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Research article

Response of carbon and nitrogen-rich metabolites to nitrogen deficiency in P_{SARK}::IPT tobacco plants

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ABSTRACT

Wild type (WT) and transgenic tobacco plants expressing isopentenyltransferase (IPT), a gene coding the rate-limiting step in cytokinin (CKs) synthesis, were grown under limited nitrogen (N) conditions. Here, we analyse the possible effect of N deficiency on C-rich compounds such as phenolic compounds, as well as on N-rich compounds such as polyamines (PAs) and proline (Pro), examining the pathways involved in their synthesis and degradation. N deficiency was found to stimulate phenolic metabolism and increase these compounds both in P_{SARK}:IPT as well as in WT tobacco plants. This suggests that nitrate (NO_3^-) tissue concentration may act as a signal triggering phenolic compound accumulation in N deficiency plants. In addition, we found the maintenance of PAs in the WT plants would be correlated with the higher stress response to N deficiency. On the contrary, the reduction of free PAs and Pro found in the P_{SARK}::IPT plants subjected to N deficiency would indicate the operation of an N-recycling mechanism that could stimulate a more efficient N utilization in P_{SARK}::IPT plants.

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1. Introduction

Plant growth and development depends on an adequate supply of nitrogen (N) in order to synthesize the amino acids (aa), proteins and nucleic acids [1]. In addition, it is of great importance in the biochemistry of compounds such as enzymes, pigments, secondary metabolites, and polyamines (PAs) [2]. N deficiency leads to wide reprogramming of primary and secondary metabolism [3], and low N has an extensive impact on the overall plant metabolism, inducing a shift from N-based to C-based compounds. For example, a typical sign of N deficiency is the depletion in aa or other N-rich compound as PAs [4,5] and the accumulation of phenolic metabolites [6,7].

Phenolic compounds are C-rich metabolites that represent the largest group of plant secondary metabolites [8]. For example phenolic compounds are important antioxidant and may play an important role as scavengers of free radicals and other oxidative species [9]. Phenolic compounds are generally synthesised through the shikimate pathway [10]. From the metabolism of carbohydrates and glycolysis, the 3-deoxy-D-arabino-heptulosonate 7-phosphate is biosynthesised by its corresponding synthase (DAHPS, EC 4.1.2.15), a key enzyme controlling the C flow towards phenolic metabolism. The pathway continues, producing the aa aromatic phenylalanine, which is afterwards deaminated by the enzyme phenylalanine ammonium lyase (PAL, EC 4.3.1.5), the key enzyme in phenolic biosynthesis. PAL catalyses the non-oxidative deamination of L-phenylalanine to form cinnamic trans-acid. Phenolic compounds are oxidatively degraded primarily by polyphenol oxidase (PPO, EC 1.10.3.2) and also by peroxidases (POX, EC 1.11.1.7). The increase in phenolic compound under low N is mainly attributed to the enhanced PAL activity [11]. It has been postulated that the appearance of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), under N deficiency could act as a signal activating PAL activity and the synthesis of phenols [12]. Others assumed a carbon-nutrient balance hypothesis, according to which secondary metabolism is directed towards C-rich metabolites, in N limited plants. Thus, this type of secondary compounds may accumulate forming a pool of C in plants subjected to N deficiency due to coordinated regulation the C and N metabolism [6,13].

PAs are known as a group of natural compounds with aliphatic N structure, that play important roles in many physiological processes, such as cell growth and development and the response to environmental stresses [14]. Spermidine (Spd) and spermine

Abbreviations: N, nitrogen; aa, amino acids; PAs, polyamines; ROS, reactive oxidative species; H_2O_2, hydrogen peroxide; Put, putrescine; Spm, spermine; Spd, spermidine; Pro, proline; Orn, ornithine; Arg, arginine; Glu, glutamate; CKs, cytokinins; IPT, isopentenyltransferase.

