Putative role of \(\gamma\)-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in \textit{Brassica napus} \(L\). 

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**ABSTRACT**

The relationship between nitrate influx, \(BnNrt2\) nitrate transporter gene expression and amino acid composition of phloem exudate was investigated during N-deprivation (short-term experiment) and over a growth cycle (long-term experiment) in \textit{Brassica napus} \(L\). The data showed a positive correlation between \(\gamma\)aminobutyric acid (GABA) in phloem exudate and nitrate uptake in the short- and the long-term experiments. The hypothesis that this non-protein amino acid could up-regulate nitrate uptake via a long-distance signalling pathway was tested by providing an exogenous GABA supply to the roots. The effect of GABA was compared with the effects of Glu, Glu and Asn, each known to be inhibitors of nitrate uptake. The results showed that GABA treatment induced a significant increase of \(BnNrt2\) mRNA expression, but had less effect on nitrate influx. By contrast, Glu, Glu and Asn significantly reduced nitrate influx and \(BnNrt2\) mRNA expression compared with the control plants. This study provides the first evidence that GABA may act as a putative long-distance inter-organ signal molecule in plants in conjunction with negative control exerted by Glu. The up-regulation effect of GABA on nitrate uptake is discussed in the context of its role in N metabolism, nutritional stress and the recent discovery of a putative role of GABA as a signal molecule in plant development.

**Key-words:** \textit{Brassica napus} \(L\); amino acids; \(BnNrt2\) genes; \(\gamma\)-aminobutyric acid (GABA); high-affinity transport system (HATS); nitrate uptake; phloem; translocation.

**INTRODUCTION**

The high-affinity transport system (HATS) is one of the two classes of transport systems involved in nitrate uptake in higher plants (Forde 2000). It operates from low external NO\(_3^–\) concentration and displays a Michaelis–Menten kinetic that is substrate saturable. On the basis of its inducibility by external nitrate, the HATS class of carriers have been further subdivided into constitutive (cHATS) and inducible (iHATS) components (Siddiqi et al. 1990), which are considered as genetically distinct and independent.

Since the first characterization of a crnA gene in \textit{Aspergillus nidulans} considered to encode an inducible HATS (Unkless et al. 1991, 1995), many genes encoding iHATS (the so-called \(Nrt2\) gene family) have been cloned in many higher plant species (Touraine, Daniel-Vedele & Forde 2001). In \textit{Arabidopsis}, seven HATS genes have been recently identified. Of these genes, \(Nrt2.1\) appears to be the most highly expressed in roots under limiting and non-limiting culture conditions and after nitrate spiking (Orsel, Krapp & Daniel-Vedele 2002; Okamoto, Vidmar & Glass 2003). The use of transgenic lines and mutants has also demonstrated that \(Nrt2.1\) is the main contributor to iHATS influx (Okamoto et al. 2003).

The regulation of the HATS nitrate transport system is thought to depend on at least two discrete and independent processes: a positive induction by external NO\(_3^–\) and a down-stream repression by N metabolites (Clarkson 1986; Siddiqi et al. 1989). These two processes recently discussed by Forde (2002) may involve local and long range signalling pathways according to the results obtained by ‘split-root’ experiments (Ohlen & Larsson 1992; Lainé, Ourry & Boucoud 1995; Lainé et al. 1998; Gansel et al. 2001).

Positive regulation by external NO\(_3^–\) of many genes involved in primary nitrogen metabolism, including nitrate uptake itself, has been widely established in many plant species at both molecular and physiological levels (Crawford & Glass 1998; Stitt & Scheible 1998; Wang et al. 2002). NO\(_3^–\) is assumed to act indirectly via a local signalling pathway on \textit{de novo} synthesis of \(Nrt2\) mRNA genes in roots (Crawford & Glass 1998; Touraine et al. 2001). However, to date, none of the components involved in this signalling pathway have been identified (Wang et al. 2002; Forde 2002).

