Effects of nitrate pulses on *BnNRT1* and *BnNRT2* genes: mRNA levels and nitrate influx rates in relation to the duration of N deprivation in *Brassica napus* L.

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Abstract

A de-repression mechanism based on the disappearance of ‘signals’ down-regulating N transporter activity has been proposed in the literature to explain the transient increase of NO$_3^-$ uptake by the roots following N deprivation in higher plants. This hypothesis was investigated at the physiological and molecular levels by measuring NO$_3^-$ influx into roots of *Brassica napus* L. grown under low or high external concentrations of KNO$_3$ following N deprivation. Parallel measurements were made of endogenous NO$_3^-$, amino acid concentrations and abundance of mRNA for *BnNRT1* and *BnNRT2*, genes encoding nitrate-inducible transport proteins. The effect of NO$_3^-$ pulsing on NO$_3^-$ transport components in N-deprived plants was also investigated by measuring influx of high- and low-affinity transport system (HATS and LATS) and assaying mRNA levels. Influx of NO$_3^-$ via HATS and LATS, and transcript levels of *BnNRT2* and *BnNRT1* decreased with the duration of N deprivation. The results suggested that the absence of de-repression of NO$_3^-$ influx and *BnNRT2* gene expression following N starvation was related to a high amino acid status. Pulsing with NO$_3^-$ induced a large increase in *BnNRT2* mRNA level, but a comparatively small increase in NO$_3^-$ influx via HATS. The level of *BnNRT1* mRNA also increased, but there was no effect on LATS uptake activity. The absence of a strict correlation between the NO$_3^-$ transport activity and the mRNA *BnNRT1* and *BnNRT2* levels is discussed in terms of possible post-transcriptional regulation by the amino acids.

Key words: *Brassica napus* L., high-affinity nitrate transporter, low-affinity nitrate transporter, nitrate influx, nitrate transporter genes.

Introduction

The availability of N for uptake by roots is one of the main factors limiting plant productivity in agricultural systems. Although the roots of higher plants can absorb simple organic compounds such as amino acids (Bush, 1993; Chapin et al., 1993), most crop species depend on mineral N forms, particularly NO$_3^-$. Until relatively recently the initial reduction of NO$_3^-$ by nitrate reductase (NR) was considered to be the main ‘growth limiting step’ in the N assimilation pathway. However, molecular studies of NR (Hoff et al., 1994) and the use of transgenic plants, with increased or decreased NR expression, have indicated little relationship between plant growth and NR level or activity. Consequently, attention has switched to the mechanisms regulating the uptake of mineral N into the roots, at both physiological and molecular levels (for review, see Crawford and Glass, 1998; Forde, 2000; Touraine et al., 2001).

It has been shown for a number of plant species that influx of NO$_3^-$ involves several different carrier systems. At low external NO$_3^-$ concentrations (< 1 mM), a constitutive high-affinity transport system (CHATS), operating at a low rate and displaying Michaelis–Menten kinetics, is regarded as genetically distinct and independent from an inducible high-affinity transport system (IHATS) that is substrate saturable and inducible (Siddiqi et al., 1990). Several full-length cDNAs with...
sequence homology to the crnA gene of *Aspergillus nidulans* (Unkles et al., 1991, 1995) encoding a high-affinity NO$_3^-$ transporter, have been characterized from *Chlamydomonas reinhardtii* (Quesada et al., 1994; Galván et al., 1996), *Hordeum vulgare* (Trueman et al., 1996; Vidmar et al., 2000), *Glycine max* (Amarasinghe et al., 1998), *Nicotiana plumbaginifolia* (Quesada et al., 1997; Krapp et al., 1998), and *Arabidopsis thaliana* (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999). In *N. plumbaginifolia*, *G. max*, *A. thaliana*, and *H. vulgare* similar patterns for the expression of NRT2 and NO$_3^-$ influx rates of HATS have recently been reported (Krapp et al., 1998; Amarasinghe et al., 1998; Zhuo et al., 1999; Vidmar et al., 2000).

At higher external concentrations of NO$_3^-$ (i.e. in the mM range) the rate of NO$_3^-$ uptake increases linearly with increasing substrate concentration (Siddiqi et al., 1990). This low-affinity transport system (LATS) has been distinguished from the active high-affinity systems by its lower sensitivity to cold temperatures and to several metabolic inhibitors (Glass et al., 1990). At the molecular level, a putative substrate inducible NO$_3^-$ transporter has been identified in *Arabidopsis thaliana* by transgenic plants with an insertional mutagen further selected for resistance to chlorate-herbicide (Tsai et al., 1993). The functional translation in Xenopus oocytes of *AtNRT1:1* mRNA has been demonstrated successfully (Tsai et al., 1993). Moreover it has been found that the corresponding mRNA was synthesized predominantly in roots of plants previously induced by NO$_3^-$ supply (Tsai et al., 1993). Lauter et al. (1996) and Zhou et al. (1998) have also characterized two tomato genes and one rape gene, respectively, for NRT1 (*LeNRT1:1, LeNRT1:2* and *BnNRT1:2*), which are inducible by NO$_3^-$. Huang et al. (1996) and Liu et al. (1999) proposed a two-gene model for the low-affinity NO$_3^-$ uptake system that may explain the discrepancy between the inducibility of NRT1 and the apparent constitutive expression of the low-affinity transport system of NO$_3^-$. Recently, another NRT1 gene (*AtNRT1:2*) has been characterized from *Arabidopsis* that encodes a constitutive low-affinity nitrate transporter (Huang et al., 1999). Consequently, the low-affinity transport system seems to have an inducible (ILATS) and a constitutive (CLATS) component, somewhat analogous to that for the high-affinity nitrate uptake system.

