

# Transition from a maternal to external nitrogen source in maize seedlings<sup>oo</sup>

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**Abstract** Maximizing  $\text{NO}_3^-$  uptake during seedling development is important as it has a major influence on plant growth and yield. However, little is known about the processes leading to, and involved in, the initiation of root  $\text{NO}_3^-$  uptake capacity in developing seedlings. This study examines the physiological processes involved in root  $\text{NO}_3^-$  uptake and metabolism, to gain an understanding of how the  $\text{NO}_3^-$  uptake system responds to meet demand as maize seedlings transition from seed N use to external N capture. The concentrations of seed-derived free amino acids within root and shoot tissues are initially high, but decrease rapidly until stabilizing eight days after imbibition (DAI). Similarly, shoot N %

decreases, but does not stabilize until 12–13 DAI. Following the decrease in free amino acid concentrations, root  $\text{NO}_3^-$  uptake capacity increases until shoot N % stabilizes. The increase in root  $\text{NO}_3^-$  uptake capacity corresponds with a rapid rise in transcript levels of putative  $\text{NO}_3^-$  transporters, *ZmNRT2.1* and *ZmNRT2.2*. The processes underlying the increase in root  $\text{NO}_3^-$  uptake capacity to meet N demand provide an insight into the processes controlling N uptake.

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## INTRODUCTION

Currently over 100 Mt of nitrogen (N) fertilizers are applied to crops each year, globally, to maximize growth and ultimately, yield, of which around 60% is applied to cereals (Heffer 2013). On average, however, cereal crops capture only 40%–50% of the applied N

(Peoples et al. 1995; Sylvester-Bradley and Kindred 2009), with the remainder lost by leaching into groundwater, in surface run-off and volatilization into the atmosphere, all of which impacts considerably on the environment (Vitousek et al. 1997). Improvements in this low N uptake efficiency could greatly reduce the economic and environmental impacts attributable to

### Abbreviations

DAI days after imbibition

DW dry weight

HATS high affinity nitrate transport system

LATS low-affinity nitrate transport system

N nitrogen

$\text{NO}_3^-$  nitrate

NR nitrate reductase

NRT nitrate transporter

RN root nitrogen

ShN shoot nitrogen

SN seed nitrogen

loss of N. One possible means to improve N uptake efficiency is to enhance a plant's capacity to take up nitrate ( $\text{NO}_3^-$ ), as this is the predominant form of N available to plants in most agricultural soils (Wolt 1994; Miller et al. 2007).

Plant  $\text{NO}_3^-$  uptake is mediated by low-affinity (LATS) and high-affinity (HATS) transport systems, which are thought to operate predominately at high or low external  $\text{NO}_3^-$  concentrations, respectively (Glass and Siddiqi 1995; Glass 2003; Glass and Kotur 2013). In *Arabidopsis*, the  $\text{NO}_3^-$  transporters (NRTs, now named NPFs (Léran et al. 2014) AtNPF6.3/NRT1.1 and AtNRT1.2/NPF4.6 have been associated with LATS  $\text{NO}_3^-$  uptake (Huang et al. 1999). However, studies have shown that AtNPF6.3 is a unique  $\text{NO}_3^-$  transporter, as it has the capacity to mediate both low- and high-affinity  $\text{NO}_3^-$  uptake, subject to its phosphorylation state (Ho et al. 2009; Parker and Newstead 2014). However, AtNPF6.3 only plays a very minor contribution to HATS  $\text{NO}_3^-$  uptake (Glass and Kotur 2013). Conversely, AtNRT2.1 and AtNRT2.2 mediate HATS  $\text{NO}_3^-$  uptake; AtNRT2.1 is thought to be responsible for the majority of HATS activity, while AtNRT2.2 makes a smaller contribution (Li et al. 2007). Although the role of NRT2.5 remains to be clarified, it is suggested that this transporter facilitates root HATS  $\text{NO}_3^-$  uptake and re-mobilization in N-starved *Arabidopsis* (Lezhneva et al. 2014). Studies in oilseed rape and maize suggest that, compared to the LATS, the HATS is responsible for much of the  $\text{NO}_3^-$  uptake, even at  $\text{NO}_3^-$  concentrations  $>1\text{ mM}$ , and is also the system that responds to N supply and demand (Malagoli et al. 2004; Garnett et al. 2013).

Studies in *Arabidopsis* have shown that, following a  $\text{NO}_3^-$  starvation period, HATS activity and transcript levels of AtNRT2.1 and AtNRT2.2 increase quickly with  $\text{NO}_3^-$  resupply, but are later repressed with prolonged exposure to sufficient  $\text{NO}_3^-$  (Zhuo et al. 1999; Okamoto et al. 2003). Although  $\text{NO}_3^-$  resupply stimulates  $\text{NO}_3^-$  uptake capacity, the internal accumulation of  $\text{NO}_3^-$  and its assimilatory products, such as amino acids, repress NRT2 transcription (Zhuo et al. 1999; Vidmar et al. 2000) and  $\text{NO}_3^-$  uptake capacity (Muller and Touraine 1992; Tischner 2000). Such observations suggest that a mechanism exists to coordinate  $\text{NO}_3^-$  uptake with plant N demand (Forde 2002). However, in most of these studies, the free N-metabolite of interest was applied exogenously, making it difficult to distinguish between the internal and external effects of these substrates.

Soon after seed imbibition, the N requirements of developing seedlings are met by the hydrolysis of finite seed protein reserves that are then transported into developing coleoptile and roots (Harvey and Oaks 1974; Watt and Cresswell 1987). When this protein reserve is exhausted, seedlings must make the transition to external N capture, in order to meet N demand and to maintain growth. This transition provides an ideal system to dissect the regulation of root  $\text{NO}_3^-$  uptake capacity.

The objective of this study was to improve our understanding of the way that cereals upregulate their  $\text{NO}_3^-$  uptake system in order to meet N demand. To this end, high temporal resolution was used between 3–15 d after imbibition (DAI), in an investigation of the root processes involved in increasing  $\text{NO}_3^-$  uptake and metabolism across the transition from seed N use to external N capture.