The down-regulation process is thought to be the result of a negative feedback mediated by long range signal(s)
from the shoot to match the N demand of the plant (Forde 2002). This assumption is supported by the results of ‘split-root’ experiments where one half of the roots is deprived of NO$_3^-$ while the other half is supplied with NO$_3^-$. These studies have shown that NO$_3^-$ uptake is only up-regulated in nitrate-fed roots suggesting the existence of a compensation effect, which could be induced by long-distance signal(s) from shoot to root (Lainé et al. 1995, 1998; Gansel et al. 2001). The main difficulty in clearly demonstrating this hypothesis is to identify the endogenous signal(s) that trigger this negative feedback. Among potential candidates, amino acids involved in the cycling of reduced N (from shoot to root) would potentially allow the integration of this regulatory process at the whole plant level (Simpson, Lambers & Dalling 1982; Cooper & Clarkson 1989; Muller, Tillard & Touraine 1995; Marschner, Kirkby & Cakmak 1996). Exogenous amino acid supply to roots or shoots results in a negative effect on NO$_3^-$ uptake in many plant species (Doddema & Otten 1979; Breterel & Arnozis 1985; Rodgers & Barneix 1988; Muller & Touraine 1992). However, these experiments have failed to distinguish between possible biochemical interconversion and assimilation of these amino acids. Recent studies using inhibitors of N assimilation in Arabidopsis thaliana and Hordeum vulgare suggested that down-regulation of the Nrt2 gene is mediated by both NH$_4^+$ and glutamine (Lee et al. 1992; Zhuo et al. 1999; Vidmar et al. 2000). In Hordeum vulgare, the use of azaserine, an inhibitor of the GOGAT activity which blocks specifically Gln assimilation, induces dramatic decrease in both Nrt2 genes expression and nitrate uptake (Vidmar et al. 2000). This result combined with the correlation obtained in Arabidopsis between nitrate influx and phloem Gln provided a strong argument in favour of a major role of Gln in long-distance down-regulation of nitrate uptake (Nazoa et al. 2003).

Although this regulatory model can theoretically explain the up- and down-regulation of nitrate uptake by a low or high supply (respectively) of amino acids to the root (Cooper & Clarkson 1989; Ismande & Touraine 1994), contradictory results have been reported in short-term N-deprivation studies associated with split-root experiments (Lainé et al. 1995; Tillard, Passama & Gojon 1998). These experiments did not result in a clear decrease in total amino acid translocation from the shoot to the nitrate-fed side of the roots (Tillard et al. 1998). Moreover, no correlation has been found between variations of nitrate influx and either quantitative or qualitative changes in downward phloem transport (Tillard et al. 1998) or the concentration of specific amino acids in roots (Lainé et al. 1995).

These ‘split-root’ experiments have provided strong evidence that not only systemic regulatory signal(s) arising from the shoot are involved in nitrate uptake regulation but also in the increase of root branching observed in the NO$_3^-$ supplied side (Drew & Saker 1975; Granato & Raper 1989; Friend, Eide & Hinckley 1990; Lainé et al. 1998). Wang et al. (2002) have recently demonstrated in rice, using ‘split-root’ experiments, that from the 37 genes rapidly up-regulated in nitrate-fed roots, four genes were involved in ethylene and/or auxin synthesis, transport and perception [auxin efflux carrier (Reh1), S-adenosyl-L-methionine synthetase (Sms), 1-aminocyclopropane-1-carboxylate oxidase (Aco2), and ethylene-responsive sensor (Ers)]. In a separate study, Zhang & Forde (2000) have proposed a dual model where root branching is locally induced by low nitrate supply (1 mM) and systemically inhibited under high nitrate supply (50 mM). Taken together, these results emphasize the complex signalling networks involved in the control of nutrient absorption by the roots and root development.

The aim of our study was to determine whether changes in the concentration of individual phloem amino acids could be correlated to variations of $^{15}$NO$_3^-$ influx and BnNrt2 mRNA expression. Two different experimental approaches in which internal N availability to the root varied, causing important influx variations, were used: (1) termination of external N supply by N-deprivation (short-term) and (2) developmental variations of N uptake over the growth cycle (long-term) between bolting-flowering and flowering-pod filling stages (Malagoli et al. 2004). The amino acids showing correlated changes in these two experiments were then tested for potential effects on HATS activity and BnNrt2 expression level.