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(Spm) and their obligate precursor putrescine (Put), are the most commonly found PAs in higher plants and could be present as free, conjugated and bound forms [14]. They are formed in the oxoglutaric acid pathway of aa biosynthesis. The first step in PAs biosynthesis in higher plants is Put synthesis from decarboxylation of either ornithine (Orn) or arginine (Arg), in reactions catalysed by the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) via agmatine (Agm). Spd and Spm are formed by the subsequent addition of an aminopropyl moiety onto Put and Spd, respectively. Intracellular free PAs pool, depend also on several processes including degradation and conjugation. PAs are oxidatively deaminated by the action of amines oxidases, include the copper diamine oxidases (DAO; EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAO reaction product from Put is γ-aminobutyric (GABA) that is formed via pyrroline. PAO yields pyrroline, diaminepropane and H_2O_2 [15]. PAs and proline (Pro) pathways are metabolically linked in their biosynthesis through the common precursor glutamate (Glu), and consequently, to the substrates Arg and Orn [16]. Glu is conversed into Pro by two reactions, catalysed by glutamate dehydrogenase and Δ^1-pyrroline-5-carboxylate synthetase (P5CS, E.C. 2.7.2.11/1.2.1.41). Another precursor of Pro synthesis is Orn, which is transaminated by ornithine-δ-aminotransferase (OAT, E.C. 2.6.1.13). The most dramatic changes in PAs and Pro metabolism and content are those brought about by nutrient starvation [17,18]. K^+ starvation in barley and Arabidopsis led to the accumulation of Put via ADC activation, also other mineral deficiencies were found to be associated with a stimulation of PAs oxidation [19,20]. N deficiency led to a depletion of the free PAs and Pro levels in different plant species as *Poa* or *Phaseolus* and possibly the use of degraded PAs and Pro as N source [4,1].

Cytokinins (CKs) are phytohormones that control the plant developmental programme and a relationship between CKs and macronutrient acquisition has been postulated [21]. Recent studies have indicated that CKs could act as long-distance messengers signalling the N status of the plant [22], thus regulating the nutrient-uptake systems [23]. Previous work indicated that tobacco plants overexpressing isopentenyltransferase (P_{SARK}::IPT), an enzyme that catalyses the limiting step in CKs synthesis, increase the amount of CKs, inhibiting the formation of ROS and preventing the oxidative stress caused by N deficiency [24,25]. Numerous studies have indicated that compounds such as phenols, PAs or Pro and its synthesis with oxidative stress such compounds being described classically as an ROS scavenger [9,26,27]. Here, we aim to determine the effects of N-deficiency on the synthesis and degradation of phenolic compounds, Pas and Pro in Wild type (WT) and P_{SARK}::IPT tobacco plants.

2. Results

2.1. Phenolic compounds, PAs and Pro

The concentration of soluble phenolic compounds was measured in WT and transgenic tobacco plants expressing P_{SARK}::IPT (Table 1). The plants were grown under normal conditions (10 mM of N) and under two N-deficient treatments (i.e. 7 and 1 mM of N). The hydroxycinnamic acid content showed a significant increase under 7 and 1 mM of N treatments in WT tobacco plants, while in transgenic plants this significant increase was detected only under severe N deficiency, i.e. the 1 mM treatment (Table 1). With respect to flavonoids and glycosides, the control treatment showed the lowest concentrations of these compounds both in the case of the WT plants as well as in the transgenic plants, showing a significant increase in these compounds with the N deficiency (Table 1). Therefore, both lines of tobacco plants, WT and P_{SARK}::IPT, registered a higher total phenol concentration in leaf, this being significant for both lines in the case of the severe N deficiency treatment (1 mM). Finally, it bears highlighting that under any of the treatments, the WT plants reached higher total phenol concentrations than did transgenic plants (Table 1).

