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ORIGINAL PAPER

Boron deficiency decreases plasmalemma H^+-ATPase expression and nitrate uptake, and promotes ammonium assimilation into asparagine in tobacco roots

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Abstract The effects of short-term boron deficiency on several aspects (growth, biomass allocation, metabolite concentrations, gene expression, enzyme activities) related with nitrate assimilation were studied in tobacco (*Nicotiana tabacum* L.) plants in order to know the early changes caused by this mineral deficiency. For this purpose, plants were grown hydroponically in a nutrient solution supplemented with 10 μM boron and then transferred to a boron-free medium for 1–5 days. Nitrate concentration decreased in both leaves and roots under boron deficiency, which was not observed in control plants. This correlated with the lower net nitrate uptake rate found in boron-deficient plants when compared to boron-sufficient ones. Results suggest that boron deficiency decreases net nitrate uptake by declining the activity of nitrate transporters rather than affecting their transcript levels. This is supported by a drop in the levels of root *PMA2* transcript during the boron deficient treatment, which could lead to a decrease in the plasma membrane H^+-ATPase activity necessary to get protons out of cell for the cotransport with nitrate inwards. In addition, boron deficiency led to an increase in root Asn content and a decline in glutamine synthetase activity when compared to control plants, which suggest that this mineral deficiency may promote ammonium assimilation via asparagine synthetase in tobacco roots.

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Keywords Asparagine · Boron deficiency · Nitrate assimilation · Nitrate transport · Nitrate uptake · Proton transport · Tobacco

Abbreviations

DW	Dry weight
GS	Glutamine synthetase
NIA	Nitrate reductase structural gene
NR	Nitrate reductase
NRT1 and *NRT2*	Nitrate transport genes
PMA2	Plasmalemma H^+-ATPase gene

Introduction

Nitrate, the most important nitrogenous source for the majority of vascular plants, can act not only as a nutrient but also as a signal for regulating N- and C-metabolism (Scheible et al. 1997a) and biomass allocation (Scheible et al. 1997c). The assimilation of nitrate requires its uptake by roots, its reduction to ammonium, and the incorporation of this cation into organic compounds (Crawford 1995). As a first step of this pathway, nitrate uptake by roots is considered to be a key process of the nitrogen metabolism. Two gene families, *NRT1* and *NRT2*, involved in the nitrate transport of low (LATS, low-affinity transport system) and high affinity (HATS, high-affinity transport system) have been characterized in vascular plants (Daniel-Vedele et al. 1998; Forde 2000; Orsel et al. 2002). Interestingly, a strong correlation has been reported between *NRT2* transcript levels and nitrate uptake (Krapp et al. 1998; Matt et al. 2001). Following uptake into root cells, intracellular nitrate is stored in vacuoles, transported to leaves or reduced to nitrite by

nitrate reductase (NR, EC 1.6.6.1). Although the reduction of nitrate takes place in both roots and leaves, in many herbaceous plants (i.e., tobacco) only a small amount is reduced to ammonium within the roots, which indicates that the most of nitrate taken up by roots is translocated via xylem to the shoot (Rufty 1997). Thus, nitrate accumulation in leaves depends mainly on the balance between the rate of nitrate uptake and its assimilation. In this way, NR plays an important role in the maintenance of this equilibrium, catalyzing the production of ammonium for amino acid biosynthesis via glutamine synthetase/glutamate synthase pathway (GS, EC 6.3.2.1/GOGAT, EC 1.4.7.1 or EC 1.4.1.14) in vascular plants (Campbell 1999). Ammonium can be also incorporated into asparagine by cytosolic asparagine synthetase (AS, EC 6.3.5.4), which can use free ammonium or glutamine as a nitrogen donor, but this pathway seems to occur mainly in the dark (Thum et al. 2003).

To date, there is no convincing evidence for a direct effect of boron on nitrate assimilation (Shelp 1993; Marschner 1995; Brown et al. 2002). Boron, an essential micronutrient for vascular plants, is involved in different processes such as vegetative growth, cell wall synthesis and structure, membrane integrity and function, phenolic metabolism, among others (for reviews see Blevins and Lukaszewski 1998; Brown et al. 2002; Bolanos et al. 2004). However, in our laboratory it was shown that leaves from tobacco plants subjected to a severe boron deficiency (boron-deprived plants for 6 weeks) had a significant decrease in magnesium, calcium, potassium and, especially, nitrate concentrations in comparison to control tobacco leaves (Camacho-Cristóbal and González-Fontes 1999). This last result suggests a possible connection between boron and nitrate assimilation in vascular plants. Therefore, the aim of the present work has been to study in detail the early changes, at both transcriptional and biochemical levels, caused by boron deficiency on several aspects of the nitrate assimilation. For this purpose, the experimental approach has been based on a short-term boron deficiency and the analyses were extended to the root as well, since this organ plays an essential role in plant mineral nutrition.

