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Full title:

Putative role of γ-aminobutyric acid (GABA) as a long distance signal in up-regulation of nitrate uptake in *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 *Brassica napus* **L.**. Our data showed a positive **correlation** between γ-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 Gln, Glu and Asn, each known to be inhibitors of nitrate uptake. Our results showed that GABA treatment induced a significant increase of *BnNrt2* mRNA expression, but had less effect on nitrate influx. By contrast, Gln, Glu and Asn significantly reduced nitrate influx and *BnNrt2* mRNA expression compared to 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 Gln**. 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: *Brassica napus* L.; nitrate **uptake**; High-Affinity Transport System (HATS); *BnNrt2* genes; GABA; amino acids; 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, Glass, Ruth & Rufty 1990), which are considered as genetically distinct and independent.

Since the first characterisation of a *crn*A gene in *Aspergillus nidulans* considered to encode an inducible HATS (Unkless, Hawker, Grieve, Campbell, Montague & Kinghorn 1991; Unkless, **Hawker, Grieve, Campbell, Montague & Kinghorn** 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 *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, Glass, Ruth & Fernando 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 & Boucaud 1995; Lainé, Ourry, Boucaud & Salette 1998; Gansel, Muños, Tillard & Gojon 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, Wu, Xia, Wu, Chen & Liu 2002). NO_3^- is assumed to act indirectly ***via*** a local signalling pathway on *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; Lainé et al. 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; Breteler & Arnozis 1985; Muller & Touraine 1992; Rodgers & Barneix 1988). However, these experiments have failed to distinguish between possible biochemical inter-conversion 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, Purves, Ratcliffe & Saker 1992; Zhuo, Okamoto, Vidmar & Glass 1999; Vidmar, Zhuo, Siddiqui, Schjoerring, Touraine & Glass 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, Vidmar, Tranbarger, Mouline, Damiani, Tillard, Zhuo, Glass & Touraine 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 (Tillard, Passama & Gojon 1998; Lainé et al. 1995). 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 (1mM) and systemically inhibited under high nitrate supply (50mM). Taken together, these results emphasise 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, Lain**é**, Le Deunff, Rossato, Ney & Ourry 2004). The amino acids showing correlated changes in these two experiments were then tested for potential effects on HATS activity and *BnNrt2* expression level.

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 24h (Fig. 1A) and then a decrease from 24h to 72h 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. 1**A and** C).

$^{15}NO_3^-$ 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 remobilisation 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 to 10h of exudation for the N-deprivation experiment and between 10 to 12h 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.

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 remobilisation is at its highest in the plant (Rossato, Macduff, Lainé, Le Deunff & Ourry 2002), total amino acid content remained low in phloem exudate compared to 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 72h (Fig. 2A) was associated with high variations in the composition of individual free amino acids (Fig. 3A, B and 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 and B). Glu, Ala and Ser remained unchanged over the 72h of N-deprivation (Fig. 3A and 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 24h to 72h after the onset of N-deprivation (Figs. 3B and 3**C**).

Over the growth cycle, the decrease in total amino acid content observed in the phloem exudates (Fig. 2**B**) was associated with high changes in the relative contribution of individual amino acid (Figs. 3D, E and F). The contribution of Gln and Asn in phloem exudate decreased two-fold during the bolting-flowering period (stages D2 to G2; Figs. 3D and 3F), whereas the relative contribution of Glu, Asp, Ala and Thr increased during the pod filling period (Figs. 3D, **E** and 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 and 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 and 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 (Figs 4**C** and **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 **to 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 1mM Gln, 1mM Glu or 1mM Asn, whereas 2 concentrations were tested for the GABA treatment, 100µM and 1mM (Fig. 5). A positive **and significant** effect on $^{15}NO_3^-$ influx was observed for 100µM **($P<0.05$)** and **no significant effect** for 1mM GABA **($P>0.05$)** treated plants compared to the control plants, while $^{15}NO_3^-$ 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 fold and 1.3 fold in response to 100μM and 1mM 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 and C).