**MATERIALS AND METHODS**

**Plant material**

Seeds of rape (Brassica napus L. cv. Capitol) were surface-sterilized with 80% ethanol and 20% hypochlorite solutions and rinsed with de-ionized water. The seeds were germinated and grown hydroponically in a climate-controlled room. The aerated nutrient solution contained 1 mM KNO$_3$, 0.4 mM KH$_2$PO$_4$, 0.15 mM K$_2$HPO$_4$, 1 mM K$_2$SO$_4$, 0.5 mM MgSO$_4$; 3 mM CaCl$_2$, 0.2 mM Fe- Na EDTA, 14 $\mu$M H$_2$BO$_3$, 5 $\mu$M MnSO$_4$, 3 $\mu$M ZnSO$_4$, 0.7 $\mu$M CuSO$_4$, 0.7 $\mu$M (NH$_4$)$_2$Mo$_7$O$_24$, and 0.1 $\mu$M CoCl$_2$, and was renewed every 2 d. The pH of the solution was maintained at 6.0 ± 0.3 by the addition of CaCO$_3$. Light measured at plant level was equal to 300 μmol m$^{-2}$s$^{-1}$ of PAR and was provided by high-pressure sodium lamps for 16 h per day. The thermoperiod was 20 ± 1 °C (day) and 15 ± 1 °C (night).

**Experimental treatments**

**Experiment 1 (N-deprivation)**

Two sets of plants were transferred to N-free nutrient solution for different durations (0, 12, 24, 48, 72 h). At each sampling time, HATS activity and BnNrt2 mRNA abundance were measured in one set of plants and amino acids content in phloem exudates was assayed with the other set.

**Experiment 2 (growth cycle)**

Plants from the field were harvested at the C$_2$ stage, screened for their tap root diameter (0.6–1.0 cm), before
being acclimated in a climate-controlled room under hydroponic culture conditions as described in the previous section. Developmental stages were estimated from the phenological calendar established by the Bayer, BASF, Ciba and Hoechst companies. Nine plants for each developmental stage of the growth cycle were used for measuring HATS influx and *BnNrt2* mRNA abundance. Six other plants were used for assaying amino acids content in phloem exudates.

**Experiment 3 (amino acid effects)**

Six pots of 25 plants were first transferred to N-free nutrient solution for 12 h before being transferred to a new nutrient solution containing 1 mM KNO$_3$ and either 100 μM GABA, 1 mM GABA, 1 mM Glu, 1 mM Glu or 1 mM Asn for 12 h. At the end of the treatments, HATS influx, *BnNrt2* mRNA abundance and root amino acids content were measured on six, two and three replicates, respectively.

**Plant harvesting, nitrogen and isotope analysis**

In order to measure nitrate influx, plants of the three experiments were grown in hydroponic tanks and were treated according to the particular experimental design. Roots were then washed twice in 1 mM CaSO$_4$ solution for 1 minute at 20 °C to remove unabsorbed K$^{15}$NO$_3$ and transferred to influx solution containing 100 μM of K$^{15}$NO$_3$ (99.9%) for 5 min. Plants were then washed twice in 1 mM CaSO$_4$ solution for 1 min at 4 °C to slow down the metabolism and remove unabsorbed tracer residing in the cell wall space. Roots and shoots were harvested separately and dried for 48 h at 60 °C before being ground to fine powder for isotope analysis. A root fraction of each plant was frozen in liquid nitrogen and stored at –80 °C for amino acids and northern blot analyses.

Nitrogen and $^{15}$N content of plant samples were measured in continuous flow using a C/N analyzer linked to an isotope ratio mass spectrometer (Roboprep CN and 20–20 mass spectrometer; Europa PDZ, Crewe, UK).

**Collection of phloem exudates and HPLC analysis of amino acid**

Phloem exudates were collected using the facilitated diffusion method by EDTA according to King & Zeevaart (1974) and Bourgis et al. (1999). Plant stems were fully cut at the crown level with a razor blade in EDTA solution (20 mM, pH 7.0). The average diameter of the stem sections was about 6 mm. Excision sections were then rinsed in a new EDTA solution (5 mM, pH 7.0) for 5 min and placed in vials containing 2 mL (N-deprivation experiment) or 8 mL (growth cycle experiment) of the same solution. Exudation was induced in the dark in a climatized chamber at 20 °C with a water-saturated atmosphere (HR = 90%). The exudation solution (5 mM EDTA, pH 7.0) was renewed every 2 h during the collection period of 12 h. The EDTA of each collected fraction was precipitated by adding 100 μL of 0.5 M HCl and stored for 1 h at –20 °C. The samples were then stored over night at 4 °C prior to centrifugation at 5000 g for 10 min. The supernatants were adjusted to pH 5.9, filtered through a 0.45 μm nylon membrane and stored at –20 °C before amino acid analyses. Free amino acids in phloem exudates were analysed by high-performance liquid chromatography (HPLC) as ophthalaldehyde derivatives on a C-18 column using a 32 Karat System (Beckman Instruments, San Ramon, CA, USA) as previously described by Murray, Hatch & Cliquet (1996). Specific amino acids were quantified using α-aminobutyric acid as an internal standard.