Materials and methods

Plant growth

Seeds of tobacco (*Nicotiana tabacum* cv. Gatersleben, a gift from Professor Mark Stitt, Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany) were sown in seedbeds filled with a mixture of perlite and vermiculite (1/1, v/v) in a growth chamber with a light/dark regime of 12/12 h, 25/20°C, 70/80% relative humidity and light intensity of 350 μmol m^{-2} s^{-1} of photosynthetically active radiation. During a 4-week period, plants were watered every 2 days with a nutrient solution containing 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 2 mM $MgSO_4$, 2 mM KH_2PO_4, 50 μM NaCl, 40 μM FeNa-EDTA, 10 μM $MnCl_2$, 2 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.5 μM Na_2MoO_4, 0.2 μM $CoCl_2$ and 10 μM H_3BO_3. Plants were transferred to a 0.2× complete nutrient solution, boron being maintained at the same concentration (10 μM) for an extra week, and during this period the nutrient medium was renewed every 2–3 days. Afterwards tobacco plants were divided into two groups: boron-sufficient (10 μM boron) and boron-deficient (boron-free medium) plants. Three plants from each treatment were harvested randomly 1, 2, 3 and 5 days after the onset of both treatments, the harvest time being always 4 h after the illumination. Shoots and roots were quickly separated, dried with paper towel, and weighed. Leaves and roots were rapidly frozen in liquid N_2 and stored at −80°C until further analyses.

Enzyme extraction and assays

Nitrate reductase (NR, EC 1.6.6.1) activity was determined in both leaves and roots as in Camacho-Cristóbal and González-Fontes (1999). To assay glutamine synthetase (GS, EC 6.3.1.2) activity, leaf or root frozen samples (0.2 g) were homogenized at 4°C in 4 volumes (v/w) of a mixture containing: 100 mM Tris-HCl (pH 7.6), 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 μM leupeptin, 2.5% (w/v) polyvinilpolypirrolidone (PVPP) and 0.5% (w/v) streptomycin sulphate. After centrifugation (8,000 *g* for 2 min at 4°C), the clear supernatant was used to measure GS activity by the transferase assay according to a modified method from de la Haba et al. (1992): The reaction mixture contained, in a final volume of 0.75 ml, 50 mM Hepes-KOH (pH 7.5), 60 mM L-glutamine, 30 mM NH_2OH, 0.4 mM ADP, 3 mM $MnCl_2$, 20 mM Na_2HAsO_4, and 50–100 μl of enzyme extract. After incubation at 30°C for 5 min, the reaction was stopped by addition of 0.75 ml of cold ferric-chloride reagent (320 mM $FeCl_3 \cdot 6\ H_2O$, 210 mM HCl, and 190 mM trichloroacetic acid). The formation of γ-glutamylhydroxamate was evaluated at 500 nm. Control assays did not contain either ADP or arsenate.

Metabolite analyses

For nitrate extraction, frozen leaves (80–100 mg) were ground to a fine powder in a mortar pre-cooled with

liquid N_2, weighed, and extracted for 30 min at 80°C in screw-cap tubes as follows: Twice with 1 ml of 80% aqueous ethanol (buffered with 5 mM Hepes-KOH, pH 7.5), once with 1 ml of 50% aqueous ethanol (buffered as before) and once with 1 ml distilled water. Between each step the extract was centrifuged (13,000 *g*, 5 min) and the four supernatants collected and combined. The ethanol/water extracts and pellets were kept at −20°C until nitrate determination. Nitrate was determined by HPLC as previously described by Camacho-Cristóbal and González-Fontes (1999).

For protein quantification, frozen material (25–50 mg) was homogenized at 4°C with four volumes (v/w) of 50 mM sodium phosphate (pH 7.0). The suspension was centrifuged (15,000 *g* for 15 min at 4°C) and protein concentration in the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Amino acids and ammonium were extracted from plant material as follows: 0.2 g of the frozen powder was homogenized twice in 1 ml of 50% (v/v) ethanol for 30 min at 4°C. Between each step, extracts were centrifuged (13,000 *g* for 5 min) and the two supernatants combined. Aliquots (1 ml) of this clear supernatant were then dansylated by adding 250 µl Na_2CO_3 [1% (w/v)] and 250 µl dansyl chloride (10 mg ml^{-1} acetonitrile). Samples were vortexed gently and incubated in the dark at 60°C for 30 min. After that, 20 µl of the dansylated extract was injected into a Spherisorb ODS-2 HPLC column (150 × 4 mm, 5 µm). Separation of dansyl-derivatives (amino acids and ammonium) was carried out at 30°C using a mobile phase consisted of two eluents: 0.5% (v/v) acetic acid and 0.0075% (v/v) triethylamin in water (eluent A) and pure acetonitrile (eluent B). The gradient program was as follows: 17.5% eluent B for 10 min (flow rate at 1 ml min^{-1}), 17.5 to 35% eluent B for 30 min (flow rate from 1 to 1.5 ml min^{-1}), and 35 to 55% eluent B for 20 min (flow rate from 1.5 to 2 ml min^{-1}). Elution was monitored at 254 nm and the amounts of amino acids and ammonium were determined by comparison with standards (Sigma, St. Louis, MO, USA).

RNA isolation, cDNA synthesis, and quantitative RT-PCR analyses

Total RNA was extracted by using TRI REAGENT®-RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Cincinnati, OH, USA) and then treated with RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Three micrograms of DNase-treated total RNA was used to prepare cDNA by reverse transcription with M-MLV reverse transcriptase (Promega) and oligo$(dT)_{18}$ primers, according to the manufacturer's protocol. Gene expression was determined by quantitative real-time PCR (MyiQ real-time PCR detection system; Bio-Rad, Hercules, CA, USA) by using *NIA2*, *NRT2*, and *PMA2* gene-specific primers and iQ SYBR Green supermix (Bio-Rad) following the manufacturer's instructions. The amplicon of tobacco elongation factor 1-α (accession number D63396) (forward primer: GCGTCAAACTGTTGCTGTTG; reverse primer: CTGCAACGTTCATTTCTTCTTCT) was used as an internal control to normalize all data.