Nitrogen uptake is a highly regulated process, the rate of uptake matching the rate at which N is required for the synthesis and expansion of new tissues (Touraine et al., 1994). One of the most commonly cited mechanisms for co-ordinating NO$_3^-$ uptake by the roots and NO$_3^-$ assimilation in the shoot is the model proposed by Clarkson (1986). It postulates that nitrogen uptake is normally down-regulated if N is freely available to the plant. The transient increase in uptake rate occurring during the first 24–48 h after N starvation is interpreted as de-repression resulting from the progressive disappearance of ‘signals’ down-regulating N-transporter activity. This model was confirmed at the molecular level in *Arabidopsis* N-starved plants and revealed at least two discrete processes: an initial *AtNRT2:1* de-repression observed 24–48 h after starvation, followed by a down-regulation corresponding to a de-induction of the gene *AtNRT2:1* under more prolonged starvation (Lejay et al., 1999). *AtNRT1* was not subjected to this de-repression process or affected by the N-status of the plant (Lejay et al., 1999).

The mechanism by which shoot demand for N, following N deprivation, regulates N uptake could rely on a common amino-N transport pool between shoot and root (Cooper and Clarkson, 1989). Using specific inhibitors of GS, GOGAT or amino transferases and externally supplied amino acids, Lee et al. (1992) showed under N starvation that treatments raising intracellular concentrations of glutamine and/or asparagine led to the suppression of net uptake of NH$_4^+$ and NO$_3^-$ by maize seedlings. Conversely, conditions, which lowered root glutamine and/or asparagine, stimulated the net uptake of NO$_3^-$. Using a GS specific inhibitor (azaserine) in *Hordeum vulgare* plants, Vidmar et al. (2000) suggested that glutamine (but not glutamate) is responsible for the down-regulation of HvNRT2 expression. Nevertheless, the model of N uptake regulation by phloem-translocated amino acids remains controversial (Lainé et al., 1995; Tillard et al., 1998). Split-root experiments have shown that N deprivation to half of the root system can be entirely and rapidly compensated for by an increase in NO$_3^-$ influx into the other half of the root system supplied with NO$_3^-$, whilst levels of amino acids in the roots were unaffected or only slightly increased.

The aims of this study were (i) to investigate the occurrence of the de-repression mechanism postulated by Clarkson (1986) for NO$_3^-$ uptake by *Brassica napus* L., at the influx and transcript levels, following N deprivation, and (ii) to measure the concurrent changes in the main N substrate pool in root and shoot tissues. The experimental approach included exposure of NO$_3^-$-deprived plants to a brief NO$_3^-$ pulse in order to alleviate the de-induction mechanism of NO$_3^-$ during starvation, so that the effect of NO$_3^-$ induction could be discriminated from other putative regulation processes. As a preliminary, the kinetics of NO$_3^-$ influx were also established in induced and non-induced plants.

**Materials and methods**

**Plant culture**

*Experiment 1 (Culture conducted in Caen, France; results in Fig. 1):* *Brassica napus* L., cv. Capitol were germinated and grown hydroponically (50 seedlings per 1 dm$^3$ plastic tank) in a greenhouse. The aerated nutrient solution contained 0.4 mM KH$_2$PO$_4$, 0.15 mM
Experiment 1 (results in Fig. 1): Experimental treatments under natural illumination until day 17 after sowing. Supplementary light (09.00±21.00 h) of 200 W SON-T and HPI/T lamps (Philips Lighting Ltd, Croydon, Surrey, UK) giving 500 μmol m⁻² s⁻¹ of photosynthetically active radiation at the height of canopy) for 16 h d⁻¹. The thermoperiod was 24 °C (day) and 18 °C (night).

Experiment 2 (Culture conducted in Aberystwyth, UK: results in Figs 2–7): Seeds of Brassica napus L. cv. Capitola were imbibed for 48 h on tissue paper saturated with 10 mM CaSO₄ and then sown into six culture units of a flowing solution culture (FSC) system incorporating automatic control of concentrations of NO₃⁻, K⁺ and H⁺ in solution (Clement et al., 1974; Hatch et al., 1986). Each culture unit contained 200 dm³ of recirculating nutrient solution and concentrations in the flowing solutions.