## RESULTS

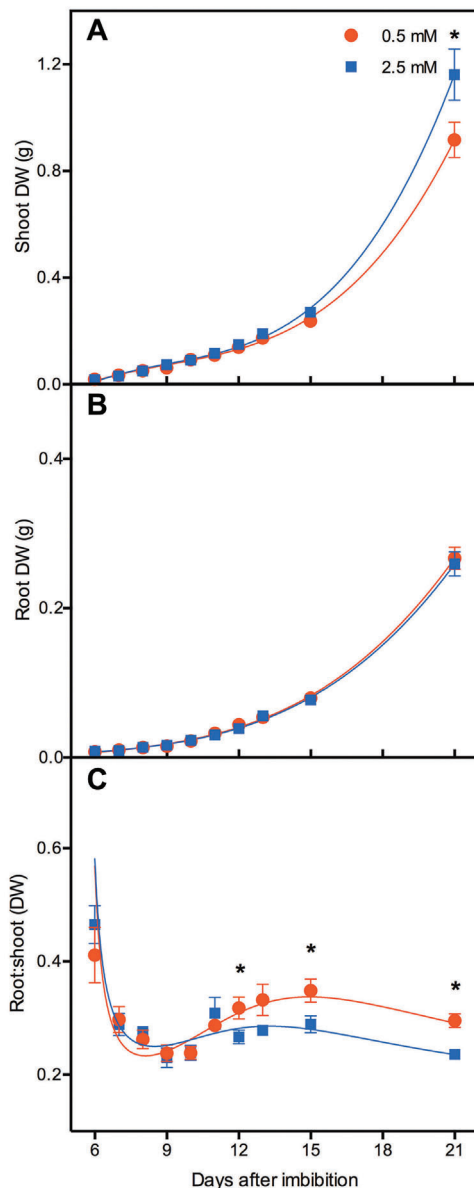
### Seedling growth

On the final sampling day (21 DAI), seedlings grown in low (0.5 mM)  $\text{NO}_3^-$  had produced 18% less shoot dry matter than those in the sufficient  $\text{NO}_3^-$  treatment (2.5 mM) (Figure 1A). External  $\text{NO}_3^-$  supply had no effect on root dry weight (DW) (Figure 1B). Root:shoot ratio was followed throughout seedling development, as changes to biomass allocation, relative to nutrient supply, signify that demand is exceeding supply (Chapin et al. 1987; Ingestad and Agren 1991). During early growth (6–8 DAI), the root:shoot decreased by half for both  $\text{NO}_3^-$  treatments (Figure 1C). Following this, the root:shoot increased, and this ratio increased further in plants grown with 0.5 mM  $\text{NO}_3^-$  compared to those in 2.5 mM  $\text{NO}_3^-$ . Nitrate treatment differences in root:shoot emerged at 12 DAI and carried through to the final harvest.

At 17 DAI, the total root length, surface area and volume did not differ between  $\text{NO}_3^-$  treatments (Figure S1). Similar trends were observed for the length, surface area and volume of axial roots and lateral roots.

### Tissue N

Independent of external  $\text{NO}_3^-$ , seed N reserves depleted by 8 DAI, with the majority depleting 5–8 DAI (Figure 2A). Shoot N% was high initially (8%) across both  $\text{NO}_3^-$  treatments, but rapidly diluted to less than 4% by 9 DAI, and stabilizing by 13 DAI (Figure 2B). Unlike

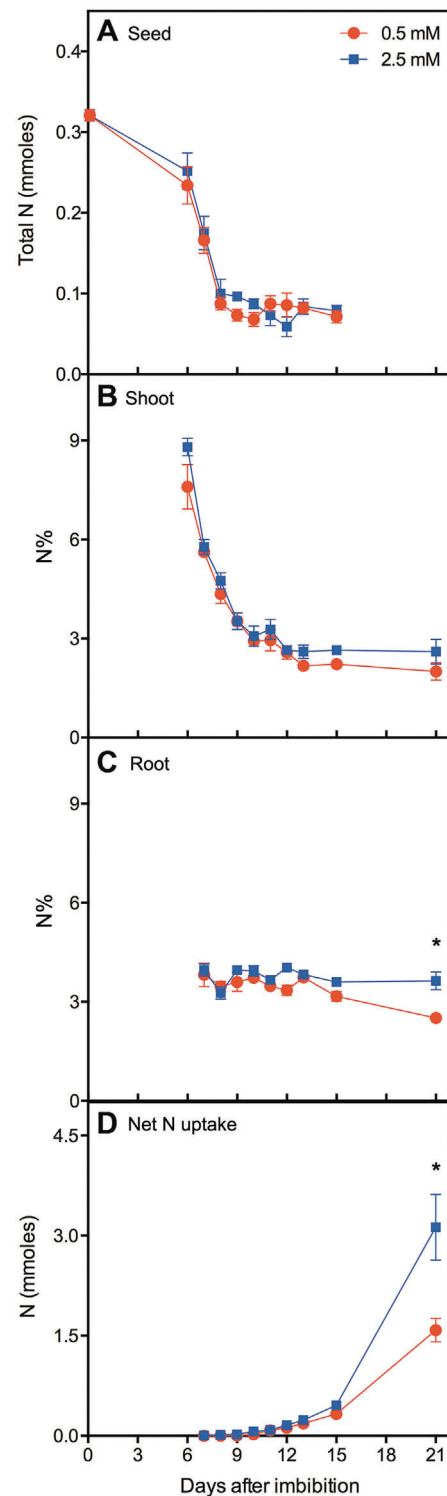


**Figure 1. Growth parameters of *Zea mays* var. B73**

Plants were grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . (A) Shoot dry weight (DW), (B) root DW, and (C) root:shoot were measured until 21 d after imbibition. Cubic polynomial functions were fitted to tissue DWs. Values generated from cubic functions were plotted along with root:shoot (solid lines). Values are means  $\pm$  SEM ( $n = 8$ ). \*Points significantly different between the two growth conditions ( $P < 0.05$ ).

the shoots, root N% was maintained at 4% throughout all sampling days across the 2.5 mM  $\text{NO}_3^-$  treatment, whereas those in the 0.5 mM treatment decreased 15–21 DAI (Figure 2C).

To examine how net N uptake was affected by external  $\text{NO}_3^-$  supply, the net N uptake was calculated



**Figure 2. Nitrogen content in *Zea mays* var. B73 tissue**

Plants were grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$  and (A) total seed N, N% in (B) shoot, and (C) root tissue was measured from dried samples. (D) Net N uptake values were calculated as described in materials and methods. Values are means  $\pm$  SEM ( $n = 4$ ). \*Points significantly different between the two growth conditions ( $P < 0.05$ ).

