentration.[24] Analyses of starch and reduced sugars were made with the anthrone reagent (Sigma-Aldrich) at an absorbance of 650 nm against a standard curve of sucrose. The starch and reduced sugar concentrations were expressed as milligrams per gram DW.

Statistical Analysis. The data compiled 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

Leaves/Roots Ratio and NUE Parameters. The leaf/root ratio was measured in WT and P_{SARK}::IPT tobacco plants. In WT plants the leaf/root ratio remained unchanged when the plants were exposed to 7 mM N, whereas a significant decline was observed when the WT tobacco plants were exposed to a severe N deficiency (1 mM) (Table 1). Nitrogen deficiency did not induce changes in the leaf/root ratio in the transgenic plants (Table 1).

Table 1. Effect of 10 mM N (Control) and N Deficiency (7 and 1 mM) on Total Leaf/Root Ratio in Two Tobacco Lines, 'WT' and 'IPT'[a]

	total leaves/root ratio	
NO_3^- treatment	WT	IPT
control	9.41 ± 0.88 a	18.80 ± 0.41
def 7 mM	9.83 ± 0.73 a	24.71 ± 1.10
def 1 mM	6.98 ± 0.30 b	19.75 ± 3.24
P value	*	NS
$LSD_{0.05}$	2.378	6.894

[a] Values are the mean ± 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), $P > 0.05$.

Both wild-type and transgenic P_{SARK}::IPT tobacco lines showed a significant decrease in TNC under severe N deficiency (1 mM), but no changes were seen in the 7 mM N treatments (Table 2). Treatments of 1 mM oN lowered the TNA in WT plants, whereas in P_{SARK}::IPT plants the TNA was reduced both under the 7 mM application and under 1 mM N (Table 2). With respect to the two NUE components, NUtE increased in both tobacco lines under the severe N-deficiency treatment (1 mM) (Table 2). Both wild-type and P_{SARK}::IPT plants showed a significant reduction of NUpE under the 1 mM N treatment with respect to control (Table 2). Similar results were found in the N uptake flux that diminished significantly with the deficient N applications in both tobacco plant lines (Figure 1).

Nicotine, Reduced Sugar, and Starch. In the WT plants, N deficiency provoked a significant increase in the nicotine amounts both under the 7 mM application and under severe deficiency (1 mM) (Figure 2). A reverse situation was seen in the P_{SARK}::IPT tobacco plants, because the N deficiency treatment significantly lowered the amounts of nicotine (Figure 2).

The WT tobacco plants showed a significant increase in reducing sugars contents under N deficiency conditions (Figure 3A). PSARK::IPT plants also showed this significant increase under the 7 mM application and under severe deficiency (1 mM) in reducing sugars contents. A similar trend was found in the quantity of starch in tobacco leaves of both lines, which showed a significant increase with N deficiency (Figure 3B).

DISCUSSION

We have shown previously that the growth of WT tobacco plants under N deficiency resulted in reduced foliar biomass and lower relative growth rates.[16] Similar results have been reported in different plant species,[25,26] confirming that plants submitted to limiting conditions of N redirect the photoassimilates toward the root zone to increase growth and intensify N uptake.[27] Our results appear to support this notion, as shown by a reduction of the leaf/root ratio in WT tobacco plants subjected to N deficiency (Table 1). On the other hand, the transgenic plants expressing P_{SARK}::IPT maintained active growth and biomass

Table 2. Effect of 10 mM N (Control) and N Deficiency (7 and 1 mM) on Total Nitrogen Content (TNC), Total Nitrogen Accumulation (TNA), Nitrogen Utilization Efficiency (NUtE), and Nitrogen Uptake Efficiency (NUpE) in Two Tobacco Lines, 'WT' and 'IPT'[a]