**RNA isolation and northern blot analysis**

Twenty micrograms of total RNA previously extracted from root tissues was (i) fractionated on 1.2% agarose gel containing formaldehyde; (ii) transferred to Hybond-N$^+$ blotting membranes (NEN Life Science, Boston, MA, USA) using 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0); and (iii) fixed onto the membranes by exposure to UV (30 s at 100 mJ). The blots were then pre-hybridized for 2 h at 60 °C in Church buffer (Church & Gilbert 1984).

The *BnNrt2* probe used in northern analyses was issued from a 643 bp cDNA fragment isolated by reverse transcriptase-polymerase chain reaction (Faure-Rabasse et al. 2002; Accession number: AF278966). The *BnNrt2* gene showed 89.3, 84.7, 73.6, 67.5, 60.3% homology with *AtNrt2.1, AtNrt2.2* and northern blot analyses.

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RESULTS

$^{15}$NO$_3$– influx and $BnNrt2$ mRNA level during N-deprivation and over the growth cycle

When plants previously fed with NO$_3$– were transferred to N-free solution, the $^{15}$NO$_3$– influx kinetic showed a transient increase (about 1.4-fold) during the first 24 h (Fig. 1a) and then a decrease from 24 to 72 h of N-deprivation. Similarly $BnNrt2$ mRNA level increased by 2.5 fold after the first 24 h of N-deprivation and decreased thereafter to a value lower than the control plants (Fig. 1a & c).

The $^{15}$NO$_3$– influx increased during the bolting period of the growth cycle, followed by a drastic decline at the flowering stage and a slight increase during pod filling. The abundance of $BnNrt2$ mRNA mirrored the evolution of $^{15}$NO$_3$– influx, except at stage E where no $BnNrt2$ mRNA was detected, suggesting that a specific regulation might exist at the transcriptional level around the flowering period when remobilization of N occurs in the plants.

Analysis of total free amino acids in phloem exudates during N-deprivation and over the growth cycle

Total free amino acid content in the exudate was maximal in the fraction collected between 8 and 10 h of exudation for the N-deprivation experiment and between 10 and 12 h for the developmental cycle experiment (data not shown). Consequently, analyses of phloem sap were performed at the optimum exudation time in terms of quantity and stability of amino acids for both experiments.

The N-deprivation caused a progressive and significant increase of total amino acid content in the phloem exudate (Fig. 2a). During the growth cycle, total amino acid contents decreased from the C2–D1 stage to the F stage suggesting that amino acids exported from the shoot were mainly used during vegetative growth to build the root (Fig. 2b) as confirmed by the dry weight increase of both tap and secondary roots (data not shown). During the pod filling period (between stages F and G4), when N remobilization is at its

Figure 1. Changes in $^{15}$NO$_3$– influx and $BnNrt2$ gene expression during the time-course of N-deprivation and over the growth cycle in *Brassica napus* L. plants. Plants were grown directly in hydroponic conditions for 2 months (N-deprivation) or were acclimated at C2 stage from the field (growth cycle) in a nutrient solution with 1 mM nitrate. For N-deprivation, plants were transferred to a solution without nitrate (time $t = 0$). In these two experiments, influx rate was measured at 100 μM with K$^{3}$NO$_3$. Values are the means of three replicates of plants (N-deprivation) or nine replicates (growth cycle). The vertical bars on nitrate influx values indicate ±SD for $n = 3$ and ±SE for $n = 9$ when larger than the symbol. For $BnNrt2$ mRNA relative expression, vertical bars indicate ±SD for $n = 2$ when larger than the symbol.
highest in the plant (Rossato et al. 2002), total amino acid content remained low in phloem exudate compared with its level at the bolting period (C2–D1–E stages).

Analysis of individual free amino acids in phloem exudates during N-deprivation and over the growth cycle

During N-deprivation, the important increase in total amino acid content in phloem exudates between 0 and 72 h (Fig. 2a) was associated with high variations in the composition of individual free amino acids (Fig. 3a–c). Modifications in the concentrations of some of the major circulating amino acids in the phloem were recorded over the N-deprivation period (Fig. 3a & b). Glu, Ala and Ser remained unchanged over the 72 h of N-deprivation (Fig. 3a & b), whereas Gln and GABA concentrations varied considerably (and inversely) during N deficiency (Fig. 3a). The contribution of minor amino acids such as Asp, Thr and Val decreased constantly or slightly from 24 to 72 h after the onset of N-deprivation (Fig. 3b & c).