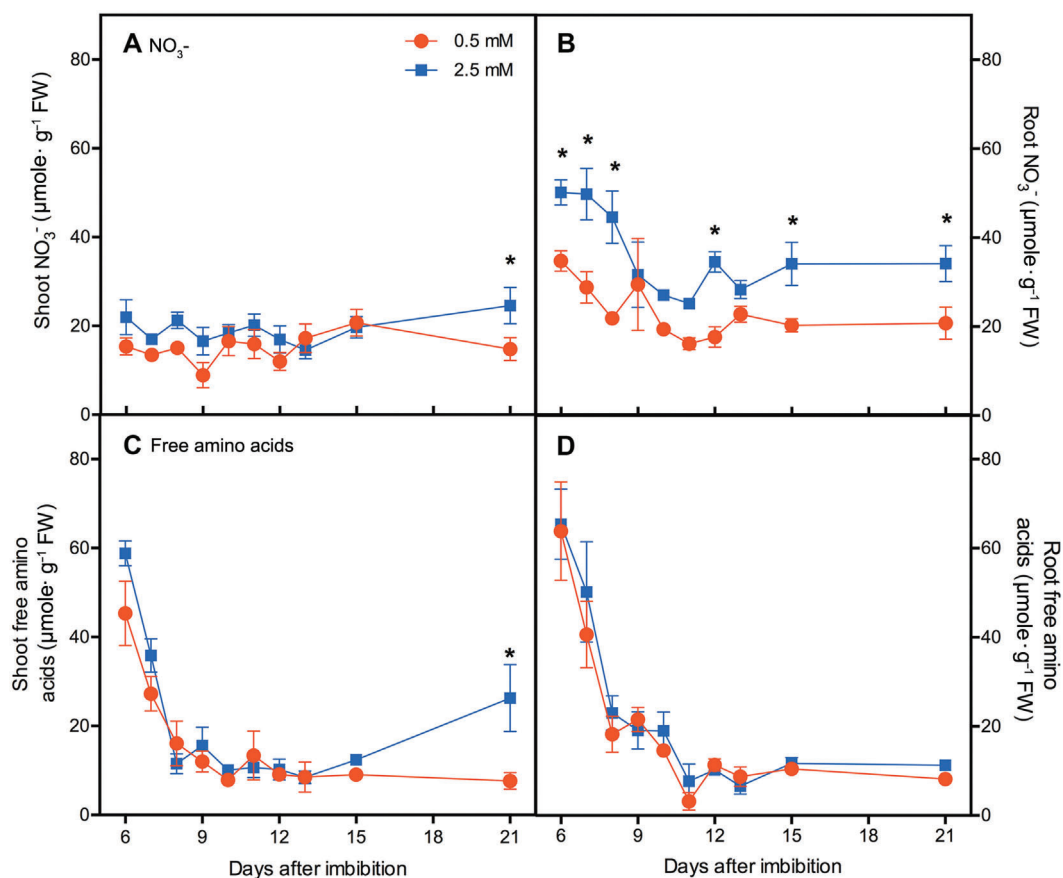
per plant, taking the seed derived N into account. Net N uptake per plant remained at baseline until 9 DAI, where it then began to steadily increase. By 21 DAI, net N uptake was influenced by  $\text{NO}_3^-$  supply, with seedlings in 0.5 mM  $\text{NO}_3^-$  capturing 50% less N than those in the 2.5 mM treatment (Figure 2D).

### Tissue $\text{NO}_3^-$ concentrations

In the 0.5 mM  $\text{NO}_3^-$  treatment, shoot  $\text{NO}_3^-$  concentrations remained stable through to the final sampling day (Figure 3A). In 2.5 mM  $\text{NO}_3^-$ , shoot  $\text{NO}_3^-$  concentrations matched those in 0.5 mM until the final sampling day, when concentrations were higher in 2.5 mM  $\text{NO}_3^-$  than in the 0.5 mM treatment. Across both  $\text{NO}_3^-$  treatments, root  $\text{NO}_3^-$  concentrations generally decreased until 11 DAI (Figure 3B). However, root  $\text{NO}_3^-$  concentrations were generally lower in seedlings grown in 0.5 mM  $\text{NO}_3^-$ , compared to those grown in the 2.5 mM treatment.

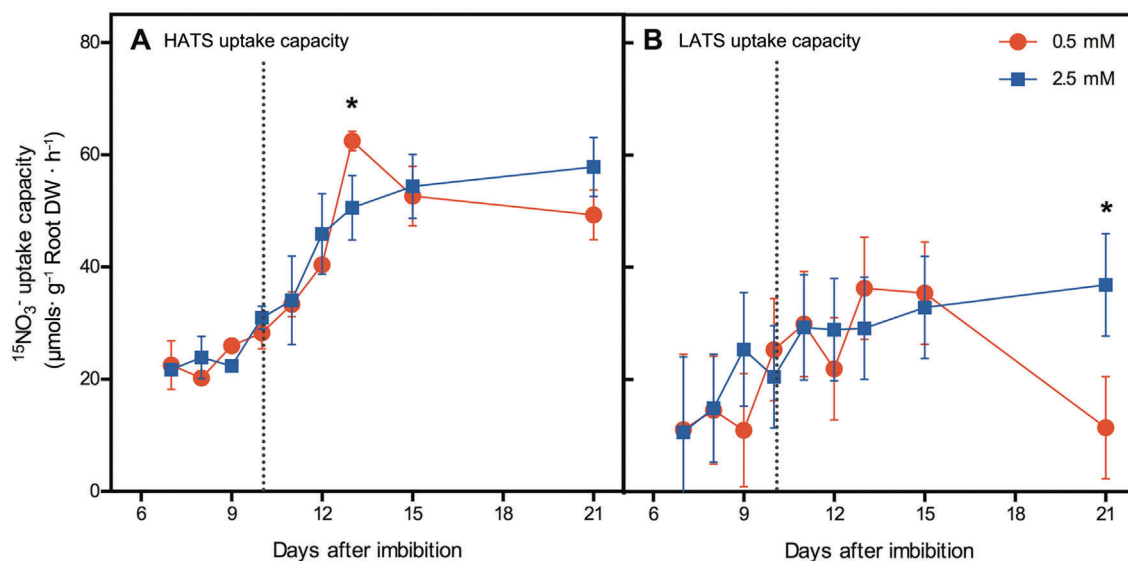
### Free amino acids

In both  $\text{NO}_3^-$  treatments, concentrations of total free amino acids in shoots (Figure 3C) and roots (Figure 3D) decreased rapidly until 8 DAI, and was similarly observed for concentrations of most individual free amino acids (Figure S2). Of the individual free amino acids that were present in high concentrations, asparagine was initially the highest in both shoot and root tissue. In seedlings grown in 0.5 mM  $\text{NO}_3^-$ , concentrations of shoot glycine, glutamine and alanine dropped until 8 DAI, then remained steady until the final sampling day, whereas concentrations in 2.5 mM  $\text{NO}_3^-$  increased 15–21 DAI. Concentrations of root glycine, glutamine and alanine shared a similar trend with root asparagine and did not differ between  $\text{NO}_3^-$  treatments across sampling days. Glutamate concentrations in shoot and root tissue dropped until 8 DAI in both  $\text{NO}_3^-$  treatments, then remained steady; this was not, however, to the same magnitude as the amino acids described above.



**Figure 3.  $\text{NO}_3^-$  and free amino acid concentrations**

Concentrations of (A) shoot  $\text{NO}_3^-$ , (B) root  $\text{NO}_3^-$ , and total free amino acid concentrations in the (C) shoot and (D) root of fresh *Zea mays* var. B73. Plants were grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . Values are means  $\pm$  SEM ( $n = 4$ ). \*Points significantly different between the two growth conditions ( $P < 0.05$ ).