The following gene-specific primers were used for the quantitative real-time PCR: *NIA2* (accession number X06134) (forward primer: GCTTGCCTGTTG GAAAACAT; reverse primer: TCGAAGTACCCC ACCTCATC), *NRT2* (accession number AJ557583) (forward primer: CACCAACGAAAGACCCTGTT; reverse primer: TTCAAGCTGTTTTGGTGCAT), and *PMA2* (accession number M80492) (forward primer: AGTGGTGAAGTTGAAGGGTCTT; reverse primer: ATGTTGCTACTATGAACTTGTCTCTTC). This last pair of primers was designed in conserved regions of *Nicotiana plumbaginifolia* and *Licopersicum esculentum* plasma membrane H^+-ATPase.

^{15}N feeding experiments

Both tobacco plants subjected to boron deprivation for 1, 2, 3 or 5 days and boron-sufficient plants were transferred from their hydroponic cultures to 0.1 mM $CaSO_4$ for 1 min and to 1.7 l-beaker containing, respectively, 0 or 10 µM boron in a fresh 0.2× nutrient solution (2.4 mM nitrate, 10 atoms% ^{15}N). After 2 h, the roots were washed for 1 min in 0.1 mM $CaSO_4$ and were separated from shoots. Shoots and roots were rapidly frozen in liquid N_2 and stored at −80°C. The frozen plant material was ground to a fine powder in a mortar with liquid N_2, and dried at 70°C for 72 h. Aliquots of the dried powder were used to analyse ^{15}N content using a continuous-flow isotope ratio mass spectrometer (model Finnigan MAT251). The total N content of the weighed aliquots was estimated with an elemental N analyser (model Fison NA1500).

Net nitrate uptake was calculated by dividing the ^{15}N content of the plant by time and root dry weight.

Statistical analysis

All analytical determinations and enzyme assays were carried out on leaves and roots from three separate plants harvested randomly, and measured in duplicate. The data shown are mean values ± SD.

Results

Effects of boron deprivation on plant growth

Tobacco plants that had been grown without boron during 5 days had smaller shoots and roots at the end of the experiment than those grown in a complete nutrient solution, this effect being more marked in roots (Fig. 1). Root growth decreased actually from the first days of boron-deficient treatment when compared to control, whereas shoot growth was only slightly affected by boron deficiency from day 3 onwards (Fig. 1).

Effects on the nitrate uptake rate and the expression of genes involved in nitrate transport

Plants growing on 2.4 mM nitrate were incubated with 2.4 mM ^{15}N-nitrate (10% enrichment) after 1, 2, 3 or 5 days of boron deprivation (Fig. 2). The feeding experiments lasted for 2 h and were carried out 4 h after the beginning of the light period. Net nitrate uptake remained steady during the boron-sufficient treatment; however, during the boron-deficient treatment net nitrate uptake declined from the onset of the experiment (Fig. 2). Specifically, net nitrate uptake decreased after 3 or 5 days of boron deprivation by 30–35% in comparison to boron-sufficient plants (Fig. 2).

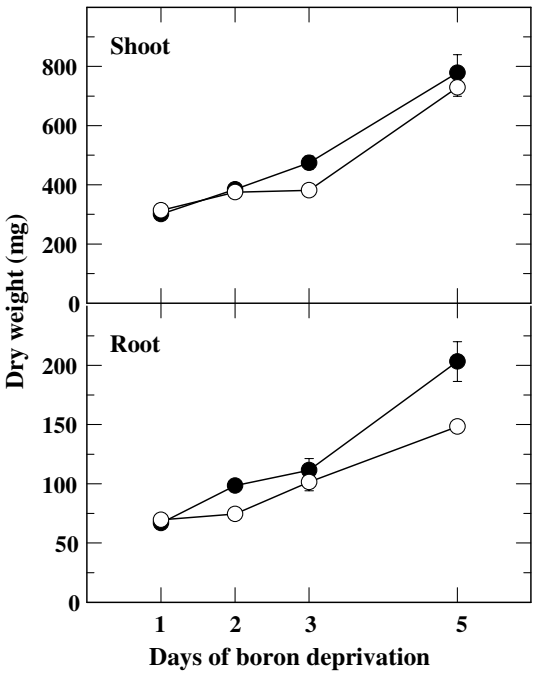

Fig. 1 Effects of boron deprivation on biomass allocation in tobacco plants. Tobacco plants subjected (*open circle*) or not (*filled circle*) to a 5-day period of boron deprivation were harvested as described in Materials and methods, and the dry weight of shoots and roots were determined. The results are given as means ± SD (n = 3 separate plants)

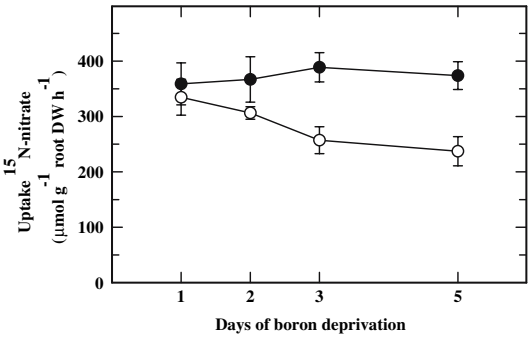

Fig. 2 Effects of boron deprivation on net nitrate uptake in tobacco plants. Tobacco plants subjected (*open circle*) or not (*filled circle*) to boron deprivation for 1, 2, 3 or 5 days were transferred for 2 h to 0.2× nutrient solution (2.4 mM nitrate, 10 atoms% ^{15}N), containing 0 or 10 µM boron, respectively. Net nitrate uptake was calculated by dividing the ^{15}N content of the plant by time and root dry weight. The results are given as means ± SD (n = 3 separate plants)

Figure 3 shows the root transcript levels of a plasmalemma H^+-ATPase protein (a, *PMA2*) and a high affinity nitrate transporter (b, *NRT2*) in boron-sufficient and -deficient plants during the 5 days of experimental period. As observed in Fig. 3, the transcript levels of *PMA2* and *NRT2* genes in boron-deficient roots were lower than those in control roots from day-1 and day-3 onwards, respectively.