In each culture unit, 200 dm³ of recirculating nutrient solution and 24 culture vessels, each containing three plants. The FSC system was located in a greenhouse, solution temperature was maintained at 20±0.5 °C and air temperature at 20±2/15±1 °C day/night (09.00–21.00 h) throughout the experiment. The plants were established under natural illumination until day 17 after sowing. Supplementary light (09.00–21.00 h) of 200 μmol m⁻² s⁻¹ PAR was provided between days 18–24 by a single 400 W SON-T lamp (Philips Lighting Ltd, Croydon, Surrey, UK) suspended 1.5 m above the surface of each culture unit. On day 24 after sowing, natural light was excluded and thereafter illumination was provided by paired 400 W SON-T and HPI/T lamps (Philips Lighting Ltd, Croydon, Surrey, UK) giving 500 μmol m⁻² s⁻¹ PAR at canopy height, over 12 h.

Nutrient concentrations in each culture unit were initially (μM): NO₃⁻, 250; K⁺, 250; H₂PO₄⁻, 50; Mg²⁺, 100; SO₄²⁻, 325; Fe³⁺, 5.4; with micronutrients as previously described by Clement et al. (1978). All culture units were drained and refilled with fresh nutrient solution of the same composition on day 18 after sowing. Nutrient concentrations were allowed to deplete by plant uptake until day 24 when automatic monitoring (every 27 min) and resupply of nutrients were introduced. Thereafter, the concentration of K⁺ was maintained at 20±2 μM in each culture unit by automatic resupply at a rate equal to the depletion of K⁺ by plant uptake. All other nutrients except NO₃⁻ were supplied automatically in fixed ratios to the net uptake of K⁺, for 1 mol of K, 0.645, 0.057, 0.045, 0.00075, and 0.522 mol of S, Mg, P, Fe, and Ca, respectively, were supplied, with micronutrients as described by Clement et al. (1978). Solution pH was maintained at 6.0±0.1 by automatic delivery of H₂SO₄ or Ca(OH)₂ to each culture unit throughout the experiment. A concentration of 20±2 μM NO₃⁻ was maintained automatically in each culture unit from day 24 until the start of the N deprivation period (day 26). Net uptake of K⁺ and NO₃⁻ was calculated on an hourly basis from the amounts required to maintain the ‘set point’ concentrations in the flowing solutions.

Experimental treatments

Experiment 1 (results in Fig. 1): On day 15 after sowing, half of the total number of plants previously grown without N was supplied with 1 mM KNO₃ for 24 h to induce the NO₃⁻ uptake system. Nitrate influx rates were measured on day 16 with induced and non-induced plants.

Experiment 2 (results in Figs 2–7): On day 26 after sowing the automatic supply of NO₃⁻ was terminated to culture units and the concentration of NO₃⁻ in these units allowed to deplete by plant uptake to <1 μM over 2 h. This point was taken as time zero (t₀) for the N-deprivation treatment.

Nitrate influx rates and mRNA abundance were measured on N-deprived plants (~N plants) at intervals during the period of N deprivation (0, 24, 48, 72, and 96 h). On each occasion additional batches of plants were exposed to a NO₃⁻ pulse 4–12 h prior to these measurements, for comparison with ~N plants. All measurements were made on three culture vessels (nine plants) for each combination treatment and time. The NO₃⁻ pulse exposure was performed by transferring plants for a period of 30 min into separate FSC units containing 200 dm³ of full (~N) nutrient solution with the addition of 100 μM NO₃⁻. Afterwards the roots were rinsed twice in 1 mM CaSO₄ solution for 1 min before returning the plants to their original ~N culture units until required for measurement of influx or mRNA. For one batch of plants, the NO₃⁻ pulse application was performed 12 h prior to the measurement of NO₃⁻ influx, and for another batch of plants the NO₃⁻ pulse exposure was performed 4 h prior to their harvest for mRNA analysis.

Measurement of NO₃⁻ influx and plant harvesting

Experiment 1 (results in Fig. 1): Influx of ¹⁵NO₃⁻ was measured on three batches of 50 seedlings on each occasion. The roots of each batch were rinsed twice for 1 min in 1 mM CaSO₄ solution at 20 °C before being immersed for 5 min in 250 ml of nutrient solution (described above for Experiment 1) containing different concentrations (10, 25, 50, 75, 100, 135, 250, 1000, 2500, 5000, 7500 μM) of K₁⁵NO₃ (99.9% atom% ¹⁵N). The extent of NO₃⁻ depletion from these solutions during the influx assays was less than 4% in each case. The roots were then rinsed twice for 1 min in 1 mM CaSO₄ solution at 4 °C before harvesting. Shoots and roots were separated, weighed and frozen in liquid nitrogen prior to freeze-drying. The freeze-dried tissues were weighed and ground to a fine powder and stored at −80 °C for subsequent analysis.