**Figure 4. Root  $\text{NO}_3^-$  uptake capacity in *Zea mays* var. B73 tissue**

(A) HATS and (B) calculated LATS  $\text{NO}_3^-$  uptake capacity of *Zea mays* var. B73 grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . HATS values are means  $\pm$  SEM ( $n = 4$ ), whereas those of LATS are calculated means  $\pm$  SED. Dotted line at 8 DAI represents the time-point when free amino acids concentrations began to stabilize. \*Points significantly different between the two growth conditions (HATS  $P < 0.05$ ; LATS  $\alpha = 0.05$ ).

### Root $\text{NO}_3^-$ uptake capacity

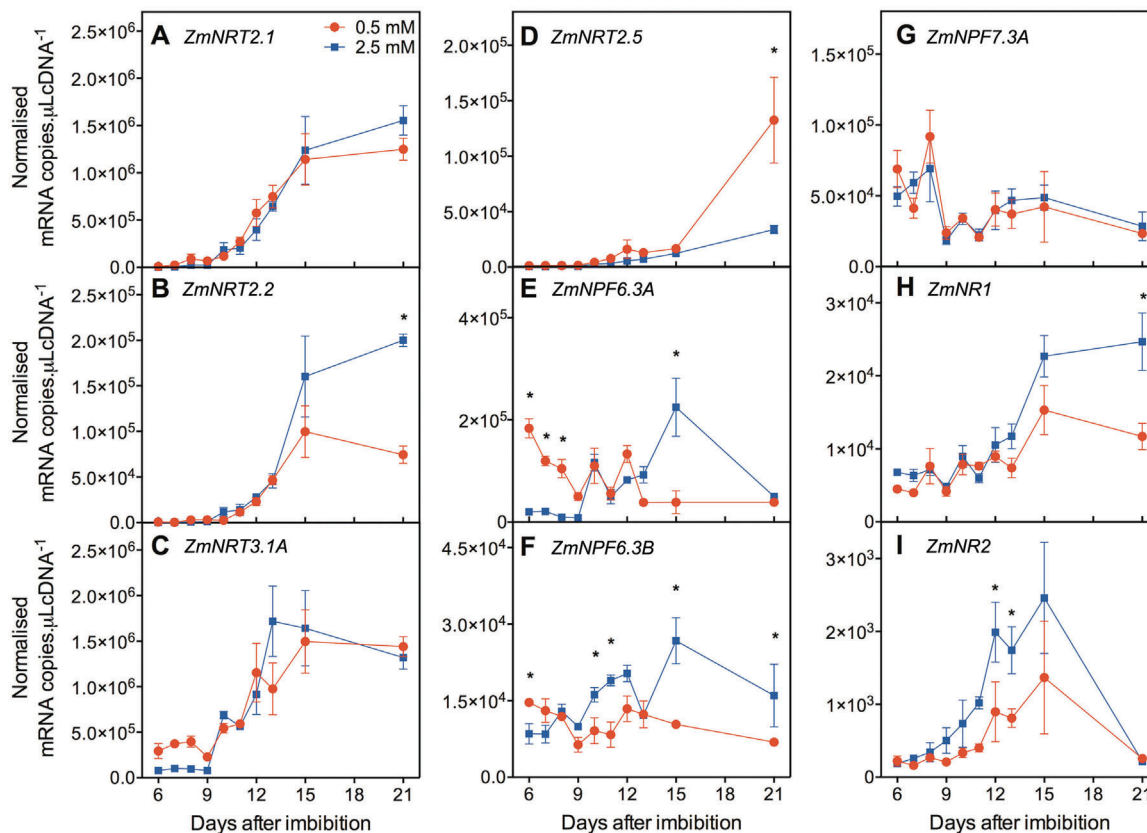
In 2.5 mM  $\text{NO}_3^-$ , HATS  $\text{NO}_3^-$  uptake capacity increased rapidly from 10 DAI, then plateaued from 15 DAI at levels three times higher than baseline activity (Figure 4A). This was similarly observed within seedlings grown in 0.5 mM  $\text{NO}_3^-$ ; however, HATS  $\text{NO}_3^-$  uptake capacity peaked 13 DAI, at levels higher than those in the 2.5 mM treatment, before stabilizing at 15 DAI. Similar trends were observed for HATS  $\text{NO}_3^-$  uptake capacity relative to root surface area for both  $\text{NO}_3^-$  treatments (Figure S3). Mean LATS  $\text{NO}_3^-$  uptake capacity began increasing after 8 DAI (Figure 4B), reaching maximum capacity, double compared to baseline activity, at 12 DAI regardless of  $\text{NO}_3^-$  supply, with peak  $\text{NO}_3^-$  uptake rates being lower than those of the HATS. In 0.5 mM  $\text{NO}_3^-$ , LATS uptake capacity decreased back to baseline activity 15–21 DAI, to half the capacity of the seedlings grown in the 2.5 mM treatment.

### Root $\text{NO}_3^-$ transporter (NRT) gene expression

Transcript levels of genes encoding putative high-affinity  $\text{NO}_3^-$  transporters *ZmNRT2.1* (Figure 5A), *ZmNRT2.2* (Figure 5B), and *ZmNRT3.1A* (Figure 5C) began increasing 10 DAI in both  $\text{NO}_3^-$  treatments. Transcript levels of *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT3.1A* increased until 15 DAI, after which transcript levels

stabilized. *ZmNRT2.2* transcript levels were lower in seedlings grown in 0.5 mM  $\text{NO}_3^-$ , compared to those in the 2.5 mM treatment, with treatment differences emerging at 21 DAI. Similar to *ZmNRT2.1*, transcript levels of *ZmNRT3.1A* increased from 10 DAI and plateaued from 15 DAI, regardless of  $\text{NO}_3^-$  supply. *ZmNRT2.5* transcript levels remained close to zero until 15 DAI, in both  $\text{NO}_3^-$  treatments. Subsequently, transcript levels in 0.5 mM  $\text{NO}_3^-$  grown seedlings increased to levels three times higher than those in the 2.5 mM treatment (Figure 5D).

Transcript levels of *ZmNPF6.3A* were highest during early growth (6 DAI) and generally decreased until 21 DAI in seedlings grown in the 0.5 mM  $\text{NO}_3^-$  treatment (Figure 5E). Conversely, *ZmNPF6.3A* transcript levels in seedlings grown in 2.5 mM  $\text{NO}_3^-$  were lowest 6–9 DAI, then began increasing from 10 DAI, peaking at 15 DAI before decreasing from 15–21 DAI. Transcript levels of *ZmNPF6.3B* in seedlings grown in 0.5 mM  $\text{NO}_3^-$  remained steady across sampling days, whereas those in the 2.5 mM treatment increased from 10 DAI and generally remained higher than those in 0.5 mM  $\text{NO}_3^-$  (Figure 5F). Transcript levels of *ZmNPF7.3A* decreased by more than half 8–9 DAI across both  $\text{NO}_3^-$  treatments, and then remained steady through the remaining sampling days (Figure 5G).