Table 1
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on phenolic compounds in two tobacco lines: 'WT' and 'IPT'. Q1

Lines/NO_3^- treatment	Compounds			
	Hydroxycinnamic acids and derivatives (mg g^{-1} DW)	Flavonoids and glycosides (mg g^{-1} DW)	Others (mg g^{-1} DW)	Total (mg g^{-1} DW)
WT				
Control	5.31 ± 0.19 c	0.52 ± 0.00 c	2.65 ± 0.06 b	8.49 ± 0.14 b
Def 7 mM	6.12 ± 0.26 b	0.59 ± 0.00 b	3.01 ± 0.27 b	9.73 ± 0.51 b
Def 1 mM	10.91 ± 0.18 a	0.92 ± 0.02 a	5.58 ± 0.45 a	17.42 ± 0.36 a
P-value	***	***	**	***
$LSD_{0.05}$	0.761	0.044	1.078	1.287
IPT				
Control	2.59 ± 0.22 b	0.42 ± 0.00 c	1.12 ± 0.16 b	4.14 ± 0.21 b
Def 7 mM	2.89 ± 0.12 b	0.37 ± 0.01 b	1.06 ± 0.07 b	4.34 ± 0.12 b
Def 1 mM	5.76 ± 0.27 a	0.56 ± 0.00 a	2.34 ± 0.06 a	8.67 ± 0.20 a
P-value	***	***	***	***
$LSD_{0.05}$	0.764	0.038	0.383	0.652

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).

PAs are a group of aliphatic amine compounds that are ubiquitous in all plant cells. We found that in WT tobacco plants, the N deficiency did not cause significant changes in the concentration of Spd and free Agm, and no detectable amounts of free Put or Spm were found under any of the treatments. On the other hand, in the transgenic P_{SARK}::IPT plants, a significant decrease in the free PAs, Put, and Spd was seen under N deficiency (Table 2) and Spm was not detected. Both WT and P_{SARK}::IPT tobacco plants showed a decrease in Pro concentration under N-deficient conditions (Fig. 1).

2.2. Phenolic compound metabolism

The activity of some of the enzymes controlling the synthesis of phenolic compounds was measured in the leaves of WT and

Table 2
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on free polyamines in two tobacco lines: 'WT' and 'IPT'.

Lines/NO_3 treatment	Free polyamines (mg g^{-1} FW)				
	Put	Spd	Spm	Agm	Total
WT					
Control	n.d	0.10 ± 0.03	n.d	3.78 ± 0.15	3.88 ± 0.12
Def 7 mM	n.d	0.05 ± 0.00	n.d	4.20 ± 0.51	4.26 ± 0.51
Def 1 mM	n.d	0.08 ± 0.00	n.d	3.05 ± 1.02	3.13 ± 1.02
P-value		NS		NS	NS
$LSD_{0.05}$		0.06		2.30	2.29
IPT					
Control	0.35 ± 0.00 a	0.23 ± 0.01 a	n.d	3.95 ± 0.62 a	4.54 ± 0.60 a
Def 7 mM	0.26 ± 0.03 b	0.07 ± 0.00 c	n.d	3.72 ± 0.37 a	4.06 ± 0.41 a
Def 1 mM	0.01 ± 0.00 c	0.14 ± 0.03 b	n.d	1.35 ± 0.37 b	1.50 ± 0.35 b
P-value	**	**		*	**
$LSD_{0.05}$	0.06	0.06		1.63	1.62

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).

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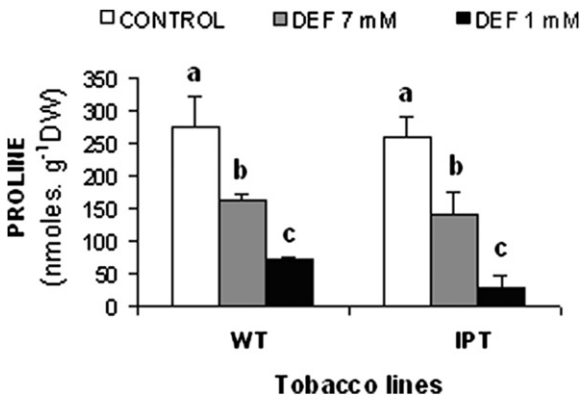

Fig. 1. Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on proline content in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the means ± SE ($n = 9$).