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

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, Tillard, Lepetit, Olive, Filleur, Daniel-Vedele & Gojon 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, Le Deunff, Lainé, Macduff & Ourry 2002). Our results for $^{15}NO_3^-$ influx and *BnNrt2* gene expression (Fig. 1A and 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 figure 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 I).**

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 (Fig. 1B and Fig. 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 (Fig. 1B and Fig. 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 inter-conversion or compartmentalisation 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 (**pre-flowering 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 24h of nitrate induction on N-deprived plants, *HvNrt2* expression decrease to undetectable levels while nitrate influx remained around its maximum level.** In this context, Rossato et al. (2002) proposed that the post-flowering decline of N

uptake could be a result of the production of methyl jasmonate (a growth regulator) by young growing tissues or senescing leaves during the flowering period. Foliar application or supply of methyl jasmonate to roots during the vegetative period induced early leaf senescence and was associated with a drastic decrease of NO_3^- uptake (Rossato et al. 2002). Alternatively, Malagoli et al. (2004) suggested that modification of carbon partitioning around the flowering period could favour newly appearing sink tissues to the detriment of the C allocated to the root. This last hypothesis implies the regulation of N uptake by C status, as demonstrated by the partial restoration of *Nrt2.1* gene expression level and NO_3^- influx after exogenous supply of sucrose to the root **at** night (Lejay et al. 1999).

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. 4 A and 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 at the transcriptional (Fig. 5 B and C) and to a lesser extent the post-transcriptional (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, Kwak, Jae, Wang & Nam 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, Gubrium, Barett, Nell & Klee 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* (Rentsch, Hirner, Schmelzer & Frommer 1996; Breitkreuz, Shelp, Fisher, Schacke & Rentsch 1999; Schwacke, Grallath, Breitkreuz, Stransky, Stransky, Frommer & Rentsch 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, **Kwart, Hummel & Frommer** 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, Rolletschej, Wobus & Weber 2001) and many authors have found that GABA is either the major phloem amino acid (Housley, Schrader, Miller & Setter 1979) or one of the minor circulating amino acid forms in the phloem (Pate, Atkins, Lhamel & McNeil 1979; Hocking 1983; Girousse, Bonnemain, Delrot & Bournoville 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, Chung, Pauls & Bown 1992; Crawford, Bown, Breitkreuz & Guinel 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 Ca^{2+}-channel receptors (GLRs) in plants (Lam, Chiu, Hsieh, Meisel, Oliveira, Shin & Coruzzi 1998) also contain part of a $GABA_B$ animal receptor sequence in their extra-cellular N-terminal region (Turano, Panta, Allard & van Berkum 2001; Turano, Muhitch, Felker & McMahon 2002). The presence of this $GABA_B$ 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 Ca^{2+}-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, Lev-Yadun, Fridmann, Arazi, Katsnelson, Zik & Fromm 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 (7 times more) GABA and low (18 times less) glutamate concentration levels in the stem. *Gad1* mutant plants in *Arabidopsis* have 5 times less GABA in the roots and exhibit shorter roots than wild type plants (Bouché, personal communication).

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: (i) 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* (Fisher et al. 1995, Rentsch et al. 1996; Breitkreuz et al. 1999; Schwacke et al. 1999); (ii) 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)**; (iii) exogenous supply of GABA induced significant increase of *BnNrt2* genes expression and, to a lesser extent, of $^{15}NO_3^-$ influx **(this study)**; (iv) GABA treatment promoted *Lemna* growth by a high and significant increase in mineral acquisition and content (Kinnersley & Lin 2000; Kinnersley & Turano 2000); (v) GABA induced the production of ethylene which is the main signal involved in root hairs et lateral roots development (Clark et al. 1999; Schiefelbein 2000); (vi) 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 Ca^{2+} signalling pathways via glutamate decarboxylase (GAD/CaM

binding domain), glutamate-gated Ca^{2+}-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).