Over the growth cycle, the decrease in total amino acid content observed in the phloem exudates (Fig. 2b) was associated with high changes in the relative contribution of individual amino acid (Fig. 3d–f). The contribution of Gln and Asn in phloem exudate decreased two-fold during the bolting-flowering period (stages D2 to F; Fig. 3d & f), whereas the relative contribution of Glu, Asp, Ala and Thr increased during the pod filling period (Fig. 3d–f). Among all the amino acids measured, the contribution of GABA showed the most similar evolution to nitrate influx over the whole growth cycle (Figs 1b & 3d).

Relationships between $^{15}$NO$_3^-$ influx and relative contribution of phloem amino acids during N-deprivation and over the growth cycle

In order to investigate the possible shoot-to-root signalling role of amino acids during N-deprivation and over the growth cycle, correlations between the relative contribution of each phloem amino acid and nitrate influx were systematically sought. The only significant correlation was obtained with GABA content ($P < 0.01$; Fig. 4a & b) both during N-deprivation and over the growth cycle. In contrast, correlations with Gln were non-significant ($P > 0.05$) in both experiments. In addition, nitrate influx and GABA were positively correlated in both the short-term and long-term experiments, whereas the trend for Gln indicated a negative correlation in the N-deprivation experiment and a positive correlation in the growth cycle experiment (Fig. 4c & d).

Effects of exogenous supply of GABA, Gln, Glu and Asn on nitrate uptake

In order to test the hypothesis that GABA could up-regulate nitrate uptake, the effect of exogenous supply of different amino acids was compared with control plants. Gln, Glu and Asn are known to act as inhibitors of nitrate uptake at the millimolar range in many species when supplied to the root nutrient solution (Muller & Touraine 1992; Vidmar et al. 2000). Therefore, roots were supplied with either 1 mM Gln, 1 mM Glu or 1 mM Asn, whereas two concentrations were tested for the GABA treatment, 100 μM and 1 mM (Fig. 5). A positive and significant effect
Figure 3. Evolution of individual amino acids present in phloem exudates of *Brassica napus* L. plants during the time course of N-deprivation and over the growth cycle. Values are the means of six plants. All vertical bars indicate ± SE for *n* = 6 when larger than the symbol.
on $^{15}$NO$_3$ influx was observed for 100 $\mu$M ($P < 0.05$) and no significant effect for 1 mM GABA ($P > 0.05$)-treated plants compared with the control plants, whereas $^{15}$NO$_3$ influx was significantly reduced by the Gln, Glu and Asn root treatments (Fig. 5a). The strongest negative effect on nitrate uptake was due to Glu (38% inhibition) followed by Asn (37% inhibition) and Gln (33% inhibition). In parallel, Northern blot analyses performed with mRNA extracted from the same amino acid treated plants confirmed previous results for all of the amino acids tested. Thus, BnNrt2 mRNA abundance was enhanced by 1.6- and 1.3-fold in response to 100 $\mu$M and 1 mM GABA treatments, respectively. The Gln, Glu and Asn treatments reduced BnNrt2 mRNA abundance by 0.5-, 0.9- and 0.8-fold, respectively (Figs 5b & c).

Endogenous concentrations of root amino acids were quantified for each treatment in order to check if GABA entered the root and to determine possible correlation between endogenous amino acid levels and nitrate influx or Nrt2 mRNA level (Table 1). After 12 h of exogenous supply, endogenous concentration of each amino acid applied increased in the root. For example, 1 mM GABA treatment increased root GABA by 2.24-fold compared with the control plants (Table 1). However, 100 $\mu$M GABA and 1 mM Glu treatments failed to increase concentrations levels of all of the four amino acids applied (Table 1). No correlation was found between changes of influx or BnNrt2 transcript levels and root amino acid concentrations or ratio between them.