P_{SARK}::IPT tobacco plants (Fig. 2). Under the severe N-deficiency treatment (1 mM), the WT plants showed a significant increase in the activity of these enzymes, with the exception of DAHP DS-Co (Fig. 2B). The 7 mM of N application only induced the reduction of DAHP DS-Mn activity with respect to the controls (Fig. 2A). In the case of the transgenic P_{SARK}::IPT plants, the enzymatic activities increased when grown under 1 mM of N, with the exception of DAHP-Co and SKDH which remain unchanged (Fig. 2B, C). When grown under 7 mM of N treatments, the activity of the enzymes did not significantly differ with respect to controls, except for the enzyme C4H that showed increased activity with respect to the N control (Fig. 2F). On the other hand, the activities of PPO and GPOX (Fig. 3A, B), enzymes involved in the degradation of phenol compounds, were lower under N deficiency treatments, this trend being significant only under the severe deficiency dosage of 1 mM in the case of the P_{SARK}::IPT tobacco plants (Fig. 3A, B).

2.3. PAs and Pro metabolism

The enzymes involved in the PAs synthesis are shown in Fig. 4. The activity of ADC, the enzyme mediating Agm synthesis, increased in WT tobacco plants grown under 1 mM of N as compared to controls and no significant differences were seen in the transgenic P_{SARK}::IPT plants grown at the N deficiency rates (Fig. 4A). A greater ODC activity was found in the WT plants grown under N deficiency, although significant differences were only seen in the 7 mM treatment. A different response was seen in the P_{SARK}::IPT plants, where ODC activity was higher under the control N treatment (Fig. 4B). The enzymes involved in the degradation of PAs, PAO and DAO followed a similar trend, with higher activities under severe N deficiency treatment 1 mM (Fig. 4C, D).

Table 3 shows the enzymatic activities responsible for the synthesis and degradation of Pro. Pro synthesis by OAT, was not appreciably affected by N deficiency. On the other hand, P5CS activity decreased with N deficiency and this decrease was significant under the 1 mM deficiency treatment (Table 3). However, in the transgenic P_{SARK}::IPT plants, the P5CS activity was significantly lower both at the 7 mM and the 1 mM rates of N deficiency (Table 3). The degradation of Pro by the enzyme PDH was