line/NO_3^- treatment	TCN (mg/g DW)	TNA (mg N)	NUtE (g DW/mg N)	NUpE (mg N g/DW root)
WT				
control	55.61 ± 4.02 a	376 ± 27.21 a	0.122 ± 0.008 b	311 ± 22.49 a
def 7 mM	59.27 ± 1.29 a (+6.5%)	320 ± 6.98 a (−14.8%)	0.091 ± 0.002 c (−25.4%)	325 ± 7.09 a (+4.6%)
def 1 mM	23.18 ± 1.18 b (−42%)	105 ± 5.35 b (−72%)	0.196 ± 0.009 a (+60.5%)	86 ± 4.40 b (−72.2%)
P value	***	***	***	***
$LSD_{0.05}$	8.760	57.14	0.026	47.93
IPT				
control	59.86 ± 2.08 a	548 ± 19.05 a	0.153 ± 0.005 b	876 ± 30.48 b
def 7 mM	53.16 ± 2.81 a (−11%)	419 ± 22.22 b (−23.5%)	0.149 ± 0.007 b (−2.6%)	1103 ± 58.48 a (+25.7%)
def 1 mM	28.48 ± 1.16 b (−47%)	234 ± 9.58 c (−57.2%)	0.289 ± 0.012 a (+88.8%)	446 ± 18.24 c (−49%)
P value	***	***	***	***
$LSD_{0.05}$	7.376	61.53	0.030	136.71

[a] Values are the mean ± 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), $P > 0.05$.

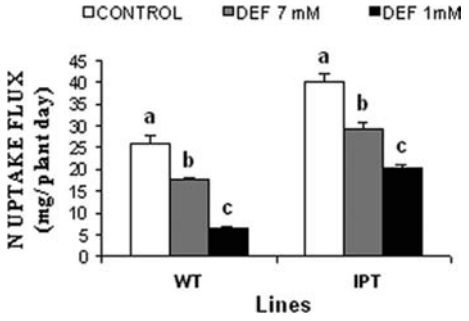

Figure 1. Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on N uptake flux of two tobacco lines: 'WT' and 'IPT'.

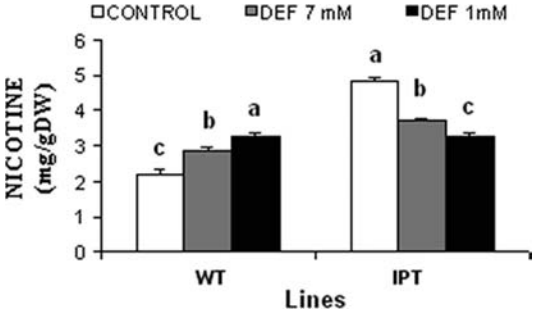

Figure 2. Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on nicotine concentration of two tobacco lines, 'WT' and 'IPT'.

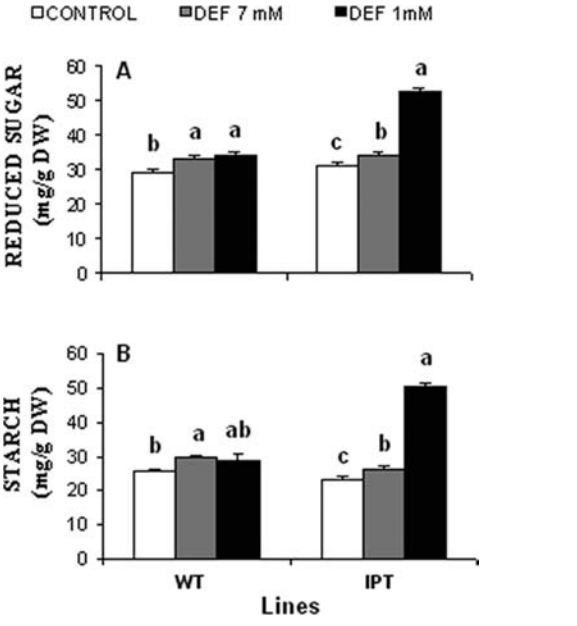

Figure 3. Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on (A) reducing sugar concentration and (B) starch concentration of two tobacco lines, 'WT' and 'IPT'.

under severe N deficiency.[16] This sustained biomass, accompanied by maintenance of the leaf/root ratio under N deficit (Table 1), would indicate that the CKs might impede the rerouting of the photoassimilates toward the root zone, which would otherwise limit N availability, favoring shoot growth.

