

RESEARCH PAPER

# Root-derived cytokinins as long-distance signals for $\text{NO}_3^-$ -induced stimulation of leaf growth

Yuni Sri Rahayu<sup>1,\*</sup>, Pia Walch-Liu<sup>1,†</sup>, Günter Neumann<sup>1</sup>, Volker Römheld<sup>1</sup>, Nikolaus von Wirén<sup>1</sup> and Fritz Bangerth<sup>2</sup>

<sup>1</sup> Institut für Pflanzenernährung (330), Universität Hohenheim, D-70593 Stuttgart, Germany

<sup>2</sup> Institut für Sonderkulturen und Produktionsphysiologie (370), Universität Hohenheim, D-70593 Stuttgart, Germany

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## Abstract

Leaf growth of many plant species shows rapid changes in response to alterations of the form and the level of N supply. In hydroponically-grown tomato (*Lycopersicon esculentum* L.), leaf growth was rapidly stimulated by  $\text{NO}_3^-$  application to  $\text{NH}_4^+$  precultured plants, while  $\text{NH}_4^+$  supply or complete N deprivation to  $\text{NO}_3^-$  precultured plants resulted in a rapid inhibition of leaf growth. Just 10  $\mu\text{M}$   $\text{NO}_3^-$  supply was sufficient to stimulate leaf growth to the same extent as 2 mM. Furthermore, continuous  $\text{NO}_3^-$  supply induced an oscillation of leaf growth rate with a 48 h interval. Since changes in  $\text{NO}_3^-$  levels in the xylem exudate and leaves did not correlate with  $\text{NO}_3^-$ -induced alterations of leaf growth rate, additional signals such as phytohormones may be involved. Levels of a known inhibitor of leaf growth, abscisic acid (ABA), did not consistently correspond to leaf growth rates in wild-type plants. Moreover, leaf growth of the ABA-deficient tomato mutant *flacca* was inhibited by  $\text{NH}_4^+$  without an increase in ABA concentration and was stimulated by  $\text{NO}_3^-$  despite its excessive ethylene production. These findings suggest that neither ABA nor ethylene are directly involved in the effects of N form on leaf growth. However, under all experimental conditions, stimulation of leaf growth by  $\text{NO}_3^-$  was consistently associated with increased concentration of the physiologically active forms of cytokinins, zeatin and zeatin riboside, in the xylem exudate. This indicates a major role for cytokinins as

long-distance signals mediating the shoot response to  $\text{NO}_3^-$  perception in roots.

Key words: ABA, cytokinins, ethylene, *flacca* mutant, hydroponic culture, leaf growth, *Lycopersicon esculentum*,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , N deprivation.

## Introduction

Nitrogen is a major nutrient for plant growth and is taken up by the roots from the soil preferentially as  $\text{NO}_3^-$  or  $\text{NH}_4^+$ . Many plants show growth inhibition when  $\text{NH}_4^+$  is supplied as the sole N source (Marschner, 1995). This  $\text{NH}_4^+$ -induced growth inhibition has been attributed to  $\text{NH}_4^+$  toxicity. However, rapid inhibition (within 12–24 h) of shoot growth by  $\text{NH}_4^+$  was detectable even when  $\text{NH}_4^+$  toxicity was minimized by using moderate  $\text{NH}_4^+$  concentrations (2 mM) in a buffered nutrient solution (Walch-Liu *et al.*, 2000; Rahayu *et al.*, 2001).

Various hormonal factors have been implicated in the regulation of plant growth responses to environmental stimuli: reduced shoot growth is frequently associated with elevated abscisic acid (ABA) concentrations as a result of certain environmental or nutritional stress conditions such as drought, salinity, soil compaction, and deficiencies of N, P, and K (Hartung *et al.*, 1999). Sole  $\text{NH}_4^+$  supply also resulted in increased ABA levels in castor bean (*Ricinus communis*) and pea (*Pisum sativum*) (Peuke *et al.*, 1994; Zdunek and Lips, 2001). The potential role of ABA as an important regulator of shoot growth is further supported

\* Present address: Jurusan Biologi, FMIPA, Universitas Negeri Surabaya, 60231 Surabaya, Indonesia.

† Present address and to whom correspondence should be sent: Biological Sciences, Lancaster University, Lancaster LA1 4YQ, UK. Fax: +44 (0)1524 843 854. E-mail: p.walch-liu@lancaster.ac.uk

Abbreviations: ABA, abscisic acid; DAS, days after sowing; DW, dry weight; FW, fresh weight; i-Ade, iso-pentenyladenine; i-Ado, iso-pentenyladenosine; Z, zeatin; ZR, zeatin riboside.

by inhibitory effects in response to external ABA applications in well-watered plants (Watts *et al.*, 1981; Robertson *et al.*, 1990).

Cytokinins are involved in the regulation of cell division and cell expansion (Ivanova and Rost, 1998; Francis and Sorrell, 2001). Inhibition of shoot growth in tomato (*Lycopersicon esculentum*) and in tobacco (*Nicotiana tabacum*) grown with  $\text{NH}_4^+$  as the sole N source was correlated with a sharp decline in cytokinin concentrations (Z+ZR) (Walch-Liu *et al.*, 2000; Rahayu *et al.*, 2001), a reaction that was rapidly reversible within 12–24 h. Evidence for the involvement of cytokinins in  $\text{NO}_3^-$ -induced shoot growth was presented by Kuiper (1988) and Kuiper and Staal (1987), who showed that exogenous cytokinin application could compensate for  $\text{NO}_3^-$  nutrition and increase the shoot/root ratio and plant growth.

Furthermore, apart from the nutritional role of  $\text{NO}_3^-$ , it has been suggested that  $\text{NO}_3^-$  may function as a signal compound triggering plant growth responses: localized  $\text{NO}_3^-$  supply can induce lateral root elongation, which has been attributed to a direct stimulatory effect of  $\text{NO}_3^-$  (but not  $\text{NH}_4^+$ ) in the external medium (Zhang *et al.*, 1999). Other studies revealed that the expression of various genes involved in the uptake and utilization of  $\text{NO}_3^-$  is triggered by the sensing of  $\text{NO}_3^-$  itself (Crawford, 1995; Forde and Clarkson, 1999; Stitt, 1999; Wang *et al.*, 2000).