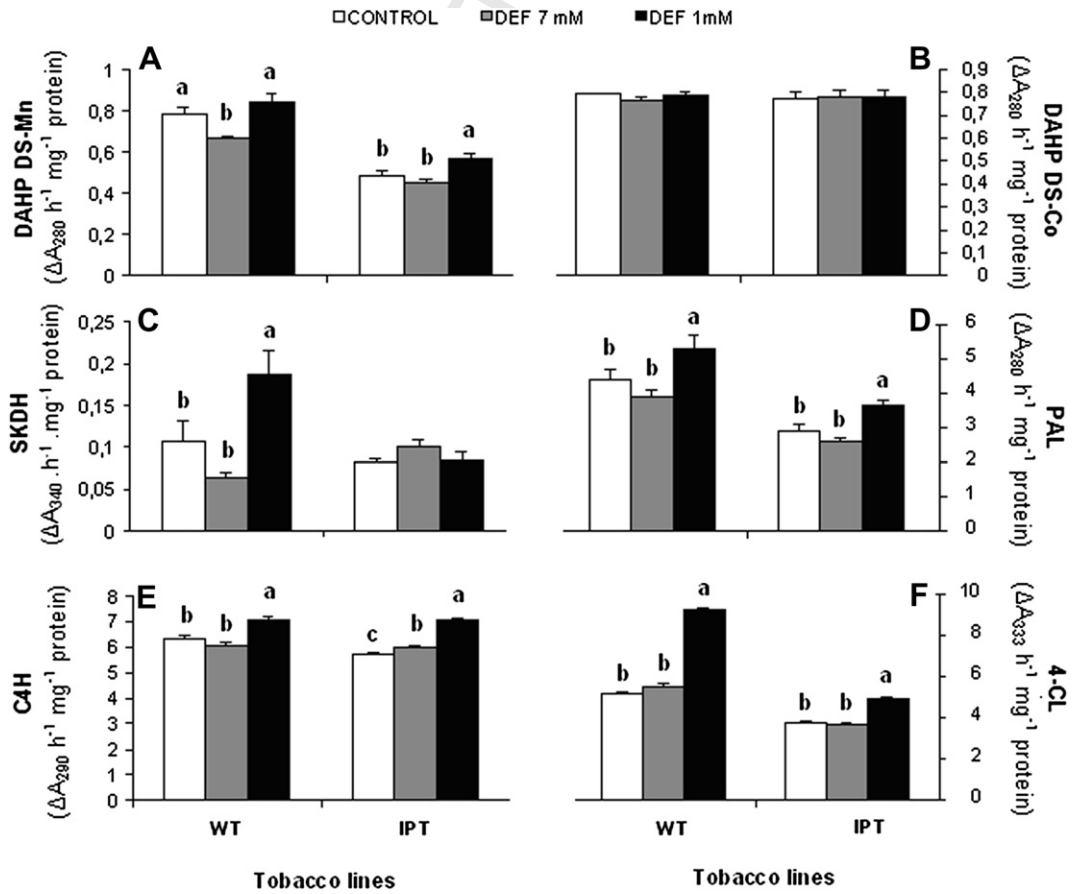

Fig. 2. Response of flavonoid and phenylpropanoid synthesis-related enzymes activity in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the mean ± SE ($n = 9$).

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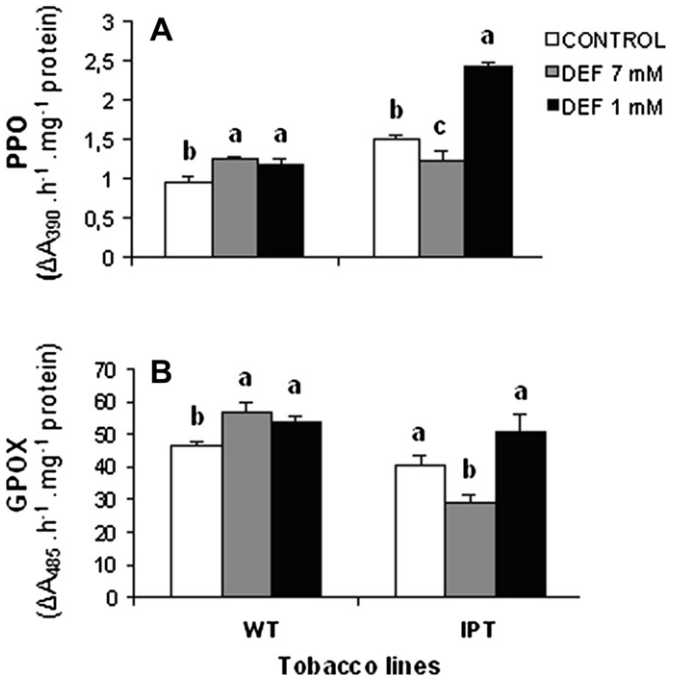

Fig. 3. Response of flavonoid and phenylpropanoid degradation-related enzymes activity in leaves of two tobacco lines: 'WT' and 'IPT' under N deficiency. Values are the mean ± SE ($n = 9$).

significantly reduced with N deficiency in both lines of tobacco plants (Table 3).

3. Discussion

N deficiency increased the concentration of phenols as well as the enzymes involved in their synthesis and degradation in WT and transgenic P_{SARK}::IPT plants (Table 1; Fig. 2). These compounds have been shown to inhibit of oxidative damage [28], and the increased activity of enzymes associated with phenol synthesis, PAL in particular, and tissue phenol concentrations have been reported in plants grown under diverse abiotic stress, including N deficit [10,11,29]. Furthermore, it has been suggested that ROS are molecules involved in signalling between the perception of stress and the expression of the enzyme PAL, and therefore an increase in ROS would act as a signal, increasing phenolic compounds synthesis. Previous results by our group have shown that under N deficiency, only WT tobacco plants increased in ROS [25], and therefore, the greater PAL activity and the higher quantity of phenols in both plant lines did not correlate with the increment in ROS. Recently, the role of N in the regulation of the synthesis of phenol compounds, and the role of nitrate (NO_3^-) in the regulation phenylpropanoid metabolism have been proposed [6]. Thus, several genes associated with phenolic metabolism are induced by N deficiency, including members of the gene families encoding enzymes such as PAL, 4CL,

Table 3
Effect of 10 mM N (control) and N deficiency (7 and 1 mM) on proline metabolism in two tobacco lines: 'WT' and 'IPT'.