The increase in nutrient-use efficiency has been described as a useful tool to improve agricultural systems.[28] Thus, because N is a determining element in tobacco yield, the selection of genotypes with high NUE is critical for the improvement of this crop. The TNC and TNA are determining parameters in the nutritional state of the plants, given that they show a direct relationship between yield and leaf content in N.[3] In our work, tobacco plants of both lines submitted to severe N deficiency (1 mM) reduced the TNC and TNA (Table 2). However, the WT plants lowered TNA (72%) by a higher proportion than the transgenic plants, with respect to control (57.2%) (Table 2). This fact is correlated with the parameters related to NUE, because both plant lines responded similarly to the N deficiency. However, in the transgenic plants the NUtE increased to 88% under severe N deficiency (1 mM), whereas in WT plants the increase was only 60% (Table 2). This would indicate that under severe N deficiency (1 mM), the transgenic plants utilize N more efficiently. Furthermore, the transgenic plants also displayed more efficient N uptake than WT plants when both lines were under severe N limitation, the NupE decreasing by 72.2% in WT plants,

whereas in the transgenic plants the decrease was only 49% (Table 2). This finding is again demonstrated by comparing the uptake flow of N of both plant lines (Figure 1). The results would explain previous observations that showed the maintenance of the biomass and foliar RGR in P_{SARK}::IPT tobacco plants under adverse growth conditions.[16] Similar results have been shown in varieties of *Brassica* under N deficiency, showing that varieties with greater NUE presented greater biomass.[29]

Tobacco quality is a complex combination of physical, chemical, and visual characteristics that are strongly influenced by N fertilization and the foliar concentration of NO_3^-.[10] Previous work has shown that the N deficiency in both tobacco lines diminished the quantity of NO_3^-.[16] This reduction noticeably affects the improvement in quality, as it reduces the quantity of nitric oxide and volatile carcinogenic compounds that are related to the quantity of this anion in the leaf. In addition to the NO_3^- concentration, another parameter that determines the quality of the tobacco leaves is the nicotine. Tropano alkaloids such as nicotine are defined as secondary metabolites that contain atomic N in their molecules, and their precursors are two amino acids such as ornithine and arginine.[11] The N deficiency provoked an increase in the quantity of nicotine in WT plants (Figure 2). This has been reported earlier in tobacco plants subjected to N deficiency that show typical symptoms of N deficiency.[30] Nicotine as a secondary metabolite could change the chemical composition of the leaf, palliating the damage caused by pathogen attack or abiotic stress.[31] The increase in nicotine amounts involves a loss in tobacco quality[3] because nicotine is the main harmful component in cigarettes, playing an essential role in the development of cardiovascular diseases and cancer.[32] However, the reduction in the quantity of nicotine shown by transgenic plants under N deficiency (Figure 2) represents better leaf quality. Finally, the increase in reducing sugars and starch in transgenic plants under severe N deficiency (1 mM) (Figure 3) would also notably favor the quality of tobacco leaf, because, in tobacco, reducing sugars have the most favorable influence on the aroma and taste during smoking and thus are important constituents for the evaluation of tobacco quality.[33] Finally, it should be pointed out that the increase of up to 69% of the soluble sugars and the starch found in the leaves of P_{SARK}::IPT tobacco submitted to severe N deficiency (1 mM) (Figure 3) would demonstrate that the photoassimilates remain in the shoot, favoring its growth while avoiding a reduction of foliar biomass as a result of N deficiency.

In short, our results appear to indicate that the overexpression of the CKs under N deficiency could be useful as a strategy to improve tobacco leaf quality, reducing costs (fertilizers, wastes, environmental burden, etc.).

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ABBREVIATIONS USED

CKs, cytokinins; IPT, isopentenyltransferase; NUE, N-use efficiency; NO_3^-, nitrate; NUpE, nitrogen uptake efficiency; NUtE, nitrogen-utilization efficiency; TNA, total nitrogen accumulation; TNC, total nitrogen content; WT, wild type.

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