A regulatory function of  $\text{NO}_3^-$  was also postulated for leaf growth in tobacco and tomato, since a mixed supply of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  stimulated leaf growth to the same extent as  $\text{NO}_3^-$  application alone and there was rapid growth inhibition within 12–24 h by sole  $\text{NH}_4^+$  nutrition (Walch-Liu *et al.*, 2000; Rahayu *et al.*, 2001). However, it remains to be established whether  $\text{NO}_3^-$  exerts a direct effect on leaf growth or via interaction with cytokinins or other phytohormones, such as abscisic acid (ABA).

In this study, putative components of the postulated signalling chain were characterized by: (i) investigating the concentration-dependency of leaf growth responses to  $\text{NO}_3^-$  as putative primary signal; (ii) comparing time-courses of N form-induced changes in concentrations of  $\text{NO}_3^-$  and hormonal factors (ABA and cytokinins) in xylem exudate and plant tissues with alterations of leaf growth in long- and short-term experiments; and (iii) assessment of ABA effects by comparing N form responses of the ABA-deficient tomato mutant 'flacca' with the isogenic wild-type line.

## Materials and methods

### Plant cultivation

Seeds were germinated on filter paper soaked in 10 mM  $\text{CaSO}_4$  solution and kept at 25 °C in the dark. After about 10 d, the seedlings were transferred to an aerated, hydroponic culture system using 2–4 plants per 3.0 l pot. During the first 24 h, the plants were supplied with saturated  $\text{CaSO}_4$  solution, and thereafter with a full-strength nutrient solution as described by Walch-Liu *et al.* (2000). Nitrogen (N) was applied either as  $\text{KNO}_3$  or  $(\text{NH}_4)_2\text{SO}_4$  at a concentration of 2 mM N.

Substantial N depletion in the nutrient solution was avoided by checking the N concentration in the nutrient solution at least once a day using a RQflex reflectometer (Merck, Darmstadt, Germany) and by adding appropriate N amounts. For  $\text{NH}_4^+$ - and N-deficiency treatments,  $\text{K}_2\text{SO}_4$  was added to compensate for potassium applied in the  $\text{KNO}_3$  variants. The nutrient solution was replaced completely every second day and pH was adjusted between 6.8 and 7.2 by the addition of  $\text{CaCO}_3$  (about 250 mg per pot). To avoid N depletion in treatments with the short-term (24 h) low  $\text{NO}_3^-$  supply [10  $\mu\text{M}$ ], single plants were cultivated in 100 l of nutrient solution, which was replaced after 12 h.

Generally, plants received a  $\text{NO}_3^-$  based preculture [2 mM N] before supplying the various long- and short-term N treatments ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$  or N deprivation) (for details see legends of different experiments). *Lycopersicon esculentum* L. cv. Moneymaker, the ABA-deficient mutant *flacca* (Stubbe, 1959) and the near-isogenic wild-type line (*Lycopersicon esculentum* Mill. cv. Rheinlands Ruhm) were used for the experiments. Plant cultivation was performed in a climate chamber under controlled environmental conditions with a 16/8 h light/dark period (light intensity of 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant level), a 23–25/18–20 °C light/dark temperature regime, and 50% relative humidity.

### Non-destructive measurements of leaf area

Leaf area was determined at the same time of day, 6 h after start of the light period, using light-sensitive paper (Knotzer, Mattersburg, Austria). The terminal leaflet of a young fully unfolded leaf was fixed to the light-sensitive paper with plastic foil. Exposure to light caused a discolouration of the paper not covered by the leaf. The border of the leaf silhouette was marked with a pencil and the leaf area was determined by subsequent digital analysis (Bio-Rad/Quantity One Program, Hercules, California, USA). Leaf growth rate was expressed as  $\text{cm}^2 \text{h}^{-1}$  and obtained by measuring the same leaf twice at different time intervals (2–24 h) as stated in the figures and legends for the different experiments.

### Plant harvest

Generally, harvests were conducted at the same time of day to exclude diurnal variations. For the short-term treatments,  $\text{NH}_4^+$  precultured tomato plants were supplied with  $\text{NO}_3^-$  for 2, 4, 12, and 24 h. After  $\text{NO}_3^-$  preculture, tomato plants were transferred to  $\text{NH}_4^+$  supply or N deprivation for 0, 2, 6, and 12 h. All plants were harvested 6 h after the start of the light period.

At harvest, plants were separated into shoots and roots for fresh weight determination. The same leaf as used for the leaf growth rate measurements was used for further analyses. The plant material was immediately frozen in liquid nitrogen, stored at –20 °C and then lyophilized. Dried samples were homogenized and kept at –20 °C until further analysis.

### Xylem exudate collection and determination of xylem root pressure exudation rate

For collection of xylem exudate, plant shoots were cut 2 cm above the root–shoot interface below the cotyledons. After 5 min, the cut stem was cleaned with deionized water to avoid contamination with the contents of wounded cells and phloem sap and then a silicon tube was fixed over the stem. The xylem exudate driven by root pressure was collected at short intervals with a Pasteur pipette during a 45 min period and immediately stored on ice and subsequently frozen at –20 °C until analyses. Since it was not possible in these experiments to measure the xylem flow rate in intact plants, xylem root pressure exudation rate was calculated by dividing the collected volume of xylem exudate by the collecting time and root fresh weight.

### *NO<sub>3</sub><sup>-</sup> analysis*

Freeze-dried leaves or root material (5–10 mg) were extracted in 1 ml deionized water. The samples were then centrifuged at 16 000 g for 1 min. Nitrate was determined reflectometrically in the supernatant and in the xylem exudate (RQflex reflectometer, Merck, Darmstadt, Germany).

### *Phytohormone analysis*

**Cytokinin and ABA:** Cytokinin and ABA concentrations in the xylem exudate and in freeze-dried leaves and roots (250 mg) were determined essentially as described by Bangerth (1994) and Bertling and Bangerth (1995) with some small modifications. Briefly, after conventional methanol (80%) extraction and evaporation of the extraction medium under vacuum, the redissolved extracts were frozen, thawed, centrifuged, and finally purified on a column combination of PVP, DEAE-Sephadex-A25, and Sep Pak C-18 cartridges. Differential elution of the hormones from this column combination allowed determination and quantification by radioimmuno assay (RIA) as described by Bohner and Bangerth (1988). All extractions and RIA-quantifications were replicated three times.