Lines/NO_3^- treatment	OAT (ΔAbs_{340} h^{-1} mg $prot^{-1}$)	P5CS (ΔAbs_{340} h^{-1} mg $prot^{-1}$)	PDH (ΔAbs_{340} h^{-1} mg $prot^{-1}$)
WT			
Control	0.187 ± 0.018	0.678 ± 0.095 a	2.982 ± 0.125 a
7 mM	0.134 ± 0.020	0.357 ± 0.030 a	2.119 ± 0.112 b
1 mM	0.184 ± 0.023	0.245 ± 0.029 b	0.122 ± 0.032 c
P-value	NS	**	***
$LSD_{0.05}$	0.060	0.176	0.289
IPT			
Control	0.213 ± 0.047	0.433 ± 0.012 a	3.895 ± 0.525 a
7 mM	0.268 ± 0.030	0.297 ± 0.026 b	2.192 ± 0.263 b
1 mM	0.214 ± 0.027	0.150 ± 0.034 c	0.924 ± 0.068 c
P-value	NS	***	***
$LSD_{0.05}$	0.105	0.075	0.998

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).

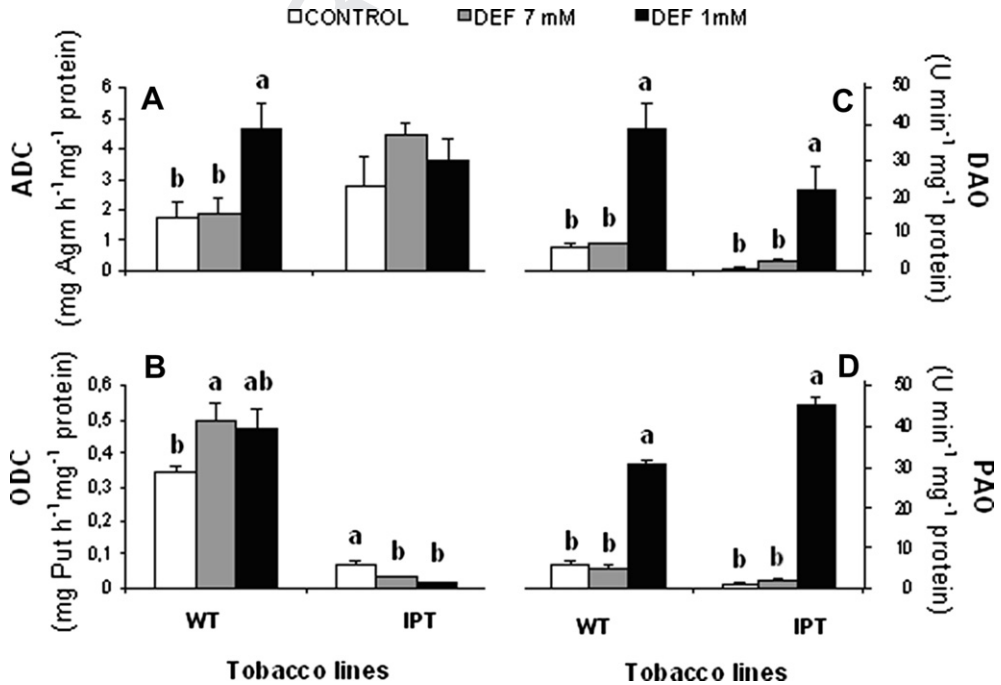

Fig. 4. Effect of 10 mM NO_3^- (control) and NO_3^- deficiency (7 and 1 mM) on arginine decarboxylase (ADC) (A), ornithine decarboxylase (ODC) (B), diamine oxidases (DAO) (C) and polyamine oxidases (PAO) (D) in leaves of two tobacco lines: 'WT' and 'IPT'. Values are the mean ± SE ($n = 9$).