MATERIALS AND METHODS

Plant material

Seeds of rape (*Brassica napus* L. cv. **c**apitol) 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 climatised room. The aerated nutrient solution contained 1mM KNO_3, 0.4mM KH_2PO_4, 0.15mM K_2HPO_4, 1mM K_2SO_4, 0.5mM $MgSO_4$, 3mM $CaCl_2$, 0.2mM Fe-Na EDTA, 14µM H_3BO_3, 5µM $MnSO_4$, 3µM $ZnSO_4$, 0.7µM $CuSO_4$, 0.7µM $(NH_4)_6Mo_7O_{24}$ and 0.1µM $CoCl_2$ and was renewed every **2** days. **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 300µmol.m^{-2}.s^{-1} of PAR and was provided by high-pressure sodium lamps for 16h 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, 72h). 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 C2 stage, screened for their tap root diameter (0.6-1.0cm), before being acclimated in a climatised 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 12h before being transferred to a new nutrient solution containing 1mM KNO_3 and either 100µM **GABA**, 1mM GABA, 1mM Gln, 1mM Glu or 1mM Asn for 12h. At the end of the treatments, HATS influx, *BnNrt2* mRNA abundance **and root amino acids content** were measured on 6, 2 **and 3** 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 1mM $CaSO_4$ solution for 1 minute at 20 °C to remove unabsorbed $K^{14}NO_3$ and transferred to influx solution containing 100µM of $K^{15}NO_3$ (99.9 %) for 5min. Plants were then washed twice in 1 mM $CaSO_4$ solution for 1 minute 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 48h 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, Sanja, Nuccio, Fisher, Tarczynski, Li, Herschbach, Rennenberg, Pimenta, Shen, Gage & Hanson (1999). Plant stems were **fully** cut at the crown level with a razor blade in EDTA solution (20mM, pH 7.0). **The average diameter of the stem sections was about 6 mm**. Excision sections were then rinsed in a new EDTA solution (5mM, pH 7.0) for 5min and placed in vials containing 2ml (N-deprivation experiment) or **8**ml (growth cycle experiment) of the same solution. Exudation was induced in the dark in a climatised chamber at 20 °C with a water-saturated atmosphere (HR = 90 %). The exudation solution (5mM EDTA, pH 7.0) was renewed every two hours during the collection period of 12h. The EDTA of each collected fraction was precipitated by adding 100µl of 0.5 N HCl and stored for 1 hour at – 20 °C. The samples were then stored over night at 4°C prior to centrifugation at 5 000 g for 10min. The supernatants were adjusted to pH 5.9, filtered through a 0.45µM nylon membrane and stored at –20 °C before amino acid analyses. Free amino acids in phloem exudates were analyzed by high-performance liquid chromatography (HPLC) as ophtaldialdehyde 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

20µg 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, USA) using 10x SSC (1.5M NaCl, 0.15M

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-hybridised for 2h 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 RT-PCR (Faure-Rabasse et al. 2002; Accession number: AJ278966). The*BnNrt2* gene showed 89.3%, 84.7%, 73.6%, 67.5%, 60.3% homology with *AtNrt2.1, AtNrt2.2, AtNrt2.3, AtNrt2.4, AtNrt2.5* respectively. **Therefore, this probe can potentially reveal the expression of several genes of *BnNrt2* gene family**. The amplified cDNA fragment was gel purified and labelled with α^{32}P [dCTP] (3000 Ci.mmol^{-1}) by using the random priming Netblot kit (New England, Biolabs, St. Quentin, Yveline, France). After addition of the probes, membranes were hybridised overnight at 60 °C in buffer containing: SDS 7%, Na_2HPO_4 0.25M, EDTA 2mM, heparin 0.2 mg.ml^{-1} and calf thymus DNA 0.1 mg.ml^{-1} (Church & Gilbert 1984). Then, the membranes were washed successively with : (1) 2x SSC, 0.1% SDS 20 min at room temperature, (2) 1x SSC, 0.1% SDS 20 min at 50°C, (3) 0.2x SSC, 0.1% SDS 20 min at 60°C, (4) 0.1x SSC, 0.1% SDS 20 min at 60°C before being analyzed. Loading of RNA was controlled **and normalised** either with **tubulin probe from *Arabidopsis*** quantified with phosphoimager (Packard Instrument Company, Rungis, France) or with an **28S +** 18S/ethydium bromide fluorescent signal measured with an image analyser (Wilbert Lourmat, France). The *BnNrt2* probe signal was quantified with phosphoimager (Packard Instrument Company, Rungis, France). The blots were then 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).