Experiment 2 (results in Figs 2–7): NO₃⁻ influx was measured 2 h prior to the end of the photoperiod, on each occasion using six culture vessels per treatment. The vessels were removed from the ~N culture units and their roots immersed for 5 min in nutrient solution of the same composition (1 dm³ per vessel) with the addition of either 100 μM (HATS activity) or 5 mM (LATS activity) of K¹⁵NO₃ labelled at 99.9 and 30 atom% ¹⁵N, respectively. Roots were rinsed twice for 1 min in 1 mM CaSO₄ solution at 4 °C and harvested immediately. Shoots and roots were separated and treated as described previously for subsequent analysis. Three additional vessels per treatment were harvested at the same time for RNA analysis.

Nitrogen, nitrate, isotope, and amino acid analysis

Nitrogen and ¹⁵N contents of plant freeze-dried samples were measured in continuous flow using a C/N analyser linked to an isotope ratio mass spectrometer (Roboprep CN and 20–20 mass spectrometer, Europa PDZ, Crewe, UK). Influx of NO₃⁻ was calculated from the ¹⁵N contents of the roots and shoots. Nitrate and amino acids were extracted from freeze-dried tissue (100 mg) with 10 ml of methanol:dichloromethane:water (60:25:15, by vol.) for 1 h. After centrifugation (10 000 g, 20 min), the pellet was re-extracted under the same conditions. The supernatants were mixed, 5 ml of dichloromethane and 5 ml of water were added, and stored overnight at 4 °C. The dichloromethane phase was discarded and the remaining upper phase containing amino acids, sugars and NO₃⁻ was collected and evaporated to dryness under vacuum at 30 °C. The residue was resuspended in 2 ml of water and filtered through a 0.45 μm membrane. 1 ml was used for the quantification of nitrate by high performance anionic chromatography (DX 100 with an Ionpac AS9 analytical column, Dionex Corporation, Sunnyvale, USA) and the other 1 ml was used for amino acid analysis. After a dilution by 10, aliquots of 15 μl each were analysed by high performance liquid chromatography (HPLC) as ophthalaldehyde derivatives on a C-18 column using Gold System 8.0 (Beckman Instruments, San Ramon, CA, USA) as previously described by Murray et al. (1996) and
specific amino acids were quantified using the α-aminobutyric acid as an internal standard.

**Cloning of BnNRT2 and BnNRT1**

BnNRT2 and BnNRT1 cDNA were obtained by the conjunction of RT-PCR, 3′ and 5′ RACE with the aid of the Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, USA). One specific pair of oligonucleotide primers was designed for BnNRT1 from the sequence of BnNRT1 gene (Muldin and Ingemarsson, 1995) and AtNRT1 gene (Tsay et al., 1993). For BnNRT2, the primers were obtained from *Hordeum vulgare* (Trueman et al., 1996). These two pairs of oligonucleotide primers: NRT1F (5′-TAC CGG GAC TGA GAC CAC CAA GAT-3′) – NRT1R (5′-GGA CTG CGC GAC CGA TAA TGT-3′) and NRT2F (5′-GGT TGC ACA TCA TCA TGG GAG TC-3′) – NRT2R (5′-GCA ACG TGC AGG CAA CTA TCA TCA CTC CC-3′), were used in an RT-PCR reaction to amplify a 736 bp and 643 bp fragments. Therefore, these resulting probes were gel purified (Quiagen Gmbh, Hilden, Germany) and cloned in a pGEM-T vector (Clontech Laboratories, Palo Alto, USA). Plasmids DNA were extracted and sequenced to check the sequence similarity.

To obtain the full-length sequence the method of 3′ and 5′ RACE was used. 1 mg Poly (A)* mRNA was used for the first and second-strands cDNA synthesis before the ligation of double strand cDNA adapter according to the manufacturer’s instructions (Clontech laboratories, Palo Alto, USA). 5′ and 3′ Rapid Amplification of cDNA ends (RACE) cloning of the BnNRT2 and BnNRT1 cDNA were conducted by using two pairs of designed primers: RACE-NRT2F (5′-GCT TCA CAC TGC CGG AAT CAT CGC AGG-3′) – RACE-NRT2R (5′-GTT GCT CCA CAA GCT GCT TGT GCT CCT-3′) and RACE-NRT1F (5′-GGC TAT GCC ATT TGC GGC TGG GCA ATC G-3′) – RACE-NRT1R (5′-GAC GGG TCC GAT GCC AAC TCG AGC CGC-3′) that produce overlapping of 5′ and 3-RACE products. Touchdown Polymerase Chain Reaction was performed with Advantage 2 Polymerase Mix (Clontech laboratories, Palo Alto, USA) for 35 cycles: 5 cycles at 94 °C for 30 s, 72 °C for 4 min; 5 cycles at 94 °C for 30 s, 70 °C for 4 min and 25 cycles at 94 °C for 30 s, 68 °C for 4 min. Amplified products were then gel purified with QIAquick Gel Extraction Kit (Quiagen Gmbh, Hilden, Germany) and cloned directly into the pGEM-Teasy cloning vector following the manufacturer’s instructions (Promega Corporation, Madison, USA). For nucleotide sequence analysis, plasmids were isolated with flexiprep kit (Amersham, Buckinghamshire, UK), sequenced with ABI PRISM dRhodamine terminator of Perkin Elmer (Perkin Elmer Applied Biosystem). Analysis of the sequenced fragment by Blast algorithm showed that the sequence of BnNRT1 (EMBL access. AJ1278966) had 96% of similarity with the clone BnNRT1 (Muldin and Ingemarsson, 1995) and that BnNRT2 (EMBL accession no. AJ293028) had 89% of similarity with AtNRT2 clone (Zhuo et al., 1999).