Figure 4. Correlations between variations of nitrate influx and contribution of GABA or Gln in phloem exudates during N-deprivation and over the growth cycle in Brassica napus L. plants. All vertical bars indicate ±SE for $n = 6$ (GABA or Gln) when larger than the symbol. All horizontal bars indicate ±SD for $n = 3$ (N-deprivation influx) and ±SE for $n = 9$ (growth cycle influx) when larger than the symbol.
DISCUSSION

Relationship between glutamine in phloem exudate and nitrate uptake during N-deprivation and over the growth cycle

It is well established that N-deprivation leads to a transient ‘de-repression’ of high-affinity nitrate influx and Nrt2 gene expression in plants with a low N status (Lejay et al. 1999; Forde 2002). In plants with a high N status, ‘de-repression’ seems to be bypassed, leading to a direct decline of nitrate uptake interpreted as ‘de-induction’ (Clarkson 1986; Siddiqi et al. 1989; Faure-Rabasse et al. 2002). Our results for $^{15}$NO$_3$– influx and BnNrt2 gene expression (Fig. 1a & c) are consistent with these previous studies.

This ‘de-repression’ is classically interpreted as a depletion of the internal amino acid pool through continued translocation, assimilation and protein synthesis (Cooper & Clarkson 1989; Lejay et al. 1999). In our N-deprivation experiment, the increase in total amino acid measured in the phloem exudate (Fig. 2a) contradicts this assumption. However, we observed that the relative contribution of Gln was inversely related to both nitrate uptake and BnNrt2 expression level although no significant correlation was found ($r^2 = 0.753$, $P > 0.05$; Fig. 4c). This lack of significant correlation with Gln can be attributed to the low number of kinetic data points ($n = 5$). Indeed, negative effect of Gln have been clearly established on gene expression and nitrate uptake in many species by exogenous supply to the root (Vidmar et al. 2000). Our results in Fig. 5 also confirmed the role of Gln as potential negative effector although no correlation was found after exogenous amino acid supply to the root between variations of nitrate influx and the root concentration of Gln or other amino acids (Table 1).

In consequence, our results support partially the hypothesis of Vidmar et al. (2000) that Gln could be the main down-regulator of nitrate uptake and a putative long-distance inter-organ signal in planta (Nazoa et al. 2003).

However, three main results of the present growth cycle experiment provide evidence which contradicts the hypothesis of N uptake down-regulation by total amino acids or Gln content: (i) the high content of total amino acids recorded in phloem exudates during the bolting period when N uptake increases (Figs 1b & 2b), which is not consistent with a main role of amino acids in down-regulation of nitrate uptake; (ii) the lack of nitrate uptake ‘de-repression’ at the beginning of pod filling (G2 stage) when total amino acid content in the phloem exudate was at its lowest value (Figs 1b & 2b); (iii) the lack of a significant correlation between $^{15}$NO$_3$– influx and relative Gln contribution in the phloem exudate ($r^2 = 0.44$, $P > 0.05$; Fig. 4d). These results must, however, be interpreted with caution. The dual role of some amino acids, both as nutrients and as signals, and their possible interconversion or compartmentalization in root tissues is potentially problematic in phloem sap analysis when attempting to correlate influx variations with amino acid composition.

In long-term experiments and non-limiting N conditions, signals other than amino acids may be involved, as suggested by the lack of BnNrt2 mRNA transcripts at stage E (preflowering stage) when nitrate uptake was at its highest level. Similar result has been previously described by Nazoa et al. (2003) in Arabidopsis around the flowering period where the expression of GUS gene driven by the AtNrt2.1 promoter give a lack of signal in the root. Likewise, Vidmar et al. (2000) reported that after 24 h of nitrate induction on N-deprived plants, HvNrt2 expression decrease to undetectable levels while nitrate influx
Values shown are the means of three independent replicates ± SD of the mean.

### Relationship between GABA in phloem exudate and nitrate uptake during N-deprivation and over the growth cycle

Our results establish for the first time the existence of a correlation between nitrate uptake and a phloem amino acid, GABA, suggesting that this amino acid could act as a shoot-to-root signal component in positive nitrate uptake regulation (Fig. 4a & b). To date, little was known about the positive effects of some amino acids on nitrate uptake after their exogenous supply to the roots (Breteler & Arnozis 1985; Muller & Touraine 1992). In our experiments, a positive effect of GABA on nitrate uptake was clearly demonstrated by exogenous supply to roots (Fig. 5a) levels. Since the common hypothesis is that nitrate uptake is under a negative feedback control (Glass 1983; Clarkson 1988), our results raised the question of whether a positive effector such as GABA could act on this stabilized retro-control. A part of the response is provided by the fact that GABA is a non-protein amino acid, mainly produced from glutamate catabolism (GABA shunt), which has been reported to accumulate to high levels in plant tissues, especially shoot tissues, after many types of abiotic and biotic stresses (Snedden & Fromm 1999; Shelp, Bown & McLean 1999; Kinnersley & Turano 2000). In consequence, N nutritional stress or important N-demand for sustaining shoot growth during bolting period could result in accumulation of GABA which in turn would induce the increase of nitrate uptake. In this assumption, presence of negative effector such as Gln will always be necessary and not contradictory in order to restore rapidly the repressed state. This type of regulation will offer the plant with the opportunity to escape and later come back rapidly to the repressed state of nitrate uptake during a stress condition.