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LEGEND OF FIGURES

Fig. 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 1mM nitrate. For N-deprivation, plants were transferred to a solution without nitrate (time t=0). In these two experiments, influx rate was measured at 100µM with $K^{15}NO_3$. Values are the means of 3 replicates of plants (N-deprivation) or 9 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**.

Fig. 2: Evolution of total amino acid content in phloem exudates during the time-course of N-deprivation and **over** the growth cycle in *Brassica napus* **L.** plants. Total amino acid content was determined in phloem exudates collected between 8-10h and 10-12h of exudation for N-deprivation and growth cycle experiments, respectively. Values are the means **of 6 plants**. All vertical bars indicate ± SE (n=6) when larger than the symbol.

Fig. 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 6 plants. All vertical bars indicate ± SE for n=6 when larger than the symbol.

Fig. 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.

Fig. 5: Effects of exogenous supply of GABA, Gln, Glu and Asn on nitrate influx (**A**) and *BnNrt2* gene expression (**B** and **C**) **on** 12h nitrate deprived plants. Plants were supplied with 1mM nitrate plus either 100μM or 1 mM GABA, 1mM Gln, 1mM Glu or 1mM Asn for 12h before measurement of $^{15}NO_3^-$ influx and *BnNrt2* transcript abundance. Influx and *BnNrt2* transcript expression values are the means of **6 and 2** pots of **25** plants **respectively**. Vertical bars indicate ± **SE** of the mean for n=**6** when larger than the symbol.