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and C4H [30,3]. Therefore, the increase in the activity of the enzymes involved in the phenolic compounds metabolism shown in this work, as well as the increase in the quantity of phenols observed in both WT and P_{SARK}::IPT plants submitted to N deficiency, appears to supports the notion of N as a possible regulator of phenolic metabolism, and that changes in the N concentrations could trigger variations in phenolic metabolism. The low concentrations of NO_3^- and total reduced N found previously both in the WT and in P_{SARK}::IPT lines subjected to N deficiency [25] would support the hypothesis that changes in NO_3^- tissue concentrations could act as a signal, triggering phenolic accumulation in N deficient plants [6] and therefore phenolic compounds could be synthesised as a C pool in plants as a response to low N concentrations.

PAs and Pro metabolism is associated with the tolerance of plants to adverse environmental conditions [18,14]. Our results show that in response to a severe N deficiency, the synthesis and degradation of PAs are stimulated and the amounts of free PAs are maintained in the WT plants (Fig. 4; Table 2). On the other hand, N deficiency induced a reduction in free PAs amounts in the transgenic P_{SARK}::IPT plants, and this reduction was associated with the increased activities of DAO and PAO (Table 2; Fig. 4C, D). In the WT plants, the maintenance of PAs was associated with the improvement of ADC activity (Fig. 4A), which has been shown to play predominant roles in the accumulation of PAs under stress conditions [31]. It has been postulated that PAs may function along the signalling processes associated with the plant responses to different stress signals [32]. Furthermore, their antioxidant effect due to a combination of their anionic and cationic-binding properties in radical scavenging has been reported [33]. Therefore, the previously noted increase in ROS in WT plants, caused by the N deficiency [25], could be involved in the maintenance of these compounds despite their high N content. However, the increased DAO and PAO activities (Fig. 4C, D) could be considered an N-recycling mechanism, given that GABA, a product of these enzymes, is subsequently transaminated and oxidised to succinic acid, which is incorporated into the TCA cycle. Thus, this pathway ensures the recycling of C and N from Put [15].

The accumulation of Pro has been associated with the response of plants to environmental stress [34,35]. Nevertheless, the reduction in Pro concentrations has been also reported during N deficiency [36,7]. WT and transgenic P_{SARK}:IPT plants displayed a reduction in Pro (Table 3, Fig. 1), that appears to be associated with the inhibition of P5CS, and would indicate a possible redirection of N towards the synthesis of other essential aa.

In conclusion, N deficiency induced changes in the primary and secondary metabolism. Our work indicates that N deficiency treatments stimulated phenolic metabolism and increased phenol contents in both WT and transgenic P_{SARK}::IPT plants. Therefore, phenolic compounds could be accumulated, indicating NO_3^- tissue concentration may act as a signal triggering phenolic compound accumulation in N deficiency plants. The maintenance of PAs in the WT plants would be correlated with the higher stress response to N deficiency. On the other hand, the reduction of free PAs and Pro found in the P_{SARK}::IPT plants subjected to N deficiency would indicate the operation of an N-recycling mechanism that could stimulate a more efficient N utilization as seen by the previously reported enhanced growth at otherwise inimical low N fertilization regimes [5].

4. Methods

4.1. 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 (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 ($n = 9$). 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.

4.2. Phenolic compound and PAs analysis by HPLC/UV

Phenolic compounds were assayed in accordance with the method of Sánchez-Rodríguez et al. [29]. The HPLC/UV analyses were carried out with an Agilent HPLC 1100 series.

For the identification of PAs, 3 g of fresh leaves was homogenized in 4 mL of 6% (v/v) cold perchloric acid (PCA), kept on ice for 1 h, and then centrifuged at 21,000 *g* for 30 min. The pellet was extracted twice with 2 mL of 5% PCA and recentrifuged. The three supernatants were pooled and used to determine the levels of free PAs. The supernatant was benzoylated in accordance with the method of Aziz and Larher [37]. The HPLC/UV analyses were carried out with an Agilent HPLC 1100 series.