**Synthesis of cDNA selective RT-PCR probes**

The cDNA selective probes were obtained by RT-PCR with a specific pair of primers for the BnNRT1 gene (NRT1F–NRT1R) and for the BnNRT2 gene (NRT2F–NRT2R). 1 μg of Poly (A)* RNA were used for reverse transcription with the M-MLV Reverse-Transcriptase (Life Technologies, Paisley, UK) and primed with each specific reverse primer according to the manufacturer’s instructions. Then PCRs were performed with 2.5 U Taq DNA polymerase (Life Technologies, Paisley, UK) in 50 μl reaction containing 1 ng of cDNA, 50 pmol of each primer, 1.5 mM magnesium chloride, and 0.2 mM dNTPs. The reactions were performed for 35 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min followed by a final extension step at 72 °C for 5 min. Amplified products of BnNRT1 (736 bp) and BnNRT2 (643 bp) were purified and cloned as described in previous section. After plasmid digestion, cDNA fragments were labelled with α-32P dCTP (3000 C; mmol−1) by using random priming NEBlot kit (New Englands Biolabs, Beverley, USA).

**Isolation of RNA and northern blot analysis**

20 μg of total RNA previously extracted from root tissue with TRI-Reagent according to the manufacturer’s instructions (MCR Euromedex, Cincinnati, USA), were fractionated on 1.2% agarose gel containing formaldehyde and transferred to Hybond-N* blotting
membranes (Amersham, Buckinghamshire, UK) using 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7), and fixed onto the membranes by backing at 80 °C for 2 h. After blotting, the blots were prehybridized for 2 h at 62 °C in the Church buffer (Church and Gilbert, 1984). After addition of the probes, membranes were hybridized overnight at 62 °C in buffer containing: SDS 7%, Na2HPO4 0.25 M, EDTA 2 mM, heparin 0.2 mg ml⁻¹, and calf thymus DNA 0.1 mg ml⁻¹ (Church and Gilbert, 1984). Then, the membranes were washed successively with: (1) 2× SSC, 0.1% SDS 20 min at 50 °C, (2) 1× SSC, 0.1% SDS 20 min at 60 °C, (3) 0.2× SSC, 0.1% SDS 20 min at 60 °C before being analysed.

Analysis of NRT1 and NRT2 transcript levels
The blots were exposed to radiographic Kodak BioMax MS film for 3–5 d at −80 °C and developed as described by the manufacturer (Eastman Kodak Company, New York, USA). The signal intensities have been quantified by image analyser (Wilbert Lourmat, France). In order to correct RNA loading differences, the ribosomal RNA 28S and 18S stained with ethidium bromide were quantified and used for the determination of NRT genes transcript levels (Fig. 4A, B).

Results

Kinetics of NO₃⁻ influx in induced and non-induced plants
At least three different systems for NO₃⁻ influx were distinguished in Brassica napus L. on the basis of the kinetic characterization (Fig. 1). Two of them were constitutive systems in non-induced seedlings. At concentrations of NO₃⁻ below 200 µM, influx rates approximated Michaelis–Menten kinetics (Fig. 1A, CHATS), with an estimated V_max of 26.3 µmol h⁻¹ g⁻¹ DW and a K_m of 15.9 µM. A second, low affinity system (Fig. 1B, CLATS) exhibited non-saturable kinetics between 1–7.5 mM NO₃⁻. When seedlings were induced by exposure to 1 mM NO₃⁻ for 24 h prior to assaying influx, NO₃⁻ influx increased across the entire range of concentrations. The concentration effects for the inducible high-affinity (Fig. 1A, IHATS) and the putative inducible low-affinity (Fig. 1B, ILATS) systems were calculated by subtracting influx measured in non-induced plants from the rates measured with induced plants. The inducible high-affinity system approximated Michaelis–Menten kinetics at substrate concentrations lower than 1 mM (Fig. 1A, IHATS), with a V_max of 135 µmol h⁻¹ g⁻¹ DW and a K_m of 85 µM. Influx attributable to the IHATS was 5-fold higher than the one associated with the CHATS. The kinetics of NO₃⁻ uptake determined at high concentrations (1–7.5 mM NO₃⁻) seems to show that the LATS system is devoid of an
inducible component (Fig. 1B, inset). Indeed, the values of ‘ILATS activity’ calculated by subtraction of the theoretical IHATS ($V_{\text{max}}$ of 135 $\mu$mol h$^{-1}$ g$^{-1}$ DW and a $K_m$ of 85 $\mu$M) from both IHATS+ILATS activity (Fig. 1B) do not show a significant difference with the values calculated for CLATS activity (Fig. 1B, inset). Taken as a whole these results confirm that 100 $\mu$M and 5 mM external concentrations of NO$_3^-$ were appropriate for assessing influx mediated, respectively, by the high- and the low-affinity systems.