Nitrogen metabolites

In leaves, nitrate and ammonium concentrations increased slightly during the boron-sufficient treatment; however, the levels of these metabolites decreased throughout the experiment after 2 and 3 days of boron deprivation, respectively (Fig. 4a, b). So, the leaf nitrate concentration was about 65% lower than the control level at the end of the boron-deficient treatment (Fig. 4a). In leaves of control plants the most abundant amino acid was glutamine (Gln) and its concentration was almost constant during the treatment (Fig. 4e). Boron-deficient leaves had higher Gln concentration than control ones during the first 2 days of the treatment; however, the level of this metabolite decreased from the day-2 of the boron deprivation and it was smaller than the control concentration after 5 days (Fig. 4e). On the other hand, asparagine (Asn), aspartate (Asp), and glutamate (Glu) concentrations were similar in boron-sufficient and boron-deficient leaves, and only light differences were observed at the end of treatments (Fig. 4c, d, f). Total leaf amino-acid contents followed virtually the same patterns than

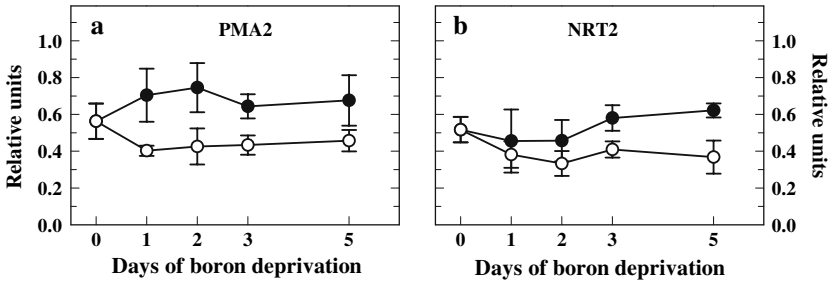

Fig. 3 **a, b** Real-time quantitative RT-PCR analysis of transcript levels for root *PMA2* and *NRT2* genes from tobacco plants subjected (*open circle*) or not (*filled circle*) to a 5-day period of boron deprivation. RNA isolation from roots, cDNA synthesis and RT-PCR analyses were performed as described in Materials and methods. The results are given as means ± SD (n = 3 separate plants)

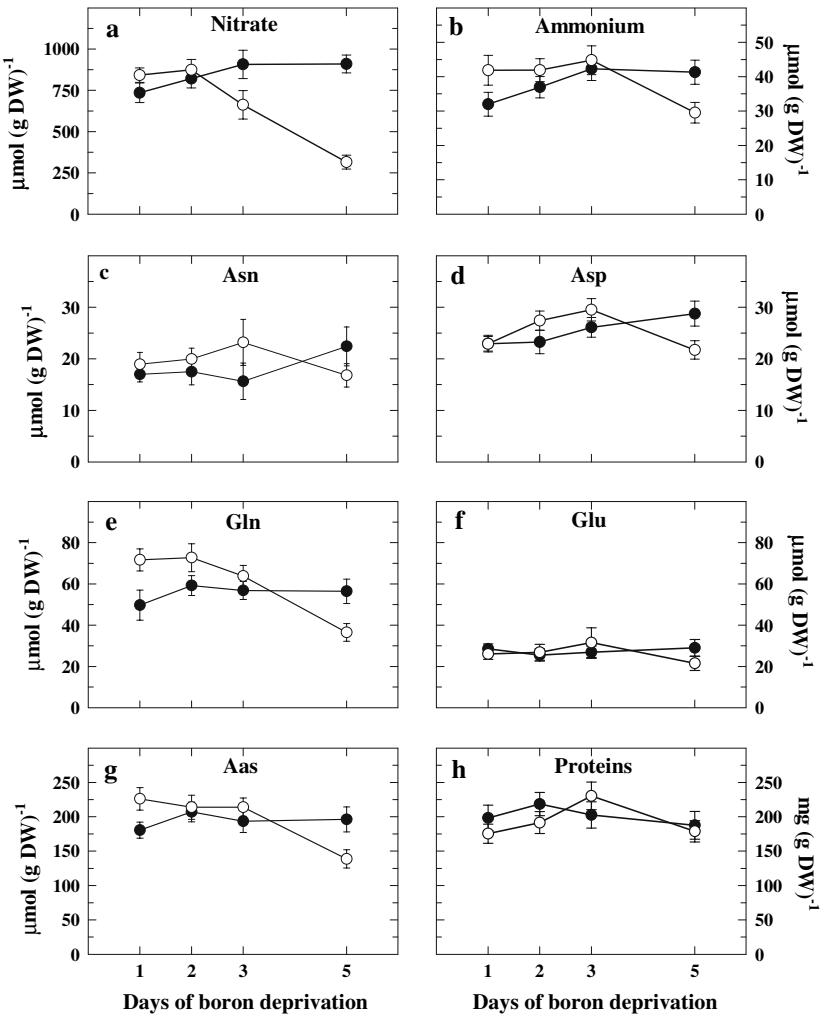

Fig. 4 Effects of boron deprivation on nitrogen metabolite concentrations in leaves of tobacco plants. Concentrations of nitrate (**a**), ammonium (**b**), asparagine (**c**), aspartate (**d**), glutamine (**e**), glutamate (**f**), total amino acids (Aas, **g**), and total soluble proteins (**h**) were determined in leaves from boron-sufficient (*filled circle*) and boron-deficient (*open circle*) plants corresponding to Fig. 1. The results are given as means ± SD (n = 3 separate plants)

those exhibited by Gln in both boron treatments (Fig. 4g). Leaf concentration of soluble proteins remained steady and at similar levels during boron-sufficient and boron-deficient treatments (Fig. 4h).