**Figure 5. mRNA transcripts levels in *Zea mays* var. B73 roots**

Transcript levels of (A) *ZmNRT2.1*, (B) *ZmNRT2.2*, (C) *ZmNRT3.1A*, (D) *ZmNRT2.5* (E) *ZmNPF6.3A* (F) *ZmNPF6.3B*, (G) *ZmNRT7.3A*, and *NADH:NR* genes, (H) *ZmNR1* and (I) *ZmNR2* in maize roots (*Zea mays* var. B73). Plants were grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . Each data point is normalized to control genes as described in materials and methods. Values are means  $\pm$  SEM ( $n=4$ ). \*Points significantly different between two growth conditions ( $P < 0.05$ ).

### Root nitrate reductase (NR) gene expression

*ZmNR1* transcript levels were similar in both  $\text{NO}_3^-$  treatments and slowly increased from 13 DAI (Figure 5H). In seedlings grown in 0.5 mM  $\text{NO}_3^-$ , *ZmNR1* transcripts reached maximum levels 15 DAI, whilst those in the 2.5 mM treatment increased to levels twice those of seedlings grown in 0.5 mM  $\text{NO}_3^-$  21 DAI, similar to trends of *ZmNRT2.2* transcript levels.

*ZmNR2* transcript levels followed a trend that was different from *ZmNR1*. In seedlings grown in 0.5 mM  $\text{NO}_3^-$ , *ZmNR2* transcript levels began increasing 10 DAI, reaching peak levels at 15 DAI, before decreasing to reach baseline at 21 DAI (Figure 5I). A similar pattern was observed in seedlings grown in 2.5 mM  $\text{NO}_3^-$ ; however, *ZmNR2* transcript levels began increasing one day earlier, at 9 DAI, and reaching levels higher than those of the 0.5 mM treatment 12–13 DAI, before returning to baseline level 15–21 DAI.

## DISCUSSION

This study exploited the seedlings transition from seed N use to external N capture to investigate the physiological processes, in particular, those within the root, to better understand how  $\text{NO}_3^-$  uptake upregulates to meet plant N demand. During early growth, root:shoot, shoot N% and concentrations of shoot and root free amino acids decreased rapidly until 8 DAI, independent of external  $\text{NO}_3^-$  supply. This is in agreement with [Srivastava et al. \(1976\)](#), and shows that external  $\text{NO}_3^-$  supply had little effect on maize seedling N content whilst growth was being supported by the seed. Although the decreasing shoot N% is due to dilution as a consequence of shoot emergence, it is likely that free amino acid concentrations decrease rapidly due to a combination of dilution and incorporation into newly-synthesized proteins.

Following seed N exhaustion and the dramatic decrease in root:shoot and free amino acid concentrations, root  $\text{NO}_3^-$  uptake capacity increased, reaching maximum rates by 12–13 DAI, when shoot N% stabilised. The rise in HATS  $\text{NO}_3^-$  uptake capacity corresponded with *ZmNRT2.1* and *ZmNRT2.2* transcript levels, suggesting that these NRTs facilitate root HATS  $\text{NO}_3^-$  uptake in maize. Numerous studies have characterized root  $\text{NO}_3^-$  uptake using <7 d old maize seedlings (Neyra and Hageman 1975; Colmer and Bloom 1998; Taylor and Bloom 1998). The results obtained from these former studies should be interpreted in light of the observation here that transcription of  $\text{NO}_3^-$  inducible *NRT2* genes and  $\text{NO}_3^-$  uptake capacity itself had not risen substantially in the developing seedling by this time. The rise in  $\text{NO}_3^-$  uptake capacity and levels of *ZmNRT2.1* and *ZmNRT2.2* transcripts corresponded with a rise in the level of *ZmNRT3.1A* transcripts, strengthening the possible association of *ZmNRT3.1A* with  $\text{NO}_3^-$  uptake in maize, as shown in *Arabidopsis* (Okamoto et al. 2006; Wirth et al. 2007). This suggests that differences in HATS activity profiles, according to the  $\text{NO}_3^-$  treatment, may also be related to reduced *ZmNRT3.1A* transcription, given the role of *NRT3.1* to regulate *NRT2*  $\text{NO}_3^-$  transport activity (Okamoto et al. 2006; Yan et al. 2011; Kotur et al. 2012). Prior to the increase in root  $\text{NO}_3^-$  capture, there was an abundance of root *ZmNPF6.3* transcripts. We hypothesize that this may provide base-level root  $\text{NO}_3^-$  uptake whilst N demand is low; then, as seedling N demand rises and is not met by NPF-mediated  $\text{NO}_3^-$  uptake, *NRT2* transcription and HATS uptake is upregulated.

Given transcript levels of *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A*, *ZmNR1*, *ZmNR2* and  $\text{NO}_3^-$  uptake capacity all increase shortly after free amino acid concentrations decrease, it is likely that this may be the physiological cue triggering the upregulation of the  $\text{NO}_3^-$  uptake system. This is supported by earlier studies which showed that NR activity rises as the products of seed protein hydrolysis deplete (Sivasankar and Oaks 1995), and those suggesting that the repression of *NRT2* transcription and root  $\text{NO}_3^-$  uptake following exogenous amino acids application may be the result of free amino acid accumulation in tissues, in particular glutamine (Zhuo et al. 1999; Vidmar et al. 2000; Nazoa et al. 2003). Indeed, in this study, root  $\text{NO}_3^-$  concentrations also decreased prior to the increase in  $\text{NO}_3^-$  uptake capacity; however, it is not believed that

the observed decrease contributes to the physiological cue, as treatment differences in root  $\text{NO}_3^-$  concentrations were not reflected in the  $\text{NO}_3^-$  uptake capacity. Additionally, shoot  $\text{NO}_3^-$  concentrations remained constant across the transition from seed N use to external N capture, which excludes the involvement of shoot  $\text{NO}_3^-$  concentrations to the cue.

Based on a study investigating N uptake across the maize lifecycle, Garnett et al. (2013) proposed that maize increases HATS  $\text{NO}_3^-$  uptake capacity, to meet N demand. The first response to meet N demand is to utilize any latent  $\text{NO}_3^-$  uptake capacity (existing root  $\text{NO}_3^-$  uptake transporters under post-transcriptional and/or post-translational regulation), followed by an increase in *ZmNRT2.1*, *ZmNRT2.2* transcription. However, in this case, this is the first time young seedlings have increased HATS  $\text{NO}_3^-$  uptake capacity to meet demand, thus not possessing any latent  $\text{NO}_3^-$  uptake capacity to utilize. Consequently, insufficient N was captured by roots to maintain maximal shoot growth rate, reflected in decreased shoot growth and increased root:shoot in low N, relative to sufficient N. Further, the observation that  $\text{NO}_3^-$  treatment differences in root:shoot emerged 12 DAI highlights how early N limitation can impact growth and development in the maize lifecycle.