**Effects of N deprivation and NO$_3^-$ pulses on influx rates and gene expression**

Plant growth (i.e. dry matter production) was not significantly affected during the first 4 d of NO$_3^-$ deprivation (Fig. 2). Consequently, effects of N deprivation on NO$_3^-$ influx and gene expression during this period were unrelated to changes in growth rate or senescence. Nitrate influx through the high-affinity systems (Fig. 3A, 100 $\mu$M) decreased progressively over the four days of N deprivation (from 125 to 30 $\mu$mol h$^{-1}$ g$^{-1}$ DW). Seventy per cent of this decline was observed during the first 48 h of N starvation. Pulsing the plants with NO$_3^-$ for 30 min, 12 h before influx was measured, reversed the trend for the first 24 h and lowered the subsequent rate of decline in influx (Fig. 3A). The NO$_3^-$ pulse application increased HATS activity during the first 24 h and resulted in a higher influx during the next three days, (77 $\mu$mol h$^{-1}$ g$^{-1}$ DW after 4 d) compared with -N plants, the difference being at least 2-fold throughout.

The activity of the low-affinity system was determined by measuring influx from 5 mM NO$_3^-$. Uptake from this higher NO$_3^-$ concentration resulted from LATS and HATS activities (Fig. 1B). Influx decreased with increasing duration of N deprivation (Fig. 3B), declining by 60% over four days. The overall trend was similar to that observed for the high-affinity system. Pulsing plants with

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**Fig. 4.** Changes in relative abundance of $BnNRT1$ (white bars), $BnNRT2$ (black bars) mRNA in roots of *Brassica napus* L. over 4 d following withdrawal of the N supply to plants. Plants either received (B, D) or did not receive (A, C) a NO$_3^-$ pulse (30 min, 100 $\mu$M NO$_3^-$) 4 h prior to the harvest of the roots for mRNA extraction and analysis by Northern hybridization (C, D). The relative transcript level of NRT genes was corrected after quantification of RNAr 28S and 18S loading differences under UV by image analyser (A, B). Data for only one repetition of three experiments is presented. The EMBL nucleotide sequence database accession numbers for $BnNRT1$ and $BnNRT2$ are AJ278966 and AJ293028, respectively.
NO$_3^-$ for 30 min, 12 h before influx was measured, increased influx through LATS+HATS by about 40%, and delayed the decline in influx by 24 h (Fig. 3B). Comparison of the differences in influx at 100 $\mu$M and 5 mM attributable to NO$_3^-$ pulsing (Fig. 3B inset) suggested that the main effect of the NO$_3^-$ pulse was to increase IHATS.

The abundance of mRNA encoding for the BnNRT1 and BnNRT2 NO$_3^-$ transporters, showing respectively 96% of homology with BnNRT1 clone (Muldin and Ingemarsson, 1995) and 89% of homology with AtNRT2:1 (Zhuo et al., 1999), were followed in ±N plants with and without exposure to the NO$_3^-$ pulse. Southern analysis (data not shown) suggested that these probes BnNRT1 and BnNRT2 recognized at least two genes of each family. Therefore, the probes used in Northern studies (described below) reveal the expression of several BnNRT1 or BnNRT2 genes. In ±N plants, transcripts levels of BnNRT1 and BnNRT2 (Fig. 4A, C) decreased as a function of the duration of NO$_3^-$ deprivation. In comparison with the plants ($t_0$), pulsing plants with NO$_3^-$ for 30 min, 4 h prior to harvest, increased by 4 the relative abundance of BnNRT1 mRNA and by 14 the relative abundance of BnNRT2 mRNA (Fig. 4B, D).

Effects of N deprivation and NO$_3^-$ pulsing on endogenous NO$_3^-$ and amino acid concentrations in shoots and roots

The effect of exposing plants to a NO$_3^-$ pulse during N deprivation on endogenous NO$_3^-$ and amino acid concentrations in shoots and roots was investigated to establish whether there was a relationship between variation in these compounds (Fig. 5) and nitrate uptake (Figs 3, 4). Generally, the effect of the NO$_3^-$ pulse was more pronounced in shoots (Fig. 5A, C) than in roots (Fig. 5B, D), and particularly so for amino acid concentrations (Fig. 5C).