**Ethylene:** The two youngest fully unfolded leaves were cut off and discarded, the remaining stems including apex and just developing leaves were weighed and then immediately transferred into 50 ml syringes. The syringes were closed with a rubber septum. After incubation for 2 h at room temperature, a 0.5 ml sample was withdrawn from the syringe and analysed for ethylene by gas chromatography using an activated alumina column and a FID detector (GC: Shimadzu, Düsseldorf, Germany).

### *Statistical analysis*

The statistical software Sigma Stat Version 2.03 (SPSS Inc., Chicago, Illinois USA) was used for analyses of variance. Comparisons between the means were conducted using the Student–Newman–Keuls test. Significant differences ( $P < 0.05$ ) among N treatments (and tomato genotypes) were marked with different letters.

## Results

### *Leaf growth as affected by the external NO<sub>3</sub><sup>-</sup> concentration*

Since it was found that the presence of NO<sub>3</sub><sup>-</sup> stimulates leaf growth, the concentration-dependency of NO<sub>3</sub><sup>-</sup> supply on leaf growth was investigated further. The effects of 10 µM and 2 mM NO<sub>3</sub><sup>-</sup> on leaf growth rate, cytokinin status, and plant NO<sub>3</sub><sup>-</sup> levels were compared over 24 h using NH<sub>4</sub><sup>+</sup> precultured plants. Supply of 10 µM NO<sub>3</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>-grown plants was sufficient to increase the leaf growth rate to approximate that of plants grown at 2 mM NO<sub>3</sub><sup>-</sup> (Table 1). The supply of 10 µM NO<sub>3</sub><sup>-</sup> already increased NO<sub>3</sub><sup>-</sup> concentrations in the xylem exudate to 50% of the level measured for plants with 2 mM NO<sub>3</sub><sup>-</sup> supply. However, leaf tissue concentrations reached only 4–5% of the 2 mM NO<sub>3</sub><sup>-</sup> treatment (Fig. 1I).

The Z+ZR concentrations in the plants supplied with 10 µM NO<sub>3</sub><sup>-</sup> also increased compared with NH<sub>4</sub><sup>+</sup>-treated plants, and in leaf and root tissue even reached significantly higher levels than at 2 mM NO<sub>3</sub><sup>-</sup> supply (Fig. 1A, B). While the concentrations of the Z+ZR precursors, i-Ado+i-Ado, in the xylem exudate were similar at 10 µM and 2 mM NO<sub>3</sub><sup>-</sup>

supply, leaf concentrations declined and root concentrations increased in the 10 µM NO<sub>3</sub><sup>-</sup> treatment (Fig. 1D, E, F).

### *N-form-dependent short-term responses of leaf growth, cytokinins, and ABA*

As shown in previous studies with tobacco (Walch-Liu *et al.*, 2000) and tomato (Rahayu *et al.*, 2001), stimulation of leaf growth by NO<sub>3</sub><sup>-</sup> supply and inhibition by the application of NH<sub>4</sub><sup>+</sup> are rapid responses occurring within 12–24 h. Time-courses of hormonal changes and NO<sub>3</sub><sup>-</sup> concentrations in plant tissues and xylem exudate as related to alterations of leaf growth were investigated in detail with two experimental set-ups:

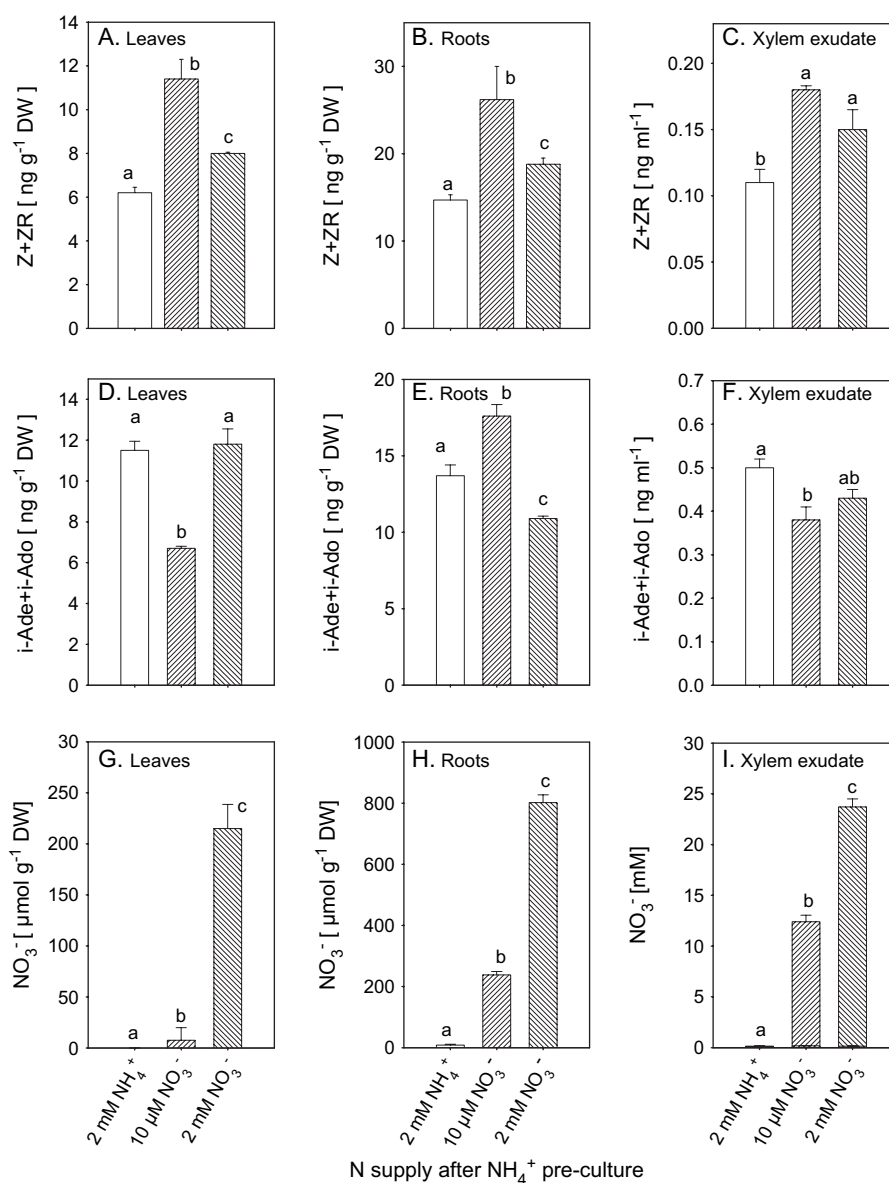
**NO<sub>3</sub><sup>-</sup>-induced stimulation of leaf growth:** Tomato plants precultured in NH<sub>4</sub><sup>+</sup> were transferred to NO<sub>3</sub><sup>-</sup> for 2, 4, 12, and 24 h and then harvested at the same time of day to exclude diurnal variations. A transient increase in leaf growth rate was detectable after 4 h with a peak at 12 h of NO<sub>3</sub><sup>-</sup> supply (Fig. 2A). This was associated with corresponding changes in the Z+ZR cytokinin fraction of leaves and xylem exudate (Fig. 2B). A rapid decline in ABA concentrations of leaves and xylem exudate within 2–4 h after commencing the NO<sub>3</sub><sup>-</sup> treatment preceded the stimulation of leaf growth. Thereafter, ABA levels partially recovered during the next 20 h (Fig. 2C). Nitrate concentrations in the xylem exudate increased within 2 h, reaching a saturation level of 30 mM 4 h after starting the NO<sub>3</sub><sup>-</sup> treatment. In leaf tissue, NO<sub>3</sub><sup>-</sup> concentrations increased continuously for the duration of the 24 h experimental period (Fig. 2D).