Furthermore, although GABA is recognized in animals as being a major inhibitory neurotransmitter by modulating conductance of ligand-gated ion-channel receptors in the central nervous system, the function of GABA in plants remains unclear (Shelp et al. 1999). Emerging literature suggests that GABA may function in plants as a potential modulator of ion transport and consequently of mineral acquisition via G-proteins as in other eukaryotes (Chen, Baum & Fromm 1994; Kinnersley & Lin 2000; Kim et al. 2001; Bouché, Lacombe & Fromm 2003). Another emerging role of GABA is that this amino acid is an inducer of stress ethylene by acting on ACC synthase gene (Kinnersley & Turano 2000). Because ethylene is the main growth regulator involved in root hair and lateral root development (Clark et al. 1999; Schiefelbein 2000) GABA could provide an amplifier of stress signal conducting to the modification of the root absorbing surface. These combined results suggest that GABA might mediate a root-specific response in mineral acquisition via the increase of root surface or conduction of ion transport, raising the question of whether GABA is transported over long distances in plants (Kinnersley & Lin 2000).

### GABA translocation during N-deprivation and over the growth cycle

Despite the ubiquitous occurrence of GABA in plant tissues (related to the location of at least two GAD isoforms: one expressed in all plant tissues – GAD2– and another that is root specific – GAD1–), the question of intercellular GABA transport is often addressed (Turano & Fang 1998; Snedden & Fromm 1999; Bouché & Fromm 2004). Recent research has shown that intercellular and inter-organ GABA transport at the whole plant level is mediated by three specific membrane transporters: AAP3, ProT1 and ProT2 (Rentisch et al. 1996; Breitkreuz et al. 1999; Schwacke, Baum & Fromm 1994; Kinnersley & Lin 2000; Kim et al. 2001; Bouché, Lacombe & Fromm 2003). Another emerging role of GABA is that this amino acid is an inducer of stress ethylene by acting on ACC synthase gene (Kinnersley & Turano 2000). Because ethylene is the main growth regulator involved in root hair and lateral root development (Clark et al. 1999; Schiefelbein 2000) GABA could provide an amplifier of stress signal conducting to the modification of the root absorbing surface. These combined results suggest that GABA might mediate a root-specific response in mineral acquisition via the increase of root surface or conduction of ion transport, raising the question of whether GABA is transported over long distances in plants (Kinnersley & Lin 2000).

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<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Glu (nmol g⁻¹ FW)</th>
<th>Asn (nmol g⁻¹ FW)</th>
<th>Gln (nmol g⁻¹ FW)</th>
<th>GABA (nmol g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>370 ± 139</td>
<td>876 ± 295</td>
<td>327 ± 123</td>
<td>494 ± 251</td>
</tr>
<tr>
<td>100 μM GABA</td>
<td>247 ± 43</td>
<td>694 ± 90</td>
<td>164 ± 40</td>
<td>460 ± 162</td>
</tr>
<tr>
<td>1 mM GABA</td>
<td>344 ± 63</td>
<td>1038 ± 305</td>
<td>231 ± 110</td>
<td>413 ± 167</td>
</tr>
<tr>
<td>1 mM Gln</td>
<td>379 ± 65</td>
<td>1266 ± 277</td>
<td>323 ± 116</td>
<td>369 ± 76</td>
</tr>
<tr>
<td>1 mM Glu</td>
<td>314 ± 80</td>
<td>887 ± 155</td>
<td>143 ± 28</td>
<td>341 ± 60</td>
</tr>
<tr>
<td>1 mM Asn</td>
<td>369 ± 74</td>
<td>850 ± 192</td>
<td>1684 ± 515</td>
<td>718 ± 240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>323 ± 130</td>
</tr>
</tbody>
</table>

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Table 1. Effects of exogenously supplied amino acids on amino acids level in plant roots

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et al. 1999). Of these transporters, AAP3 and ProT2 are constitutively expressed in roots and all tissues, respectively, and Prot1 is induced after drought and salt stresses (Fischer et al. 1995). Furthermore, germination experiments have suggested that GABA is the phloem amino acid form translocated from cotyledons to the growing axis (Cho & Splittstoesser 1972; Desmaison & Tixier 1986; Golombek et al. 2001) and many authors have found that GABA is either the major phloem amino acid (Housley et al. 1979) or one of the minor circulating amino acid forms in the phloem (Pate et al. 1979; Hocking 1983; Girousse et al. 1991; Nazoa et al. 2003).