HATS  $\text{NO}_3^-$  uptake capacity peaked at 13 DAI in 0.5 mM  $\text{NO}_3^-$  grown plants, at levels exceeding those in the 2.5 mM treatment, then began to decrease. We speculate this reflects that plants grown in 0.5 mM  $\text{NO}_3^-$  required greater  $\text{NO}_3^-$  uptake capacity to meet their N demand, and transcript levels of putative  $\text{NO}_3^-$  transporters involved in root  $\text{NO}_3^-$  uptake did not differ because release of post-translational control of uptake capacity facilitating N demand to be met. In this case, the post-translational control may possibly occur by changing the way *ZmNRT2.1* and *ZmNRT3.1A* is assembled in the plasma membrane (Wirth et al. 2007), potentially influencing HATS activity. Similarly, there is evidence of post-translational control after 13 DAI, in that the HATS activity reduces whilst transcript levels of *ZmNRT2.1* and *ZmNRT2.2* continue to increase 13–15 DAI.

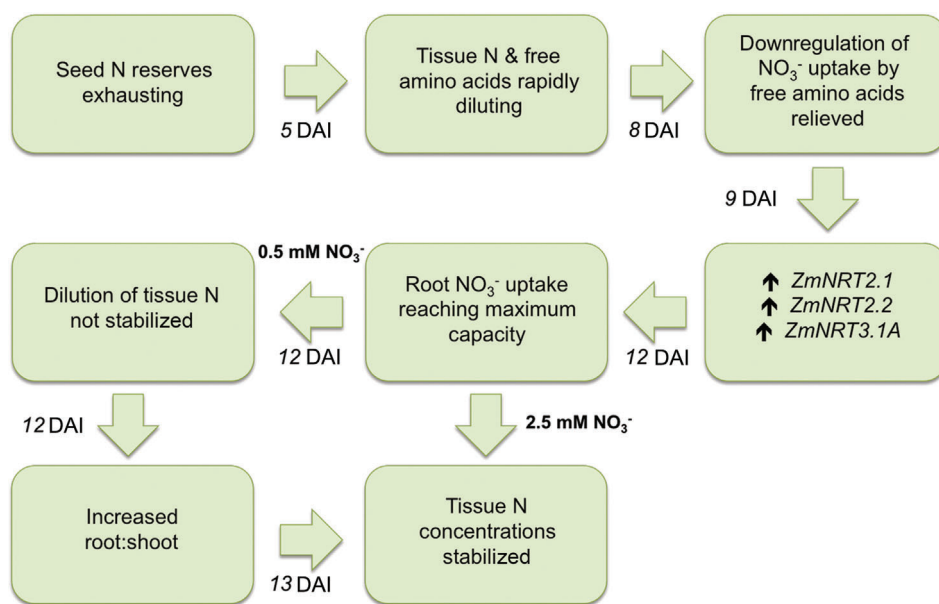
*ZmNRT2.5* was the only *NRT2* that was upregulated in low N, supporting its possible involvement in responses to low N limitation (Garnett et al. 2013, Lezhneva et al. 2014); however, the transcript profile of *ZmNRT2.5* does not correspond with  $\text{NO}_3^-$  uptake capacity. In *Arabidopsis* roots, the expression of *AtNRT2.5* is localized to

the epidermis and cortex of roots at the root hair zone and facilitates HATS  $\text{NO}_3^-$  uptake in N-starved plants (Kotur et al. 2012; Lezhneva et al. 2014). Conversely, the rice NRT2.5 orthologue is mainly expressed in xylem parenchyma cells of the root stele and plays a key role in root to shoot  $\text{NO}_3^-$  transport in low  $\text{NO}_3^-$ , suggesting that it may be the inducible HATS component involved in  $\text{NO}_3^-$  loading into the xylem (Tang et al. 2012). Given the discrepancy between *Arabidopsis* and cereals in terms of the localization and proposed function of NRT2.5, further investigation is required to clarify the role of this protein, at least in cereals.

Transcript profiles of *ZmNPF6.3A*, *ZmNPF6.3B* differ to root  $\text{NO}_3^-$  uptake capacity. *ZmNPF6.3A* transcript levels in plants grown in 2.5 mM  $\text{NO}_3^-$  are greater than those in 0.5 mM  $\text{NO}_3^-$  at 15 DAI, whilst for *ZmNPF6.3B*, these  $\text{NO}_3^-$  treatment difference exist 10–21 DAI. NPF6.3 may be facilitating root to shoot translocation here, as described in *Arabidopsis* by Léran et al. (2013). With greater  $\text{NO}_3^-$  concentrations in root tissue in 2.5 mM  $\text{NO}_3^-$ , relative to 0.5 mM, a greater requirement for root to shoot  $\text{NO}_3^-$  translocation exists, to facilitate assimilation. However, as the role of NPF6.3 remains convoluted (Glass and Kotur 2013), further evidence is required to assume its role in both *Arabidopsis* and cereals.

Apart from  $\text{NO}_3^-$  in the roots, there was little difference in free amino acid or  $\text{NO}_3^-$  tissue concentrations between  $\text{NO}_3^-$  treatments. The root  $\text{NO}_3^-$  concentration being higher in the 2.5 mM  $\text{NO}_3^-$  treatment, but not the shoots, is thought to reflect that higher  $\text{NO}_3^-$  availability, but suggest root to shoot transport of  $\text{NO}_3^-$  is limiting and supports the hypothesis regarding NPF6.3 above. This hypothesis supports that both the shoot-free amino acid and  $\text{NO}_3^-$  concentrations were higher in the 2.5 mM  $\text{NO}_3^-$  treatment only at 21 DAI, suggesting that it was only at that time that supply of N to the shoot was meeting the demand cause by growth.

Despite the differences between  $\text{NO}_3^-$  treatments in net N uptake and growth, the processes leading to and involved in the upregulation of the  $\text{NO}_3^-$  uptake system were found to be similar. Seed N content and free amino acid concentrations decreased rapidly until 8 DAI, followed by an increase in root  $\text{NO}_3^-$  uptake capacity, corresponding with a rise in *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A* transcript levels. Then, as seedlings developed and N demand increased, low N supply had an impact on growth, as reflected in an increase in root:shoot, up to values beyond those for seedlings in sufficient N. This observation may be explained on the basis that during early growth, low N supply may satisfy



**Figure 6. Proposed model**

Proposed model outlining the way that maize (*Zea mays* L.) seedlings manage the transition from seed N use to external  $\text{NO}_3^-$  capture in order to maintain plant N status.



the N demand of seedlings, whereas later, N demand may exceed low supply, suggesting that small differences in N uptake and growth early in growth become magnified over time as a consequence of increasing N demand.