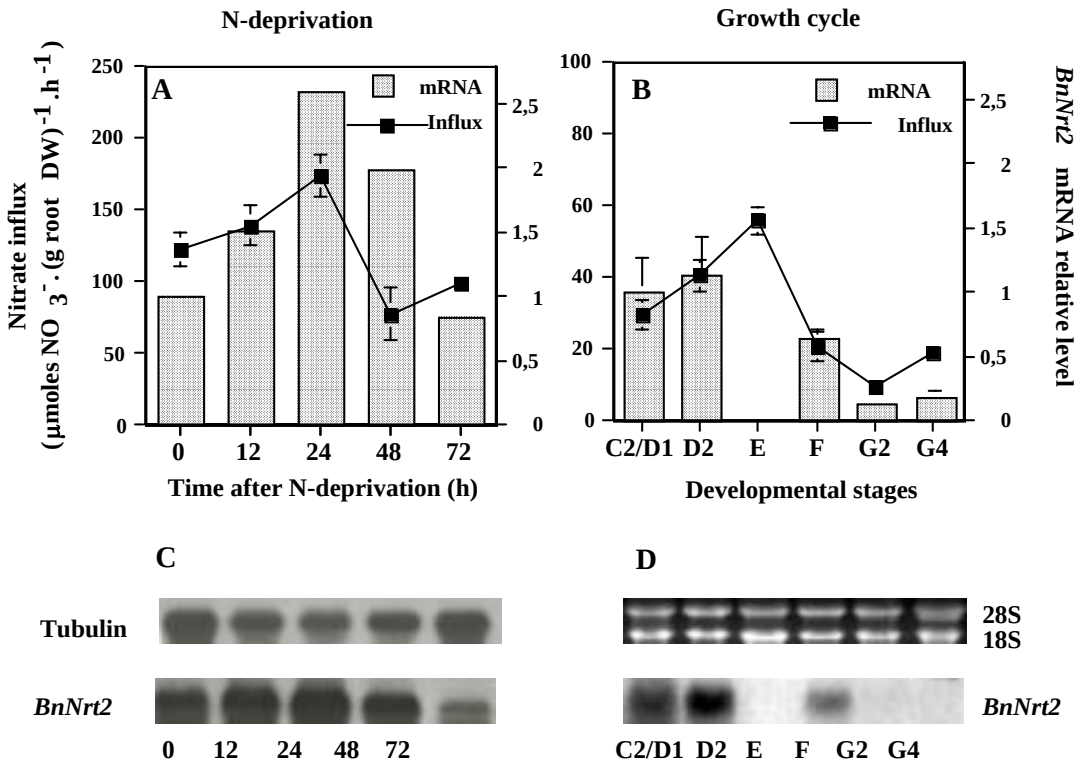
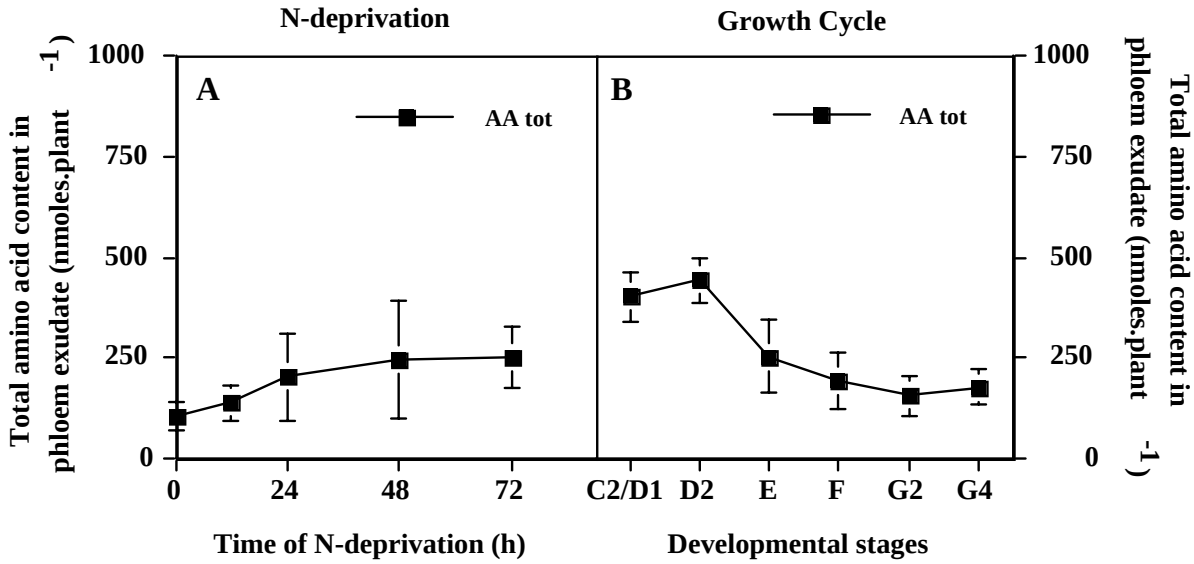