The concentration of NO$_3^-$ was initially higher in the shoots than in the roots (Fig. 5A, B), but following termination of the N supply it decreased more rapidly in the roots (Fig. 5B), reaching negligible levels after 2 d, compared with 4 d in the shoots. Pulsing the plants with NO$_3^-$, 12 h prior to harvest, accelerated the depletion of NO$_3^-$ in the shoots between 24–48 h of N deprivation, but had no other effect on endogenous NO$_3^-$ concentrations in shoots or roots.

Surprisingly, total amino acid concentrations increased in both shoots (Fig. 5C) and roots (Fig. 5D) of ±N plants during the first 48 h of N deprivation, but decreased

![Fig. 5. Changes in endogenous NO$_3^-$ and total amino acid concentrations in the shoots (A, C) and roots (B, D) of Brassica napus L. plants as a function of the time of NO$_3^-$ deprivation. Plants either received (filled squares) or did not receive (open squares) a NO$_3^-$ pulse (30 min, 100 $\mu$M NO$_3^-$) applied 12 h prior to harvest. Vertical bars indicate ±SD of the mean for $n=3$ when larger than the symbol.](image-url)
thereafter. Pulsing the plants with NO$_3^-$ did not affect the trend in amino acid concentration in the roots, although the absolute values were invariably slightly lower compared with those in ±N plants (Fig. 5D). By contrast, NO$_3^-$ pulsing markedly accelerated the decrease in total amino acid levels in the shoots (Fig. 5C). In terms of specific amino acids in the roots, the largest decrease attributable to NO$_3^-$ pulsing was in glutamate followed by glutamine, after 48 h of N starvation (Fig. 6A, B), whereas the sum of the other amino acids increased slightly (Fig. 6C).

The dynamics of the physiological attributes and molecular components of NO$_3^-$ transport as affected by N deprivation and by N deprivation + NO$_3^-$ pulsing are summarized on a relative basis in Fig. 7. In the case of N deprivation, the effects are relative to plants at $t_0$, the start of the N deprivation period (Fig. 7A, B), whilst the NO$_3^-$ pulsing effect is relative to the corresponding plants without NO$_3^-$ pulsing (Fig. 7C, D). Expression of the results in this form highlights the positive effect of NO$_3^-$ pulsing on mRNA BnNRT2 levels (Fig. 7D).

**Discussion**

*Nitrate uptake systems in Brassica napus L.*

Nitrate influx in *Brassica napus* L. appears to involve at least three kinetically different transport systems, including constitutive low-affinity, inducible and constitutive high-affinity systems (Fig. 1A, B). At high nitrate concentrations, this kinetic study does not reveal the existence of an inducible low-affinity transporter system as previously reported by Touraine and Glass (1997) in *Arabidopsis thaliana*. Wang et al. (1998) and Liu et al. (1999) had suggested that the protein carrier AtNRT1.1 may also be an important component of both the high-affinity and the low-affinity nitrate uptake systems and that AtNRT1.1 may be a dual-affinity nitrate transporter in *Arabidopsis thaliana*. In addition, unexpected expression of this transporter in rapidly dividing cells prompted Guo et al. (2001) to re-examine AtNRT1.1 functions. They suggested that this nitrate transporter supports the growth of nascent organs in roots and shoots and that it could also play a role in the induction of the flowering.

**Effect of N deprivation**

The concept that N uptake is regulated at the whole plant level to match the N demand associated with growth is widely accepted (Touraine et al., 1994). Although the mechanism of regulation at this level is not fully understood, several authors have suggested that the size, composition or rate of internal recycling of the free amino acid pool within the plant might be involved (Cooper and Clarkson, 1989; Marschner et al., 1996). One of the simplest experimental approaches for altering the internal availability of N in roots and shoots is the termination of the external N supply to the plant. In the short-term (up to 4 d), it has been hypothesized that N deprivation might progressively up-regulate the capacity of the N uptake system and its transporters (Clarkson, 1986). This up-regulation would be the result of depletion of the internal NO$_3^-$ and amino acid pools through continued translocation, assimilation and protein synthesis in growing tissues (Cooper and Clarkson, 1989).

In the present study with *Brassica napus* L. growth was reduced only after 5 d of NO$_3^-$ deprivation although
photosynthesis was affected sooner (Figs 2, 7). A comparison between the concentrations of N compounds occurring in *B. napus* plants grown under field conditions (Colnenne et al., 1998) and those measured in the present study (total N higher than 6% DW, high NO$_3^-$ and amino-acid concentrations), suggests that the latter had optimum N status at the start of the period of N starvation. Consequently, it is likely that N uptake systems were in a down-regulated state at $t_0$, and hence the effects of N deprivation on their activity were expected to be significant. However, at the carrier level and at the levels of putative gene expression the results showed that NO$_3^-$ uptake activity was not up-regulated shortly after the withdrawal of the external supply. Indeed, contrary to the results obtained by Lejay et al. (1999) in *Arabidopsis*, no correlative de-repression of both activity of high-affinity NO$_3^-$ uptake systems and *BnNRT2* expression was observed during the entire period of N deprivation in *Brassica napus*. N starvation resulted in decreased activity of both low- and high-affinity NO$_3^-$ uptake systems and the expression of *BnNRT1* and *BnNRT2*.