Given the order of the physiological processes involved in  $\text{NO}_3^-$  uptake and assimilation observed, a model is proposed that outlines the key processes managing the transition from seed N use to external N capture (Figure 6). It is believed that this model captures the way in which seedlings manage this transition, thereby maintaining control of plant N status during early vegetative growth, and further describes how this transition can be influenced by  $\text{NO}_3^-$  supply. This model highlights how rapidly the plant's system responds to N supply and demand, even with steady external N supply, which is important when investigating the regulation of these plant processes. Here, *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT3.1A* transcription increased 9–15 DAI, following the decrease in free amino acid concentrations and resulted in a tripling in HATS activity, before reaching maximum capacity. Further investigation into the global transcriptional profiles at time points within this period may lead to the identification of genes following similar/opposite expression patterns to these HATS associated genes, as well as genes that may be upstream of, and, regulating the NRTs. Execution of such an investigation with high-temporal resolution could contribute to systems biology approaches to elucidating the regulation of N uptake, by mapping components involved in N-signalling and N uptake, and the order they may lie within the hierarchy of the signalling network (Krouk et al. 2010; Gutiérrez 2012). Among 0.5 mM  $\text{NO}_3^-$  grown seedlings, changes to root:shoot, relative to those in the 2.5 mM treatment, occurred 12 DAI, when root  $\text{NO}_3^-$  uptake capacity almost reached maximum rates. Although this highlights how early in development N limitation impacts growth; this period offers the opportunity to use global transcription analysis on root and shoot tissue to identify potential regulators of biomass allocation.

The development of such a model in seedlings is important for future research efforts, as it relates well to processes in older maize plants that increase root  $\text{NO}_3^-$  uptake capacity to meet N demand (Garnett et al. 2013). This means that studies investigating the upregulation of  $\text{NO}_3^-$  uptake capacity in steady-state

N conditions, rather than N-starvation, can be explored in cereal crops using young seedlings that are less than 2 weeks old, rather than growing them for longer periods of time, which consumes more time, space and resources. Although the proposed model is generally applicable to maize, the corresponding DAI of transitional steps are likely specific to our particular experiment and may change with environment and plant species. The model may also be applicable to other stages of the plant lifecycle during which root  $\text{NO}_3^-$  uptake capacity increases to meet demand, keeping in mind that any latent  $\text{NO}_3^-$  uptake capacity will preferentially be utilized before *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A* transcription. It is worth investigating how the processes within this study are influenced by seed N content, not only using seeds with differing initial N content, but also excising seed at different time-points during seeding establishment, in order to determine how the quantity of maternal N source influences the transition. Wider ranges of external N supply could also be used to further validate the model, as well as different maize varieties. By understanding how maize upregulates root  $\text{NO}_3^-$  uptake, we identify potential controls/cues (free amino acid concentrations, HATS activity and the associated NRTs). It is anticipated that such controls/cues can become targets for manipulation, in order to increase root  $\text{NO}_3^-$  (e.g., greater free amino acid to protein conversion to lower total free amino acid concentration).

## MATERIALS AND METHODS

### Plant growth

Maize seeds (*Zea mays* L. var. B73) of similar size were imbibed in aerated RO- $\text{H}_2\text{O}$  for 24 h at room temperature, after which they were transferred onto filter paper moistened with 0.5 mM  $\text{CaCl}_2$  (3 d, 26 °C, dark). Germinating seedlings were then transferred to one of eight 120 L ebb and flow hydroponic systems, with a complete fill/drain cycle of 30 min (four separate systems for each  $\text{NO}_3^-$  treatment). Individual seedlings were grown on mesh collars within tubes (300 × 50 mm). This allowed the roots to remain separate from adjacent seedlings while allowing free access to solution. The hydroponic system was situated in a controlled environment room with a day/night cycle of 14 h at 26 °C/10 h at 20 °C, with a flux density at canopy

level of  $650 \mu\text{mol m}^{-2} \text{ s}^{-1}$  and relative humidity of 60%. The nutrient solution was a modified Johnson's solution (Johnson et al. 1957) containing (in mM): 0.5  $\text{NO}_3^- \text{ N}$ , 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P for the 0.5 mM  $\text{NO}_3^-$  treatment, and 2.5  $\text{NO}_3^- \text{ N}$ , 1.8 K, 0.6 Ca, 0.5 Mg, 0.5 S, 0.5 P for the 2.5 mM  $\text{NO}_3^-$  treatment. The choice of  $\text{NO}_3^-$  concentrations was based on those used by Garnett et al. (2013), who suggested that 0.5 mM was the threshold concentration eliciting a major response in the maize  $\text{NO}_3^-$  uptake system. Both treatment solutions also contained (in  $\mu\text{mol/L}$ ): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, and 100 Fe (as FeEDTA and ethylenediamine-N, *N'*-bis(2-hydroxyphenylacetic acid) (FeEDDHA)). Iron was supplemented twice weekly with the addition of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  ( $8 \text{ mg L}^{-1}$ ) to avoid deficiency (Cramer et al. 1994). Solutions were maintained between 19–21 °C using a refrigerated chiller. Solution pH was maintained between 5.8–6.0 and nutrient solutions were changed every 7 d. Concentrations of  $\text{NO}_3^-$  were monitored using a  $\text{NO}_3^-$  electrode (TPS, Springwood, Qld, Australia) and maintained at the target concentration  $\pm 5\%$ . Other nutrients were monitored using an inductively coupled plasma optical emission spectrometer (ICP-OES: ARL 3580 B, ARL, Lausanne, Switzerland) and showed limited depletion between solution changes.

### Measurement of root traits

On sampling days, maize seedling roots were separated from the remainder of the plant, blotted, and weighed to obtain fresh weights (FW). Seedling roots were then scanned as a digital image (Epson Expression 10000XL), and root parameters (length, surface area, volume, average diameter and root tip number) were determined from scanned root images using WinRHIZO Pro root image analysis software (V.2005b, Regent Instruments, Quebec, Canada). Axial roots comprise both primary and seminal roots, whereas lateral roots are defined as the roots developing from axial roots (Figure S4). Lateral roots were differentiated from the axial roots with WinRHIZO, using a distinguishing diameter of 0.677 mm (verified for both  $\text{NO}_3^-$  treatments). The number of axial roots was counted manually from digital images, whereas the lateral root number was calculated by subtracting the number of axial roots from the total number of root tips. Although the number of tips included the points where the root was cut, this was negligible (<2%). The average lateral and

axial root length was calculated by dividing the total length of the respective root type by the total number of that root type. Lateral root density was calculated by dividing the total number of root tips by total axial root length.