**Inhibition of leaf growth in the absence of NO<sub>3</sub><sup>-</sup>:** Tomato plants were exposed to NH<sub>4</sub><sup>+</sup> supply or complete N deprivation for 0, 2, 6, and 12 h after NO<sub>3</sub><sup>-</sup> preculture and harvested at the same time of day to exclude diurnal variations. Inhibition of leaf growth was already detectable for both N treatments 6 h after the withdrawal of NO<sub>3</sub><sup>-</sup> and was particularly obvious under N deprivation (Fig. 3A). The inhibition of leaf growth rate was preceded by declining concentrations of cytokinins (Z+ZR) in the xylem exudate and in young growing leaves, while the hormone levels in older leaves remained unaffected (Fig. 3B). Similar trends were noticeable for ABA concentrations (Fig. 3C). The NO<sub>3</sub><sup>-</sup> concentration in the xylem exudate had

**Table 1.** Short-term effects of NO<sub>3</sub><sup>-</sup> on leaf growth

Different NO<sub>3</sub><sup>-</sup> concentrations were supplied for 24 h to tomato plants precultured on NH<sub>4</sub><sup>+</sup> for 5 d. Leaf growth rate was measured at 29 DAS. Data are mean values and standard error of the means ( $n=4$ ).

N treatment	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	
N concentration	2 mM	10 µM	2 mM
Leaf growth rate (cm <sup>2</sup> h <sup>-1</sup> )	0.14±0.013 a	0.18±0.018 ab	0.20±0.012 b



**Fig. 1.** Short-term effects of  $\text{NO}_3^-$  on cytokinin levels. Different  $\text{NO}_3^-$  concentrations were supplied for 24 h to tomato plants precultured on  $\text{NH}_4^+$  for 5 d. Concentrations of Z+ZR (A–C), i-Ade+i-Ado (D–F), and  $\text{NO}_3^-$  (G–I) in leaves, roots, and xylem exudate were measured at 29 DAS. Data represent mean values and standard error of the means ( $n=4$ ).

already dropped from 14 mM to 0.3 mM 2 h after  $\text{NO}_3^-$  withdrawal from the nutrient solution.

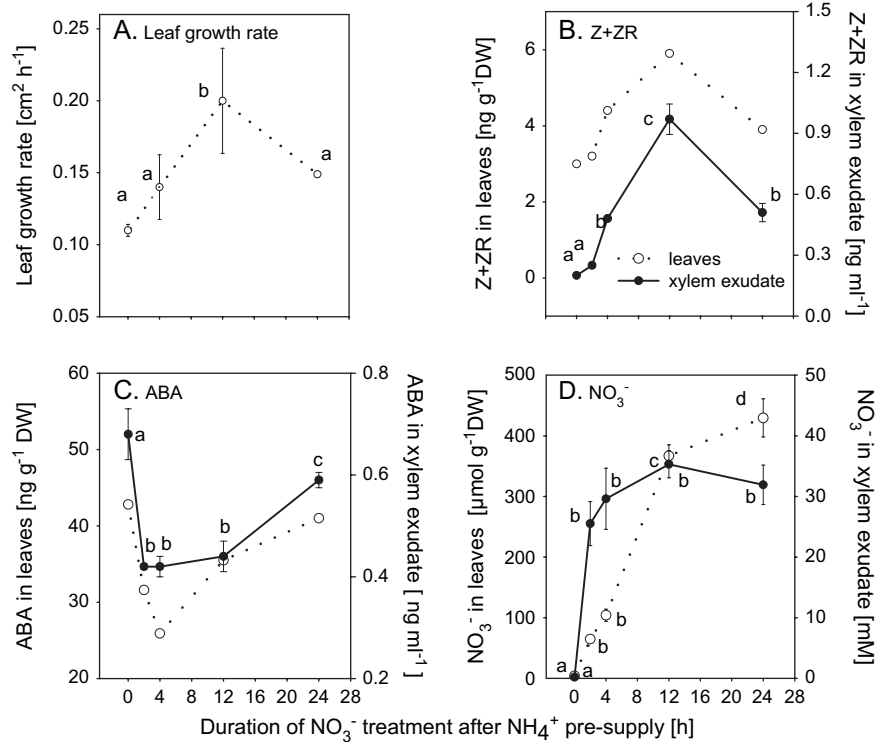
#### *$\text{NO}_3^-$ -induced oscillations in leaf growth and changes in translocation of cytokinins in the xylem*

Nitrate application over prolonged time periods (4–7 d) triggered an oscillation in leaf growth rate with a 48 h interval, which was not detectable in  $\text{NH}_4^+$ -treated plants (Fig. 4A). This oscillation was associated with corresponding changes in Z+ZR levels in the xylem exudate (Fig. 4B), while Z+ZR concentrations in the leaf tissue did not show comparable changes (Fig. 4B). There was no detectable oscillation of ABA concentration in leaves, xylem exudate

or  $\text{NO}_3^-$  concentration in xylem exudates (Fig. 4C, D). The oscillation pattern was also not reflected in the xylem exudation flow rate (Fig. 4E).