Fig. 1 : Beuve et al . 2004



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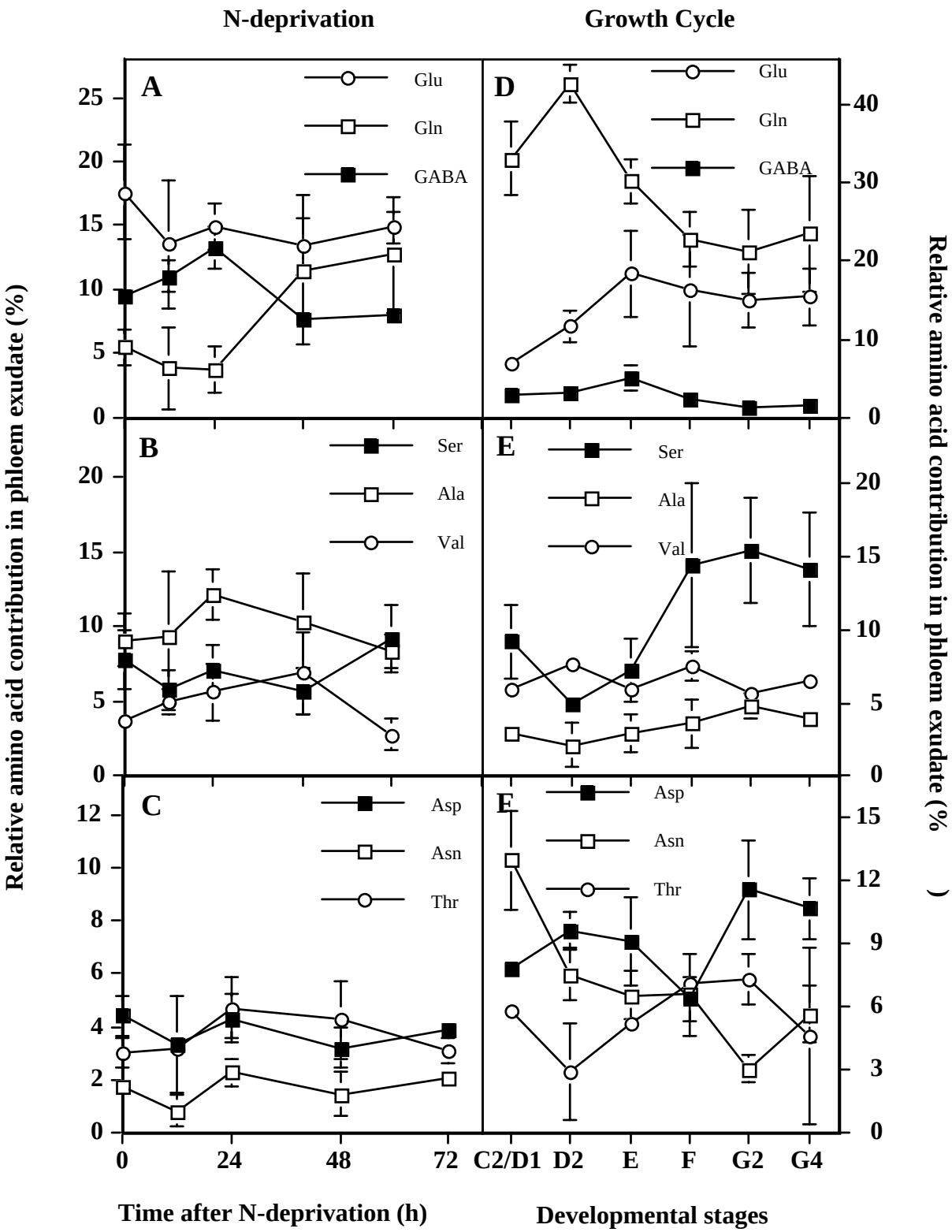