4.3. Pro concentration

For the determination of the free-Pro concentration, leaves were homogenized in 5 mL of ethanol at 96%. The insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 3500 *g* for 10 min and the supernatant was preserved 4 °C for the Pro determination [38].

4.4. Enzyme extractions and assays

For determination of DAHPS (DS-Mn, DS-Co) and phenylalanine PAL activities, 0.2 g whole fresh leaf was homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifugated at 15,000 *g* for 15 min at 4 °C. The supernatant was passed through a Sephadex G-25 column (24 × 100 mm) previously equilibrated with the same buffer. DAHPS activity was assayed using a modified method of Morris et al. [39]. PAL activity was measured by a method of Tanaka et al. [40].

For determination of SKDH and PPO activities, whole fresh leaf was homogenized in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 15,000 *g* for 15 min at 4 °C. Shikimate dehydrogenase (SKDH, EC1.1.1.25) activity was determined according to Ali et al. [41]. PPO assay was performed in mixture containing 2.85 mL of 50 mM potassium phosphate buffer (pH 7.0), 50 μL of 60 mM catechol and 0.1 mL of supernatant.

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Increase in absorbance was read over 3 min at 390 nm [42]. For determination of cinnamate 4-hydroxylase (C4H, EC 1.14.13.11), fresh sample of leaf was homogenized in 200 mM potassium phosphate buffer (pH 7.5) containing 2 mM of 2-mercaptoethanol. Homogenates were centrifuged at 10,000 *g* for 15 min at 4 °C. C4H activity was assayed by using the method described by Lamb and Rubery [43]. For 4-coumarate coenzyme A ligase (4CL, EC 6.2.1.12) activity was performed. The extract buffer was 0.05 M Tris–HCl (pH 8.8) containing 14 mM mercaptoethanol and 30% glycerol. The activity was determined with the spectrophotometric method, using caffeic acid as the preferred phenolic substrate [44]. For determination of guaiacol peroxidase (GPOX, EC 1.11.1.7) the extract buffer was 50 mM Tris–HCl (pH 7.5) containing 5 mM mercaptoethanol, 2 mM DTT (dithiothreitol), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA-Na. Homogenates were centrifuged at 16,500 *g* for 30 min at 4 °C. GPOX activity was determined by monitoring guaiacol oxidation at 485 nm [45].

ADC and ODC activities were determined according to Xu et al. [46] with some modifications. Plant material (1.5 g) was homogenized in 50 mM potassium phosphate buffer (pH 6.3) containing 5 mM EDTA, 0.1 mM PMSF, 40 μM pyridoxal phosphate (PLP), 5 mM DTT, 20 mM ascorbic acid, and 0.1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 *g* for 40 min at 4 °C and the supernatant was dialyzed at 4 °C, against 3 mL of 10 mM potassium phosphate buffer (pH 6.3) containing 0.05 mM PLP, 1 mM DTT, 0.1 mM EDTA for 24 h in darkness. The dialyzed extract was used for enzyme assay. DAO and PAO activities were determined by measuring the generation of H_2O_2, a product of the oxidation of PAs, as described by Xu et al. [46], with some modifications. Plant material (0.5 g) was homogenized in 100 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was used for enzyme assays.

P5CS, extraction was carried out according to Sumithra et al. [47]. Leaves were homogenized with extraction buffer containing 100 mM Tris–HCl (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM PMSF and then centrifuged at 10,000 *g* for 15 min. The supernatant was used for enzyme assays. For OAT and proline dehydrogenase (PDH, E.C. 1.5.99.8) extraction, leaves were homogenized in 100 mM K-phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12,000 *g* for 20 min (4 °C) [48]. OAT was assayed according to Charest and Phan (1990) in 0.2 M Tris–KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition the enzyme extract. PDH activity was assayed by the reduction of NAD^+ at 340 nm.

4.5. 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 ($P > 0.05$).

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