#### *$\text{NO}_3^-$ -induced stimulation of leaf growth, xylem translocation of cytokinins, and ethylene production in the ABA-deficient tomato mutant 'flacca'*

The ABA-deficient mutant of tomato (*flacca*) and the near-isogenic wild-type line was used to assess the potential involvement of ABA in the regulation of N-form-dependent responses in leaf growth further. Compared with  $\text{NH}_4^+$  nutrition,  $\text{NO}_3^-$  supply increased plant biomass and leaf area in both the *flacca* mutant and the wild-type



**Fig. 2.** Short-term effects of  $\text{NO}_3^-$  on leaf growth and cytokinin, ABA, and  $\text{NO}_3^-$  levels. Nitrate was supplied at a concentration of 2 mM N and for 2, 4, 12, and 24 h to tomato plants precultured on  $\text{NH}_4^+$  for 3 d. Leaf growth rate (the value at  $t=0$  represents the leaf growth rate [ $\text{cm}^2 \text{h}^{-1}$ ] measured at continuous  $\text{NH}_4^+$  supply in a time interval of 24 h) (A), concentrations of Z+ZR (B), ABA (C), and  $\text{NO}_3^-$  (D) in leaves and xylem exudate were measured at 27 DAS. Data represent mean values and standard error of the means ( $n=4$ ). ABA and Z+ZR concentrations in leaf material represent single measurements obtained from four pooled leaves, since leaf material of single leaves was insufficient for analysis.

line (Fig. 5A, B), although biomass production of the *flacca* mutant was on average about 2.5 times less than the wild type (Fig. 5A, B). Increase in leaf growth of both tomato genotypes coincided with higher levels of Z+ZR in xylem exudate (Fig. 5D), while Z+ZR levels in the leaves were not affected by the N form (Fig. 5C). In wild-type plants, ABA concentrations increased in leaves and xylem exudate in response to  $\text{NH}_4^+$  application, while ABA levels in the *flacca* mutant were not affected by the supply of different N forms and were similar to wild-type plants supplied with  $\text{NO}_3^-$  (Fig. 5E, F). Moreover, the *flacca* mutant exhibited morphological symptoms characteristic of excessive ethylene production, such as curling of the shoot tip and leaf epinasty (results not shown). Accordingly, ethylene evolution originating from stems of the *flacca* mutant was more than 700% of that of the wild-type plants (Table 2). However, ethylene production in both *flacca* mutant and wild type was not affected by the supply of different N forms (Table 2).

#### Xylem root pressure exudation rates

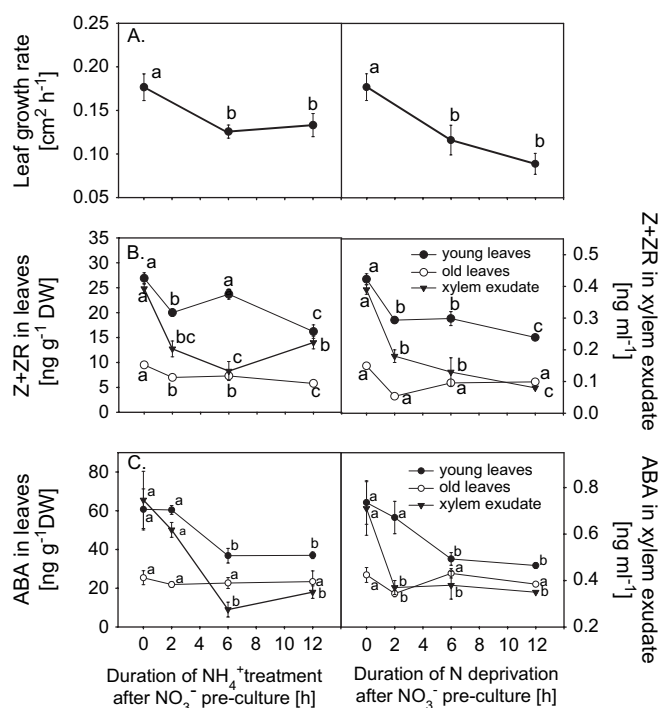
The flow rates of xylem root pressure exudation in short-term experiments were not affected by the different N treatments. Values ranged from an average of  $0.4 \text{ ml h}^{-1} \text{ g}^{-1}$  root FW in the short-term  $\text{NO}_3^-$  treatment (Fig. 2;

except for the continuous  $\text{NH}_4^+$  treatment,  $t=0$ , with  $0.24 \text{ ml h}^{-1} \text{ g}^{-1}$  root FW) to  $0.21 \text{ ml h}^{-1} \text{ g}^{-1}$  root FW for the short-term  $\text{NH}_4^+$  treatment and N-deprivation experiments (Fig. 3). Measurements of xylem root pressure exudation rate, as performed in the present study, do not necessarily represent rates in the intact plant, since the influence of transpiration was not considered. However, in the short-term experiments, changes in leaf area were probably not large enough to exert a significant influence on transpiration. Moreover, Beck and co-worker (Wagner and Beck, 1993; Beck, 1996) showed that cytokinin translocation in the xylem may be fairly independent of xylem water flux. Therefore, the concentration changes of signal compounds in the xylem exudate are likely to reflect real alterations in root-to-shoot translocation.

## Discussion

#### Does $\text{NO}_3^-$ directly regulate leaf growth?

A direct role of  $\text{NO}_3^-$  as a signal molecule has been demonstrated for various physiological and morphological plant responses, such as the stimulation of lateral root elongation in nutrient-rich patches (Zhang *et al.*, 1999) or  $\text{NO}_3^-$ -dependent gene expression of enzymes involved in



**Fig. 3.** Short-term effects of  $\text{NH}_4^+$  and N deprivation on leaf growth and cytokinin, ABA, and  $\text{NO}_3^-$  levels. Tomato plants precultured on  $\text{NO}_3^-$  for 14 d were either supplied with  $\text{NH}_4^+$  at a concentration of 2 mM N or N was completely withheld for 0, 2, 6, and 12 h. Leaf growth rate (A), concentrations of Z+ZR (B), ABA (C), and  $\text{NO}_3^-$  (D) in leaves and xylem exudate were measured at 29 DAS. Data represent mean values and standard error of the means ( $n=4$ ).