The absence of up-regulation induced by N starvation in the short-term has also been reported by Siddiqi et al. (1989) in *Hordeum vulgare*, where N deprivation of plants previously fed with NO$_3^-$ caused a decrease in NO$_3^-$ influx during the first 24 h to levels similar to those in plants which had not been exposed to NO$_3^-$ Under these experimental conditions, it is suggested that the unexpected repression of NO$_3^-$ influx in *Brassica napus* was due to a rapid assimilation of nitrate pools during the first 48 h of N starvation, resulting in increased levels of free amino acids. This phenomenon may be an adaptive mechanism enabling fast-growing species like *B. napus* to maintain rapid growth during a short period of N starvation. Beyond 48 h of N deprivation it is probable that, in the absence of an external NO$_3^-$ signal, the de-induction mechanism suggested by Clarkson (1986), more than counterbalanced the effect of decreasing levels of amino acids, leading to a decline in nitrate influx and expression of *BnNRT* genes.

**The effect of a NO$_3^-$ pulse during N deprivation: maintaining NO$_3^-$ induction?**

Because several components of the NO$_3^-$ uptake and assimilation processes are known to be substrate-inducible (Crawford, 1995; Stitt, 1999), the application of a NO$_3^-$
pulse during N deprivation would be expected to increase the potential activity of the NO$_3^-$ transport systems, via increased transcription and translation of genes encoding for transporters (Siddiqi et al., 1990; Redinbaugh and Campbell, 1991). The results showed that a NO$_3^-$ pulse during N deprivation increased the activity of HATS and the mRNA $BnNRT2$ level compared with untreated (−N) plants. This component of NO$_3^-$ pulse-induced up-regulation appears to be inversely correlated with endogenous nitrate levels. By contrast, it was found that the LATS activity was unaffected by NO$_3^-$ pulsing, despite the increase in mRNA $BnNRT1$ level. This suggests that these two families of NO$_3^-$ transporters are differentially regulated with respect to NO$_3^-$ per se.

**Relationships between influx rates and gene expressions**

The responses measured in mRNA $NRT2$ levels and NO$_3^-$ influx to N deprivation were generally similar in trend. However, they differed in magnitude (Fig. 7). For example, the relative increase in $BnNRT2$ mRNA level associated with exposure to a NO$_3^-$ pulse was greater than the corresponding increase in NO$_3^-$ influx mediated by the HATS. As reported in *Nicotiana plumbaginifolia* by Fraisier et al. (2000), the present results suggest that the HATS activity may be subject to a post-transcriptional regulation. The observation that the concentration of free amino acids (notably Gln) was high during the first 48 h of N deprivation suggests that the amino acid pool could be involved in this post-transcriptional regulation. However, although this interpretation would appear to support the findings of Fraisier et al. (2000), it is possible that mRNAs for other genes encoding for components of the HATS were not picked up by the probe used in the current study.

The increase in endogenous free amino acids levels coinciding with a decrease in NO$_3^-$ influx measured in present work, together with the results obtained with an external supply of glutamine in *Nicotiana plumbaginifolia* (Krapp et al., 1998) and in *Hordeum vulgare* (Vidmar et al., 2000) support the hypothesis that amino acids are involved in the transcriptional and/or post-transcriptional regulation of influx during the first 48 h of N deprivation. However, the case for their involvement after 48 h is far less convincing. In the plants exposed to a NO$_3^-$ pulse, the decrease in amino acid concentrations (notably Gln and Glu) between 48 h and 120 h of N deprivation coincided with a decline in HATS activity. This suggests that ‘signals’ other than amino acids are implicated in the regulation of NO$_3^-$ uptake at this time. A detailed quantification of the translocatory flux and cellular compartmentation of amino acids in parallel with measurements of influx and $NRT2$ mRNA abundance is required before the speculative hypothesis for the transcriptional or/and post-transcriptional role of endogenous amino acids in the regulation of NO$_3^-$ uptake by *Brassica napus* can be confirmed or refuted.

In conclusion, these results indicate that regulation of NO$_3^-$ influx activity in *Brassica napus* during N starvation involves several components acting differentially over time. These include the putative negative feedback regulation by amino acids at transcriptional and/or post-transcriptional levels, linkage to the endogenous nitrate pool and the inductive effect of exogenous NO$_3^-$ on the expression of NO$_3^-$ transporter genes.

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**References**