In roots, nitrate content increased slightly during the boron-sufficient treatment and decreased from the onset of the boron deprivation; in fact, nitrate concentration in boron-deficient roots was about 30% lower than that in control roots at the end of the treatments (Fig. 5a). During the first 3 days of both treatments, no significant differences were observed between ammonium concentration in boron-deficient and -sufficient roots; however, at day-5, ammonium concentration was higher in boron-deficient roots than in boron-sufficient ones (Fig. 5b). On the other hand, Asp, Gln, Glu, and total protein levels were similar in boron-sufficient

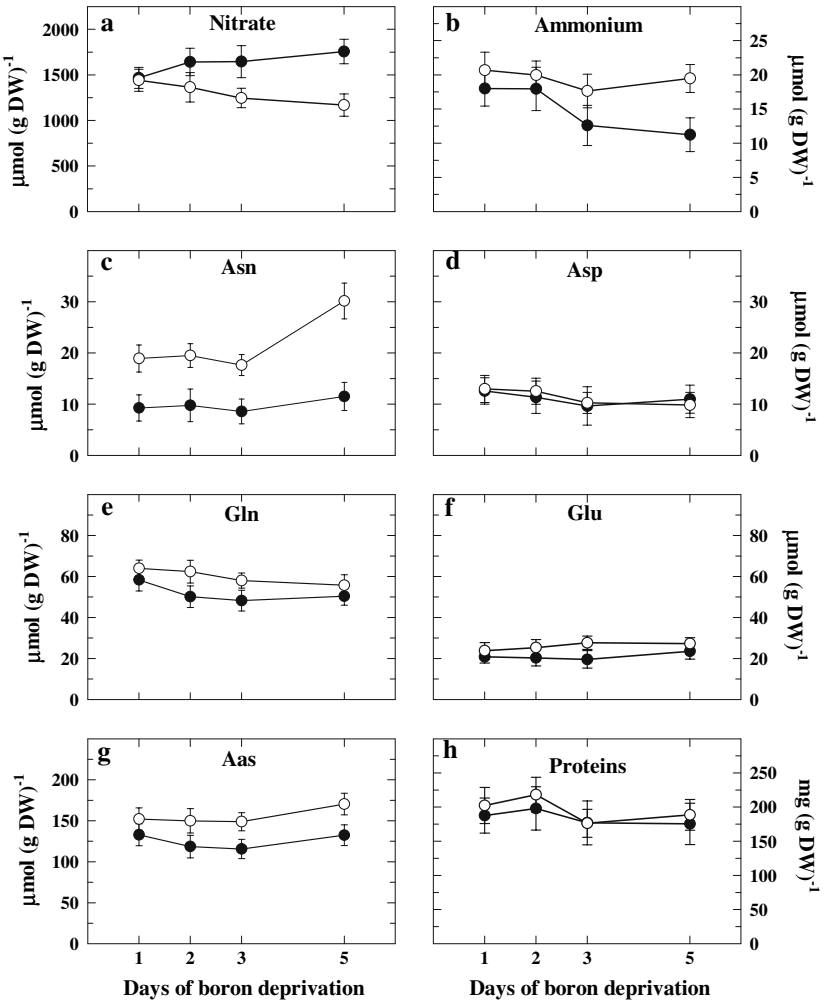

Fig. 5 Effects of boron deprivation on nitrogen metabolite concentrations in roots of tobacco plants. Concentrations of nitrate (**a**), ammonium (**b**), asparagine (**c**), aspartate (**d**), glutamine (**e**), glutamate (**f**), total amino acids (Aas, **g**), and total soluble proteins (**h**) were determined in roots from boron-sufficient (*filled circle*) and boron-deficient (*open circle*) plants corresponding to Fig. 1. The results are given as means ± SD ($n = 3$ separate plants)

and -deficient roots (Fig. 5d–f, h). It is also important to point out that the short-term boron deficiency led to an increase in root Asn content when compared to control plants, this effect being more marked at the end of the treatment (Fig. 5c). This result is consistent with the higher total amino acid content of boron-deficient roots in comparison to boron-sufficient ones (Fig. 5g).

NR and GS activities and *NIA* transcript levels

In both boron-sufficient and boron-deficient plants, leaf and root NR activities decreased slightly from the onset of the treatments, and no marked differences were observed as a consequence of the boron deficiency (Fig. 6a, c). It is noticeable that NR activity was lower in roots than in leaves of tobacco plants (Fig. 6a, c).

Although leaf GS activity did not markedly change with boron treatments (Fig. 6b), root GS activity decreased from the onset of boron deficiency when compared to control plants (Fig. 6d). Thus, GS activity was about 50% lower in boron-deficient roots than in boron-sufficient ones at the end of the treatments (Fig. 6d).