N uptake ( $\text{NO}_3^- + \text{NO}_2^-$  transporters) and in N assimilation (glutamine synthetase/glutamate synthase) (Aslam *et al.*, 1993; Redinbaugh and Cambell, 1993). However, in this study, the  $\text{NO}_3^-$ -induced stimulation of leaf growth was not closely related to changes in  $\text{NO}_3^-$  translocation in the xylem exudate or  $\text{NO}_3^-$  concentrations in leaves. While the initially increased leaf growth rates declined again after 12 h of  $\text{NO}_3^-$  supply, the  $\text{NO}_3^-$  concentrations in the leaves continued to increase or reached a saturation level in the xylem exudate after 4 h (Fig. 2A, E). The  $\text{NO}_3^-$ -induced increase in leaf growth also did not depend on a substantial  $\text{NO}_3^-$  accumulation in the leaf tissue: a  $\text{NO}_3^-$  supply of 10  $\mu\text{M}$  stimulated the leaf growth rate comparable to 2 mM  $\text{NO}_3^-$ , although  $\text{NO}_3^-$  concentration in the leaves only reached 4–5% of that of plants supplied with 2 mM  $\text{NO}_3^-$  (Table 1; Fig. 1G). These findings are in contrast with those of Scheible *et al.* (1997), who found a strong correlation between  $\text{NO}_3^-$  concentration in the shoot and shoot/root ratio in tobacco. Furthermore, an oscillation in leaf growth induced by prolonged  $\text{NO}_3^-$  application was not associated with corresponding changes in  $\text{NO}_3^-$  concentrations of the xylem exudate (Fig. 4D).

Although the stimulation of leaf growth by traces of  $\text{NO}_3^-$  in the external medium (10  $\mu\text{M}$ ) (Fig. 1) supports the idea of  $\text{NO}_3^-$  as a ‘signal molecule’, the above data suggest that  $\text{NO}_3^-$  itself may not be the direct trigger in mediating

the N-form-induced changes in leaf growth. However, it cannot be excluded that the presence of  $\text{NO}_3^-$  in the xylem or leaves may be necessary for the function of secondary signals, such as hormonal factors regulating leaf growth *ad loci*.

#### ABA is not involved in the regulation of $\text{NO}_3^-$ -induced stimulation of leaf growth

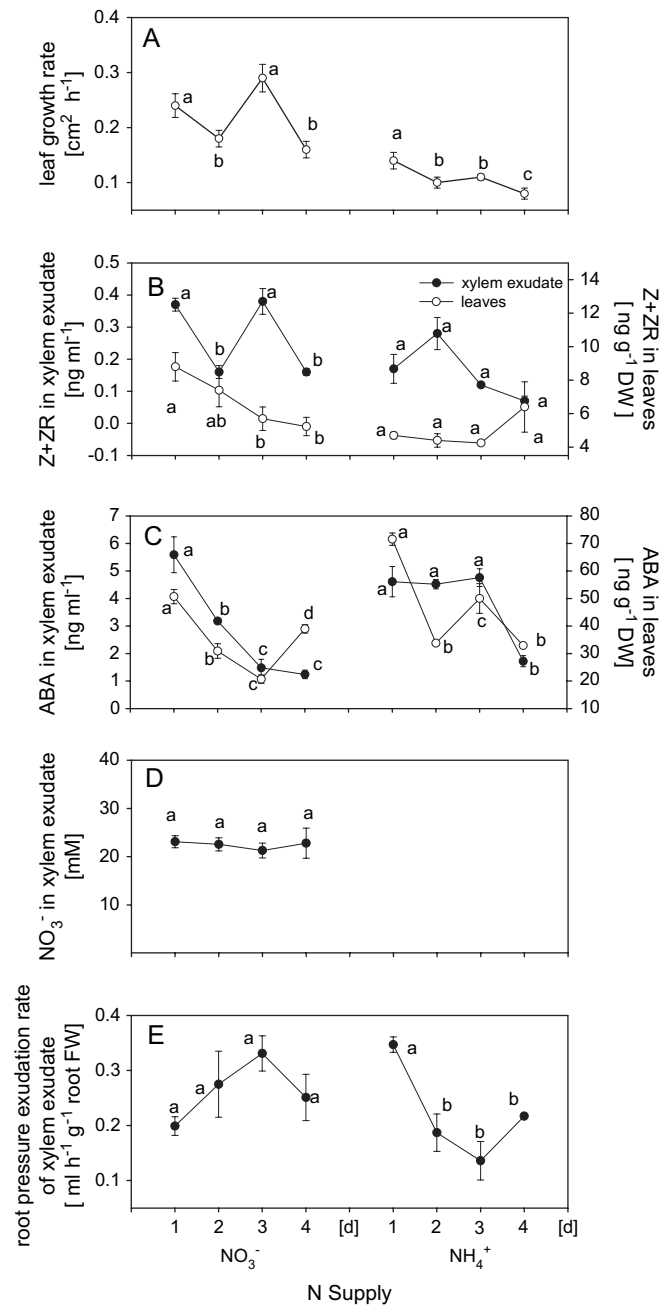
Compared with  $\text{NO}_3^-$  nutrition, long-term  $\text{NH}_4^+$  supply increased the ABA levels in leaves and in the xylem exudate (Fig. 5E, F). This increase coincided with a reduction in plant biomass production (Fig. 5A), which could be mainly attributed to an inhibition of leaf area (Fig. 5B). Similar findings were reported for castor bean (*Ricinus communis*; Peuke *et al.*, 1994) and pea (*Pisum sativum*; Zdunek and Lips, 2001), supplied with  $\text{NH}_4^+$  as the sole nitrogen source. Therefore, it was suggested that increased internal ABA accumulation might be responsible for the inhibition of shoot growth in plants continuously fed with  $\text{NH}_4^+$ . However, in this study, short-term (2–12 h) supply of  $\text{NH}_4^+$  or N deprivation revealed a rapid inhibition of leaf growth in  $\text{NO}_3^-$  precultured plants (within 6 h) (Fig. 3A), which was associated with declining levels of both cytokinins and ABA in the xylem exudate and in the tissue of young leaves (Fig. 3B, C). Thus, the decreased leaf growth rate could not be attributed to an inhibitory effect of ABA accumulation. Also the oscillation of leaf growth rate in plants continuously supplied with  $\text{NO}_3^-$  was not related to corresponding changes in ABA levels in the xylem exudate and leaf tissue (Fig. 4A, C). Moreover, compared with  $\text{NO}_3^-$  supply,  $\text{NH}_4^+$ -nutrition also inhibited leaf growth of the ABA-deficient tomato mutant ‘*flacca*’ showing a low level of residual ABA production, which did not respond to the application of different N forms (Fig. 5B). Furthermore, daily applications of (10  $\text{mg l}^{-1}$ ) ABA completely ‘normalized’ the vegetative phenotype of *flacca* (F Bangerth, unpublished results).