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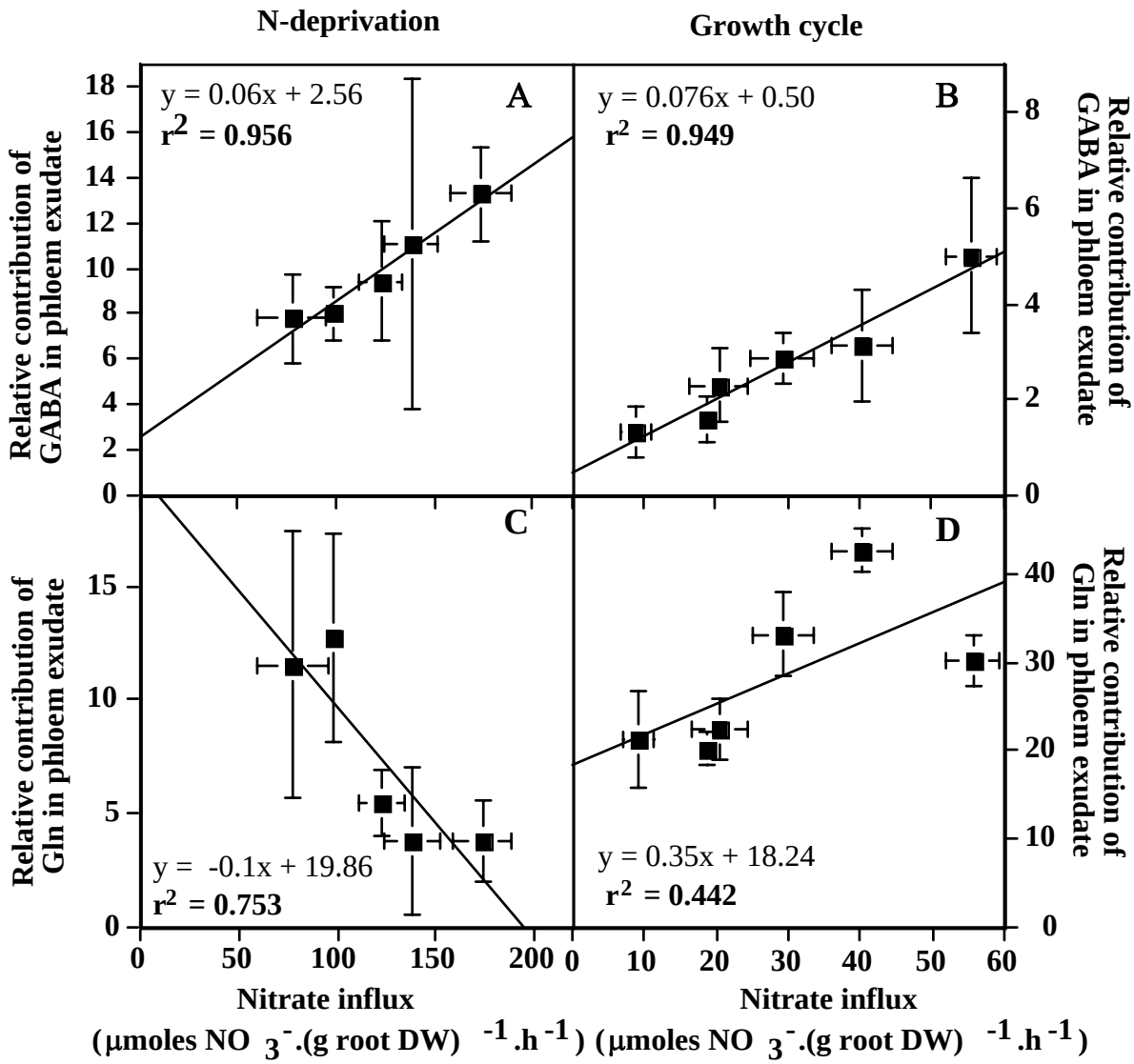
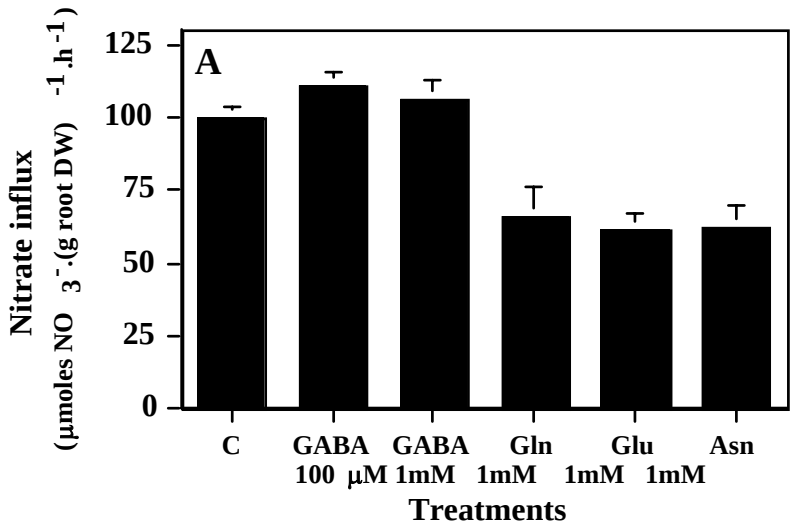
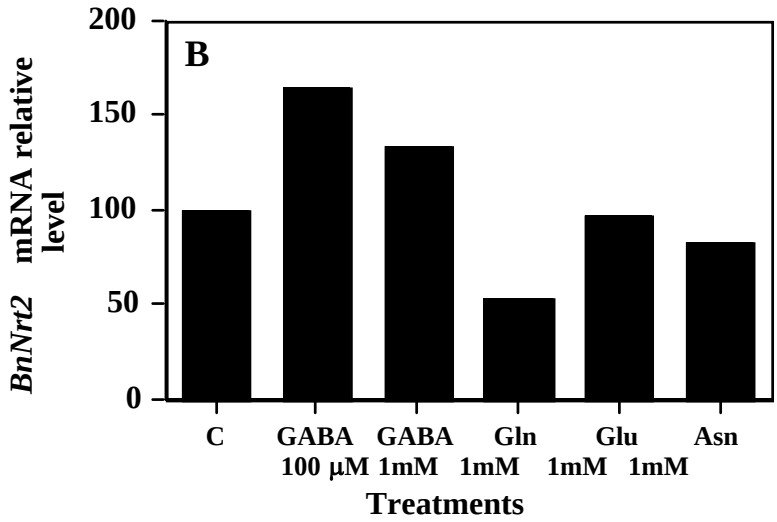