Leaf *NIA* transcript levels were lower under boron deficiency than in boron-sufficient conditions, which correlated with the leaf nitrate concentration (Figs. 4a, 7a). However, the levels of *NIA* transcripts in roots were virtually unaffected by boron deficiency, which is consistent with the much lower differences in root nitrate concentration between boron-deficient and boron-sufficient plants (Figs. 5a, 7b).

Discussion

It is widely known that root growth is more sensitive than shoot growth to boron deficiency (Fig. 1), this effect leading to a higher shoot/root ratio (Blevins and Lukaszewski 1998; Camacho-Cristóbal and González-Fontes 1999).

As explained in Introduction, in herbaceous plants like tobacco, the accumulation of nitrate in leaves depends mainly on the balance between the rate of nitrate uptake and its reduction in leaves, since most of the nitrate taken up by roots is transported to the

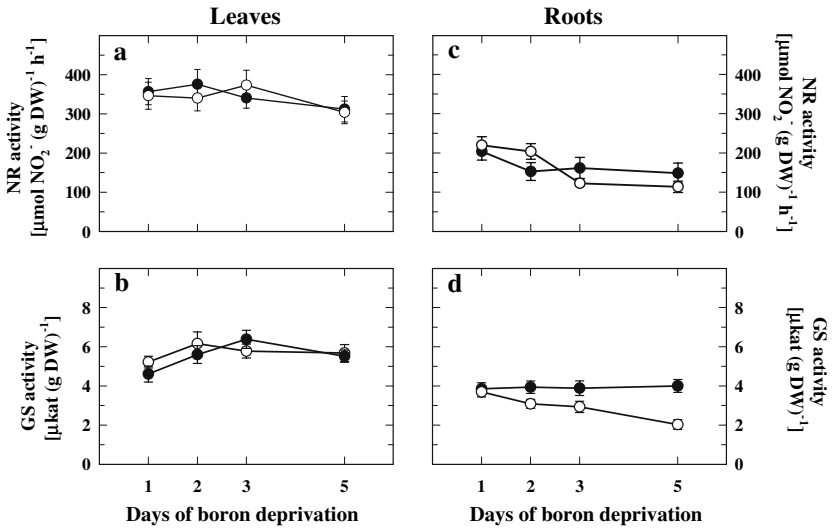

Fig. 6 Effects of boron deprivation on NR and GS activities in leaves and roots of tobacco plants. Maximal NR (**a**, **c**) and GS (**b**, **d**) activities were determined in leaves (**a**, **b**) and roots (**c**, **d**) from boron-sufficient (*filled circle*) and boron-deficient (*open circle*) plants corresponding to Fig. 1. The results are given as means ± SD (n = 3 separate plants)

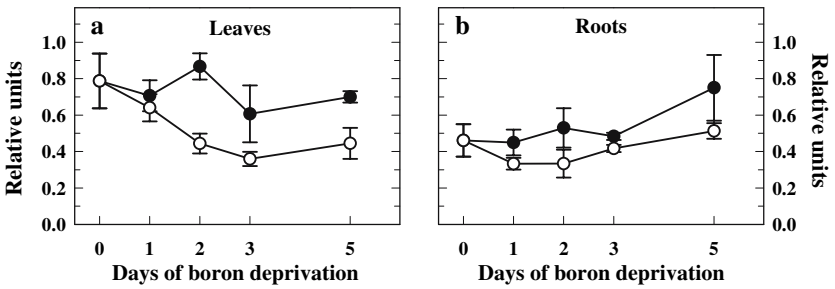

Fig. 7 Real-time quantitative RT-PCR analysis of transcript levels for *NIA* gene from leaves (**a**) and roots (**b**) of tobacco plants subjected (*open circle*) or not (*filled circle*) to a 5-day period of boron deprivation. RNA isolation, cDNA synthesis and RT-PCR analyses were performed as described in Materials and methods. The results are given as means ± SD (n = 3 separate plants)

leaves, where carbon skeletons and the assimilatory power necessary for its assimilation are utilized more directly (Rufty 1997). Accordingly, both nitrate reductase (NR) activity and glutamine synthetase (GS) activity—enzyme that catalyzes ammonium incorporation into Glu to produce Gln—were smaller in roots than in leaves (Fig. 6). Recently we have shown that short-term boron deficiency did not provoke a decrease in either the leaf or the root concentrations of macronutrients such as magnesium, calcium, potassium or phosphate (Camacho-Cristóbal et al. 2005); however, in the present work boron deficiency led to a decline in root and, especially, leaf nitrate contents (Figs. 4a, 5a). During the short-term boron-deficient treatment, a clear correlation was found between net nitrate uptake and leaf and root nitrate concentrations; specifically, these parameters began to decrease clearly from the second day onwards in boron-deficient plants (Figs. 2, 4a, 5a). Hence, these results suggest that the lower leaf and root nitrate contents in boron-deficient plants would be a consequence of their lower net nitrate-uptake rate. There is evidence supporting a strong correlation between nitrate uptake and *NRT2* transcript levels (Krapp et al. 1998; Matt et al. 2001). This correlation was clearly observed from day-3 onwards since these parameters were lower in boron-deficient roots than in boron-sufficient ones (Figs. 2, 3). Nevertheless, at day-2 root *NRT2* transcript levels were similar in both boron treatments, whereas net nitrate-uptake rate was lower in boron-deficient roots than in boron-sufficient ones (Figs. 2, 3b). At first glance, this result could be explained by a drop in (1) the transcript levels of other nitrate transporters and/or (2) the activity of NRT2 transporters (post-transcriptional regulation). It is well known that secondary active transport of nitrate across the plasma membrane is powered by the coupling to a primary proton gradient generated by plasmalemma H^+-ATPases (McClure et al. 1990a, b). Thus, one can expect that a decrease in H^+-ATPase activity could lead to a reduced nitrate transport across the plasma membrane. In the present work we show a rapid decrease of root *PMA2* transcript levels under boron deficiency (Fig. 3a), which could imply a drop in the plasmalemma H^+-ATPase activity (Schon et al. 1990; Ferrol et al. 1993) and, as a consequence, a lower nitrate uptake (Fig. 2). In experi-