Although exogenous ABA application shows inhibiting effects on shoot growth in some plants (Watts *et al.*, 1981; Robertson *et al.*, 1990), the results of the present study provide strong evidence that ABA is not directly involved in the signalling pathway of leaf growth, as ABA levels do not rapidly respond to the application of different N forms or to N deprivation. Increased ABA levels in plants continuously deprived of N or exclusively supplied with  $\text{NH}_4^+$  may rather reflect a stress response related to metabolic disorders due to prolonged N deprivation or  $\text{NH}_4^+$  supply (Peuke *et al.*, 1994; Palmer *et al.*, 1996; Fig. 5E, F).

#### Ethylene is not involved in regulating $\text{NO}_3^-$ -induced stimulation of leaf growth

Compared with wild-type plants, the *flacca* mutant was characterized by excessive ethylene production (Table 2).



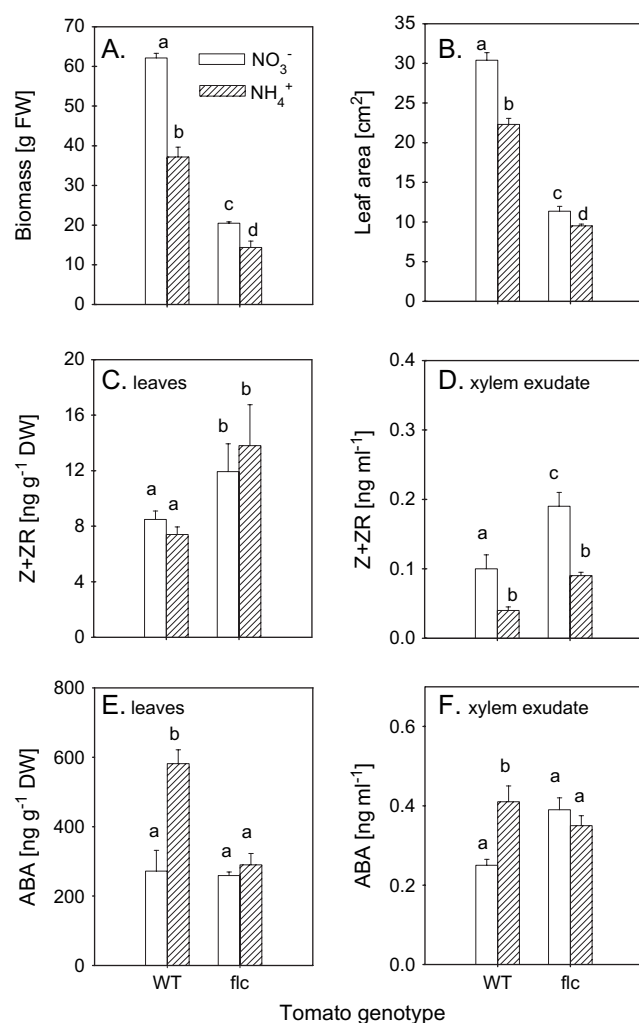


**Fig. 4.** Long-term effects of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  on leaf growth and cytokinin, ABA, and  $\text{NO}_3^-$  levels. Nitrate and  $\text{NH}_4^+$  were supplied to tomato plants at a concentration 2 mM N. Leaf growth rate (A), concentrations of Z+ZR (B), ABA (C), and  $\text{NO}_3^-$  (D) in xylem exudates and root pressure exudation rate (E) were measured at 30–34 DAS. Day 1 indicates the first day of measurements taken after the plants were long-term adapted for 3 d to the exposed N treatments. Data represent mean values and standard error of the means ( $n=4$ ).

Consistent with this finding, it was reported that ethylene production was enhanced in shoots of *flacca* (Tal *et al.*, 1979; Sharp *et al.*, 2000) and in whole plants of an ABA-deficient mutant of *Arabidopsis* (Rakinita *et al.*, 1994) grown under well-watered conditions. In both wild-type plants and in the *flacca* mutant, ethylene production was not affected by the application of different N forms (Table 2).

Nitrate-induced stimulation of shoot growth was observed in the *flacca* mutant in the presence of high ethylene levels, and also in wild-type plants with low ethylene production (Fig. 5B; Table 2).

These findings suggest that, similar to ABA, ethylene is also not directly involved in regulating leaf-growth responses to application of different N-forms.



**Fig. 5.** Long-term effects of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  on plant growth, cytokinin, and ABA levels of the ABA-deficient *flacca* mutant. Nitrate and  $\text{NH}_4^+$  were supplied to tomato plants at a concentration 2 mM N for 8 d. Plant biomass (A), leaf area (B), Z+ZR concentrations in leaves (C) and xylem exudate (D), and ABA concentrations in leaves (E) and xylem exudate (F) of the *flacca* mutant (flc) and the isogenic wild-type line (WT) were measured at 30 DAS. Data represent mean values and standard error of the means ( $n=4$ ).

#### Are cytokinins mediating the $\text{NO}_3^-$ signal in leaf growth?

In accordance with previous studies conducted with tomato and tobacco (Walch-Liu *et al.*, 2000; Rahayu *et al.*, 2001),  $\text{NO}_3^-$ -induced stimulation of leaf growth coincided with elevated Z+ZR levels in the xylem exudate and in the tissue of growing leaves (Figs 1, 2, 4, 5; Table 1). In addition, there are several reports for cotton (*Gossypium hirsutum*), stinging nettle (*Urtica dioica*), and barley (*Hordeum vulgare*) demonstrating that  $\text{NO}_3^-$  supply increased cytokinin levels of previously N-starved plants (Wagner and Beck, 1993; Samuelson and Larsson, 1993; Yong *et al.*, 2000). Similar to the sole  $\text{NH}_4^+$  supply, N deprivation led to a rapid decrease in leaf growth (Fig. 3A, B; Sattelmacher

**Table 2.** Long-term effects of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  on ethylene levels of the ABA-deficient *flacca* mutant

Nitrate and  $\text{NH}_4^+$  were supplied to tomato plants at a concentration 2 mM N for 8 d. Ethylene evolution of the *flacca* mutant and the near-isogenic wild-type line were measured at 30 DAS. Data represent mean values and standard error of the means ( $n=4$ ).