Fig. 4 : Beuve et al. 2004





C. Northern analysis

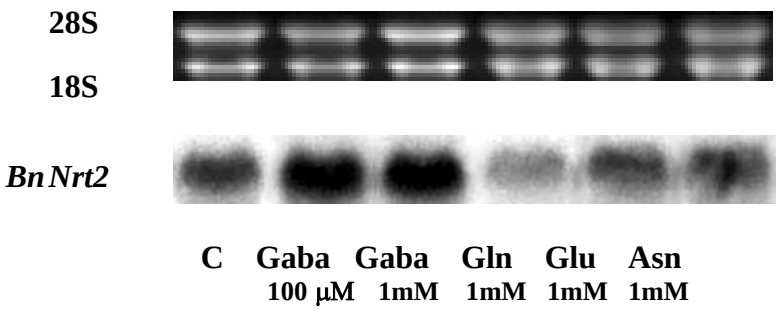


Fig. 5 : Beuve et al. 2004

Table I: Effects of exogenously supplied amino acids on amino acids levels in plant roots

Values shown are the means of three independent replicates ± SD of the mean

Treatment	Amino Acid				
	Asp	Glu	Asn	Gln	GABA
			nmol g fresh wt		
Control	370 ± 139	876 ± 295	327 ± 123	506 ± 168	494 ± 251
100 µM GABA	247 ± 43	694 ± 90	164 ± 40	460 ± 126	308 ± 85
1 mM GABA	344 ± 63	1038 ± 305	231 ± 110	413 ± 167	1105 ± 237
1 mM Gln	379 ± 65	1266 ± 277	323 ± 116	753 ± 277	369 ± 76
1 mM Glu	314 ± 80	887 ± 155	143 ± 28	341 ± 60	361 ± 39
1 mM Asn	369 ± 74	850 ± 192	1684 ± 515	718 ± 240	323 ± 130