ments with cultures of sunflower cells Ferrol et al. (1993) suggested that the regulation of plasmalemma H^+-ATPase by boron treatment was due to changes in its activity rather than in the amount of this protein. In addition, in the present work we have observed a strong transcriptional regulation of plasmalemma H^+-ATPases by boron deficiency in tobacco roots (Fig. 3a).

As set out above, nitrate from cytosol is reduced to ammonium to be incorporated into carbon skeletons for amino-acid synthesis. The first step in nitrate assimilation is the reduction of this anion to nitrite in a reaction catalyzed by the enzyme NR, which is highly regulated at transcriptional and post-transcriptional levels (Campbell 1999; Kaiser and Huber 2001). Some evidences show that both expression and activity of NR are induced by nitrate, decreased by Gln or related metabolites, and increased by carbohydrates (Kaiser and Huber 2001; Stitt et al. 2002). Leaf NR activity was similar in plants treated with or without boron (Fig. 6a); nevertheless the levels of nitrate concentration and *NIA* transcripts decreased notably in boron-deficient plants after 2 days of deficiency in comparison to control plants (Figs. 4a, 7a). Thus, as previously described by other authors (Ferrario et al. 1996; Scheible et al. 1997b; Man et al. 1999), there were no evidences for a relation between the tissue nitrate concentration and the post-transcriptional regulation of NR. There was no correlation between NR activity and *NIA* transcript levels either (Galangau et al. 1988). However, in leaves, a relation between nitrate concentration and levels of *NIA* transcripts was observed (Figs. 4a, 7a; Galangau et al. 1988; Matt et al. 2001; Stitt et al. 2002). Therefore, in roots, there was no relation between nitrate concentration and levels of *NIA* transcripts as found clearly in leaves (Figs. 5a, 7b). Nevertheless, since most of the nitrate may be located at the vacuole (Miller and Smith 1996), the cytosolic nitrate concentration could be decisive for controlling NR synthesis and activity rather than the total tissue-nitrate concentration (Man et al. 1999).

Ammonium, the end form of inorganic nitrogen prior to the synthesis of organic nitrogen compounds, is mainly incorporated into glutamine through the combined action of GS and GOGAT (Lam et al. 1996), this step being essential to avoid that eventually this ion rise to toxic levels. Moreover, it is widely known that ammonium can be also metabolized by AS to produce Asn (Lam et al. 1996). A very early effect caused by boron deficiency was a rapid and clear increase in root Asn concentration when compared to control (Fig. 5c), which would imply an enhancement of AS transcripts and activity. On this matter it will be very interesting to evaluate whether the increased Asn levels in roots under boron deficiency are correlated with a higher expression of asparagine synthetase gene(s). Indeed, recently, an increase in AS mRNA along with Asn content in tomato roots subjected to cadmium toxicity has been reported (Chaffei et al. 2004). Our results seem to suggest that under boron deficiency the ammonium incorporation into Asn via AS may grow in importance in tobacco roots and, therefore, this enzyme would act as a complement to GS/GOGAT pathway under this stress condition. This is in agreement with the lower root GS activity found in boron-deficient roots when compared to control ones from day-1 onwards of the treatment (Fig. 6d). Therefore, the decreasing root GS activity under boron deficiency could be attributable to the accumulation of Asn, which has been reported to lead to a drop in both levels of GS transcripts and activity (Oliveira and Coruzzi 1999). Consistent with our hypothesis, an inverse relation between GS activity and Asn level has been reported in *Lotus japonicus* (Harrison et al. 2003) and *Medicago truncatula* (Carvalho et al. 2003) nodules, as well as in *Lycopersicon esculentum* plants subjected to cadmium toxicity (Chaffei et al. 2004).

In conclusion, the present work shows that boron deficiency has clear and quick effects on several aspects of the nitrate assimilation in tobacco plants. On the one hand, this mineral deficiency seems to decrease net nitrate uptake by repressing root *PMA2* gene expression and, consequently, by decreasing the proton electrochemical gradient across the plasma membrane. Moreover boron deficiency seems also to repress *NRT2* gene expression and, on the other hand, to promote the assimilation of ammonium by AS, which leads to a rapid increase in Asn content and a decrease in GS activity in tobacco roots.