Genotype	Wild-type		<i>flacca</i> mutant	
N treatment	$\text{NO}_3^-$	$\text{NH}_4^+$	$\text{NO}_3^-$	$\text{NH}_4^+$
Ethylene evolution ( $\text{nl g}^{-1} \text{h}^{-1}$ )	$4.7 \pm 0.35$ a	$5.8 \pm 0.30$ a	$36.5 \pm 5.30$ b	$34.5 \pm 0.45$ b

and Marschner, 1978; Palmer *et al.*, 1996) already detectable 6 h after removal of  $\text{NO}_3^-$  from the growth medium (Fig. 3). Inhibition of leaf growth was preceded by a drop in cytokinins in the xylem exudate and in young leaves, which was already detectable 2 h after the start of the N treatments (Fig. 3). Accordingly,  $\text{NO}_3^-$  application resulted in a rapid stimulation of leaf growth within 4 h of supplying  $\text{NO}_3^-$ , and was associated with a corresponding increase in the Z+ZR translocation in the xylem (Fig. 2). The  $\text{NO}_3^-$ -induced increase in leaf growth and Z+ZR concentrations in the plant only required very low external  $\text{NO}_3^-$  concentrations (10  $\mu\text{M}$ ) (Table 1; Fig. 1). Interestingly, Z+ZR concentrations in the leaves of plants supplied with 10  $\mu\text{M}$   $\text{NO}_3^-$  were even higher than with 2 mM  $\text{NO}_3^-$  supply. This increase in Z+ZR concentrations in the leaves could not be attributed to an increased Z+ZR supply by the xylem (Fig. 1A, C). Since the concentration of the Z+ZR precursor, i-Ade+i-Ado, declined in the leaves at 10  $\mu\text{M}$   $\text{NO}_3^-$  supply to the same extent as the Z+ZR levels increased (Fig. 1D), it seems likely that the conversion rates of i-Ade+i-Ado at low  $\text{NO}_3^-$  supply differ from conversion rates at high  $\text{NO}_3^-$  supply.

Prolonged  $\text{NO}_3^-$  application, but not  $\text{NH}_4^+$  supply, induced oscillations in leaf growth rate with an interval of 48 h. These oscillations were associated with corresponding alterations in Z+ZR translocation in the xylem exudate, but not with changes in Z+ZR levels in leaves (Fig. 4A, B). These 48 h oscillations are distinct from the well-known 24 h circadian rhythms and may be determined by the properties of the system rather than by external factors (Volkenstein, 1983). As far as is known, cytokinins have not, to date, been implicated in either diurnal or longer period oscillations. The factors triggering the above fluctuations in Z+ZR in the xylem exudate are unknown and further specific experiments are needed to elucidate the regulatory mechanisms behind this system. Nitrate may act as a constant positive feed-forward signal leading to increased Z+ZR translocation in the xylem. Subsequently, an as yet hypothetical negative feed-back signal may temporarily repress Z+ZR synthesis in the roots and thus Z+ZR in the xylem exudate. Thereafter, the negative feed-back signal is reduced leading to a de-repression of the positive



feed-forward signal etc. A likely candidate for the negative feed-back signal may be IAA via the basipetal IAA-transport pathway. This hormone has been shown repeatedly to suppress cytokinin biosynthesis in roots (Bangerth, 1994; Li *et al.*, 1995) and thus could explain the above changes in cytokinin xylem exudate. Alternatively, this IAA/CK interaction could take place in above-ground tissue (Kaminek *et al.*, 1997; Nordström *et al.*, 2004).

All experiments conducted in this paper indicated a consistent association between Z+ZR translocation in the xylem exudate and leaf growth rate (Figs 1–5; Table 1). Further evidence for an involvement of cytokinins in  $\text{NO}_3^-$ -induced stimulation of leaf growth comes from studies with the direct leaf application of cytokinins, which could mimic the stimulating effect of  $\text{NO}_3^-$  supply. (Kuiper, 1988; Kuiper and Staal, 1987). There is also evidence that  $\text{NO}_3^-$ -inducible genes responded much faster to cytokinin application than to  $\text{NO}_3^-$  (Sakakibara *et al.*, 1998; Taniguchi *et al.*, 1998). These genes encode protein homologues to bacterial two-component regulators, which act as signal transduction intermediates in different signalling pathways. Alterations in xylem translocation of Z+ZR may induce corresponding changes of Z+ZR concentrations in the leaf apoplast. Recently, a cytokinin receptor in plants was reported. This receptor may sense apoplastic cytokinins via an extracellular cytokinin-binding domain (Yamada *et al.*, 2001; Inoue *et al.*, 2001). Downstream of the cytokinin-sensing system, cytokinin-induced response regulators may control the expression of genes which are involved in leaf growth. A role for cytokinins in the regulation of leaf growth by promoting cell expansion (Ulvskov *et al.*, 1992; Downes *et al.*, 2001) and cell division (Soni *et al.*, 1995) were previously reported. To conclude, from this and the present work, it is very likely that cytokinins are involved as a 'mediating hormonal signal' in the  $\text{NO}_3^-$ -induced stimulation of leaf growth.

From an evolutionary point of view, the observed rapid alterations of leaf and shoot growth depending on the form and amount of N supply may be part of the well-known adaptive responses of plant growth to nutrient limitation, characterized by increased root/shoot ratios. Promotion of root growth at the expense of shoot growth by sensing  $\text{NO}_3^-$  concentrations in the rhizosphere could be an advantage for the exploitation of a larger soil volume under conditions of N deficiency, but also in the presence of nutrients with limited mobility in soils, such as  $\text{NH}_4^+$ .

## Conclusion

This study presents further evidence that  $\text{NO}_3^-$  regulates leaf growth via a root-to-shoot signalling cascade. In the roots,  $\text{NO}_3^-$  acts as a stimulator that increases the synthesis of Z+ZR and elevates the rate of the Z+ZR translocation in the xylem, which finally increases the rate of leaf growth. It

seems rather unlikely that  $\text{NO}_3^-$  by itself is the primary signal in the investigated process. Similarly ABA and ethylene are not involved in the  $\text{NO}_3^-$ -induced increase in leaf growth rate. To prove these statements, further investigations have to be conducted including the interaction of  $\text{NO}_3^-$  with other phytohormones in the shoot, characterization at the molecular genetic level, and analysis of mutants and/or transgenic plants with altered expression of the related genes.

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