Putative shoot-to-root signalling role of GABA

The potential role of GABA as a signal molecule was first proposed by Snedden & Fromm (1999) on the basis of the important GABA efflux from the high cellular concentrations induced after different types of stresses (Mayer, Cherry & Rhodes 1990; Chung, Bown & Shelp 1992; Snedden et al. 1992; Crawford et al. 1994). Until recently, this hypothesis remained speculative due to the lack of identification of GABA receptors in plants. However, sequence analyses have shown that the identified glutamate-gated Ca²⁺-channel receptors (GLRs) in plants (Lam et al. 1998) also contain part of a GABA recep tor sequence in their extra-cellular N-terminal region (Turano et al. 2001, 2002). The presence of this GABA sequence located before the two putative Glu-binding domains in the N-terminal region strongly suggests that in addition to functioning with glutamate (Kang & Turano 2003), some of these GLRs in plants probably also interact with GABA (Kinnersley & Lin 2000; Turano et al. 2001; Bouché et al. 2003). Because these receptors were first proposed to be involved in plant development (Lam et al. 1998) the question of whether Glu and GABA could act on plant development via these Ca²⁺-channel receptors was raised. A partial response has been provided by the recent findings of Kang & Turano (2003) who demonstrated that the AtGLR1.1 receptor could function as a regulator of C and N metabolism in Arabidopsis. In addition, results obtained with transgenic lines (Baum et al. 1996) and mutants of GAD (Bouché, personal communication) demonstrated developmental alterations in shoot and root development according to Glu and GABA tissue levels. Indeed, transgenic tobacco plants over-expressing either GAD or GAD lacking the CaM-binding domain, undergo severe developmental abnormalities such as reduced stem growth with very high (seven times more) GABA and low (18 times less) glutamate concentration levels in the stem. Gad1 mutant plants in Arabidopsis have five times less GABA in the roots and exhibit shorter roots than wild type plants (Bouché, personal comm.).

The following points are consistent with the involvement of GABA not only as an intercellular signalling molecule (short distance) but also as an inter-organ signalling molecule in plants (long distance), and especially as a shoot-to-root signal in nitrate uptake regulation: (1) the present study and references cited above confirms that GABA is a translocated molecule in phloem exudate and that it undergoes intercellular and inter-organ transport at the whole plant level via the action of two constitutive specific membrane transporters, AAP3 and ProT2 (Fischer et al. 1995, Rentsch et al. 1996; Breitkreuz et al. 1999; Schwacke et al. 1999); (2) a strong correlation was observed in this study between GABA content in phloem exudates and changes in nitrate influx during N-deprivation and over the growth cycle in Brassica napus (this study); (3) exogenous supply of GABA induced significant increase of BnNr2 genes expression and, to a lesser extent, of ²¹³NO⁻ influx (this study); (4) GABA treatment promoted Lemma growth by a high and significant increase in mineral acquisition and content (Kinnersley & Lin 2000; Kinnersley & Turano 2000); (5) GABA induced the production of ethylene which is the main signal involved in root hairs et lateral roots development (Clark et al. 1999; Schiebelbein 2000); (6) GABA is produced from glutamate issued from the GS/GOGAT cycle via the 'GABA shunt' and provides a direct metabolic link between nitrogen, carbon and energetic metabolism (Krebs cycle) (Snedden & Fromm 1999; Shelp et al. 1999; Bouché et al. 2003).

A key challenge for future researches in nitrate uptake will be to elucidate the role of GABA in Ca²⁺ signalling pathways via glutamate decarboxylase (GAD/CaM binding domain), glutamate-gated Ca²⁺-channel receptors (GLR) and ACC/ethylene synthesis. A clear priority is to investigate the direct or indirect influence of GABA on nitrate uptake and/or mineral acquisition in relation to root development (root hairs and lateral roots).

REFERENCES


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