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References

Blevins DG, Lukaszewski KM (1998) Boron in plant structure and function. Annu Rev Plant Physiol Plant Mol Biol 49:481–500

Bolanos L, Lukaszewski K, Bonilla I, Blevins D (2004) Why boron? Plant Physiol Biochem 42:907–912

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254

Brown PH, Bellaloui N, Wimmer MA, Bassil ES, Ruiz J, Hu H, Pfeffer H, Dannel F, Römheld V (2002) Boron in plant biology. Plant Biol 4:205–223

Camacho-Cristóbal JJ, González-Fontes A (1999) Boron deficiency causes a drastic decrease in nitrate content and nitrate

reductase activity, and increases the content of carbohydrates in leaves from tobacco plants. Planta 209:528–536
Camacho-Cristobal JJ, Maldonado JM, González-Fontes A (2005) Boron deficiency increases putrescine levels in tobacco plants. J Plant Physiol 162:921–928
Campbell WH (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu Rev Plant Physiol Plant Mol Biol 50:277–303
Carvalho HG, Lopes-Cardoso IA, Lima LM, Melo PM, Cullimore JV (2003) Nodule-specific modulation of glutamine synthetase in transgenic *Medicago truncatula* leads to inverse alterations in asparagine synthetase expression. Plant Physiol 133:243–252
Chaffei C, Pageau K, Suzuki A, Gouia H, Ghorbel MH, Masclaux-Daubresse C (2004) Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. Plant Cell Physiol 45:1681–1693
Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7:859–868
Daniel-Vedele F, Filleur S, Caboche M (1998) Nitrate transport: a key step in nitrate assimilation. Curr Opin Plant Biol 1:235–239
de la Haba P, Cabello P, Maldonado JM (1992) Glutamine-synthetase isoforms appearing in sunflower cotyledons during germination. Effects of light and nitrate. Planta 186:577–581
Ferrario S, Valadier MH, Foyer CH (1996) Short-term modulation of nitrate reductase activity by exogenous nitrate in *Nicotiana plumbaginifolia* and *Zea mays* leaves. Planta 199:366–371
Ferrol N, Belver A, Roldan M, Rodriguez-Rosales MP, Donaire JP (1993) Effects of boron on proton transport and membrane properties of sunflower (*Helianthus annuus* L.) cell microsomes. Plant Physiol 103:763–769
Forde BG (2000) Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta 1465:219–235
Galangau F, Daniel-Vedele F, Moureaux T, Dorbe MF, Leydecker MT, Caboche M (1988) Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light–dark regimes and nitrate supply. Plant Physiol 88:383–388
Harrison J, de Crescenzo MAP, Sene O, Hirel B (2003) Does lowering glutamine synthetase activity in nodules modify nitrogen metabolism and growth of *Lotus japonicus*? Plant Physiol 133:253–262
Kaiser WM, Huber SC (2001) Post-translational regulation of nitrate reductase: mechanisms, physiological relevance and environmental triggers. J Exp Bot 52:1981–1989
Krapp A, Fraisier V, Scheible WR, Quesada A, Gojon A, Stitt M, Caboche M, Daniel-Vedele F (1998) Expression studies of *Nrt2:1Np*, a putative high affinity nitrate transporter: evidence for its role in nitrate uptake. Plant J 14:723–732
Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:569–593
Man HM, Abd-El Baki GK, Stegmann P, Weiner H, Kaiser WM (1999) The activation state of nitrate reductase is not always correlated with total nitrate reductase activity in leaves. Planta 209:462–468
Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, San Diego, pp 379–396
Matt P, Geiger M, Walch-Liu P, Engels C, Krapp A, Stitt M (2001) Elevated carbon dioxide increases nitrate uptake and nitrate reductase activity when tobacco is growing on nitrate, but increases ammonium uptake and inhibits nitrate reductase activity when tobacco is growing on ammonium nitrate. Plant Cell Environ 24:1119–1137
McClure PR, Kochian LV, Spanswick RM, Shaff JE (1990a) Evidence for cotransport of nitrate and protons in maize roots. I. Effects of nitrate on the membrane potential. Plant Physiol 93:281–289
McClure PR, Kochian LV, Spanswick RM, Shaff JE (1990b) Evidence for cotransport of nitrate and protons in maize roots. II. Measurement of NO_3^- and H^+ fluxes with ion-selective microelectrodes. Plant Physiol 93:290–294
Miller AJ, Smith SJ (1996) Nitrate transport and compartmentation in cereal root cells. J Exp Bot 47:843–854
Oliveira IC, Coruzzi GM (1999) Carbon and amino acids reciprocally modulate the expression of glutamine synthetase in arabidopsis. Plant Physiol 121:301–309
Orsel M, Filleur S, Fraisier V, Daniel-Vedele F (2002) Nitrate transport in plants: which gene and which control? J Exp Bot 53:825–833
Rufty TW (1997) Probing the carbon and nitrogen interaction: a whole plant perspective. In: Foyer CH, Quick WP (eds) A molecular approach to primary metabolism in higher plants. Taylor & Francis, London, pp 255–273
Scheible WR, González-Fontes A, Lauerer M, Müller-Röber B, Caboche M, Stitt M (1997a) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell 9:783–798
Scheible WR, González-Fontes A, Morcuende R, Lauerer M, Geiger M, Glaab J, Gojon A, Schulze ED, Stitt M (1997b) Tobacco mutants with a decreased number of functional *nia* genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. Planta 203:304–319
Scheible WR, Lauerer M, Schulze ED, Caboche M, Stitt M (1997c) Accumulation of nitrate in the shoot acts as a signal to regulate shoot–root allocation in tobacco. Plant J 11:671–691
Schon MK, Novacky A, Blevins DG (1990) Boron induces hyperpolarization on sunflower root cell membranes and increases membrane permeability to K^+. Plant Physiol 93:566–571
Shelp BJ (1993) Physiology and biochemistry of boron in plants. In: Gupta UC (ed) Boron and its role in crop production. CRC Press, Boca Raton, pp 53–85
Stitt M, Müller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot 53:959–970
Thum KE, Shasha DE, Lejay LV, Coruzzi GM (2003) Light- and carbon-signaling pathways. Modeling circuits of interactions. Plant Physiol 132:440–452