### Determination of tissue N and cumulative net N uptake

To determine total tissue N content, sampled plants were blotted and the roots, shoots, and seed were separated and dried (5 d, 60 °C), weighed and ground to a fine powder (Clarkson et al. 1996). The total amount of N within each sample was determined using an isotope mass spectrometer (Sercon, Crewe, Cheshire, UK). Cumulative net N uptake, taking into account the amount of seed-derived N within the plant, was calculated using the formula below, where  $T_n$  denotes the day of sampling and  $T_o$  is the time point before imbibition. Nitrogen in shoots (ShN) and roots (RN) were added to derive total plant N. Subsequently, the cumulative amount of seed N (SN) calculated to be translocated into the seedling ( $T_o-T_n$ ) was subtracted from total plant N:

$$\text{Net N uptake}_{T_n} = (\text{ShN}_{T_n} + \text{RN}_{T_n}) - \text{SN}_{T_o-T_n}$$

### Amino acid analysis

Concentrations of free amino acids in root and shoot tissue were determined using liquid chromatography electrospray ionization-mass spectrometry as described by Boughton et al. (2011).

### Tissue $\text{NO}_3^-$ determination

To extract  $\text{NO}_3^-$  from plant tissue, 20 mg of homogenous finely ground frozen plant tissue was added to 1 mL MQ- $\text{H}_2\text{O}$  and boiled in a water bath (20 min, 95–100 °C). The boiled samples were then cooled and centrifuged (12,000 g, 15 min). For analysis, 50  $\mu\text{L}$  supernatant was added to 200  $\mu\text{L}$  5% w/v salicylic acid in  $\text{H}_2\text{SO}_4$  and incubated (20 min, RT). Then, 125  $\mu\text{L}$  of the mixture containing the sample and 5% w/v salicylic acid in  $\text{H}_2\text{SO}_4$  was added to NaOH (2.375 mL, 2 N) and incubated (20 min, RT). Processed samples were then loaded into 96-flat well plates (200  $\mu\text{L}$ /well) (Greiner Bio-One, Vic, Australia), read at an absorbance of 410 nm (POLARstar Optima, BMG Labtech, Germany), and compared against  $\text{KNO}_3$  standards.

### NO<sub>3</sub><sup>-</sup> uptake capacity measurement

On sampling days, between 11:00 and 13:00 h, plants were transferred to nutrient solutions that matched the growth solutions within the same controlled environment conditions. Roots were then rinsed for 5 min with the same nutrient solution, but with either 100 μM or 1,000 μM NO<sub>3</sub><sup>-</sup>, followed by 10 min of exposure to the same solution, but with <sup>15</sup>N-labelled NO<sub>3</sub><sup>-</sup> (<sup>15</sup>N 10%). The concentration of 100 μM NO<sub>3</sub><sup>-</sup> was used because it is close to saturation of the HATS and 1,000 μM would include activity of both, HATS and some LATS (Siddiqi et al. 1990; Kronzucker et al. 1995a; Crawford and Glass 1998). At the end of the <sup>15</sup>N incubation flux period, roots were rinsed for 2 min in matching, but unlabelled, solutions. Two identical solutions were used for this rinse to allow an initial 5 s rinse to remove labeled solution adhering to the root surface. The flux timing was based on that used by Kronzucker et al. (1995b) and chosen to minimize <sup>15</sup>N efflux back into the solution. Roots were then blotted and separated from shoots, dried (5 d, 60 °C), weighed and ground to a fine powder (Clarkson et al. 1996). The amounts of <sup>15</sup>N in the plant samples were determined using an isotope mass spectrometer (Sercon, Crewe, Cheshire, UK). Nitrate uptake capacity was calculated on the basis of <sup>15</sup>N content in the plant. Mean LATS uptake capacity for a given time-point was calculated by subtracting the mean 100 μM NO<sub>3</sub><sup>-</sup> uptake capacity value from that of 1,000 μM NO<sub>3</sub><sup>-</sup> uptake capacity, at the same time-point and NO<sub>3</sub><sup>-</sup> treatment (Okamoto et al. 2003). Error bars for calculated LATS NO<sub>3</sub><sup>-</sup> uptake capacity plots (1,000 μM – 100 μM) represent the standard error of difference (SED) between NO<sub>3</sub><sup>-</sup> treatments (0.5 mM and 2.5 mM NO<sub>3</sub><sup>-</sup>), which was calculated using the equation below:

$$SED_{1000\ \mu\text{M}-100\ \mu\text{M}} = \sqrt{\sigma^2(1000\ \mu\text{M}) + \sigma^2(100\ \mu\text{M})}$$

$\sigma$  represents the standard error of a mean (SEM) of 100 μM or 1,000 μM NO<sub>3</sub><sup>-</sup> uptake capacity measurement, and is calculated using the below equation:

$$\sigma = \frac{SED(0.5\ \text{mM vs. } 2.5\ \text{mM})}{\sqrt{2}}$$

HATS NO<sub>3</sub><sup>-</sup> uptake capacity was also calculated relative to the root surface area. Root surface area (cm<sup>2</sup>) of dried roots exposed to the <sup>15</sup>N flux was calculated

from the original FWs using the linear relationship between root FW and surface area (Figure S5). Individual regressions were determined for each NO<sub>3</sub><sup>-</sup> treatment.

### Real-time quantitative PCR (Q-PCR)

On sampling days, root material was harvested between 11:00 and 13:00 h (5–7 h after start of light period). Whole roots were excised and snap frozen in liquid N<sub>2</sub> and stored at –80 °C. Homogenous finely-ground frozen root tissue (100 mg) was added to 1 mL TRIzol-like reagent, containing 38% v/v phenol (equilibrated pH 4.3, Sigma-Aldrich, Australia), 11.8% w/v guanidine thiocyanate, 7.6% w/v ammonium thiocyanate, 3.3% v/v sodium acetate (3 M, pH 5), 5% v/v glycerol, and made up to 100% v/v with MQ-H<sub>2</sub>O. Extraction of RNA was performed using the method of Chomczynski (1993). Extracted RNA was DNase treated (Ambion, USA) according to the manufacturer's instructions. The integrity of the RNA was confirmed by 1.2% w/v agarose gel electrophoresis. cDNA synthesis was performed with 1 μg of DNase treated total RNA, using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Q-PCR was carried out as outlined in Burton et al. (2008) and Garnett et al. (2013). Four control genes (*ZmGaPDh*, *ZmActin*, *ZmTubulin* and *ZmEIF1*) were used to calculate the normalisation factor. Primer sequences were the same as those used by Garnett et al. (2013), except for *ZmNPF6.3B* (GRMZM2G161459: forward primer: 5'-GTCATCAGCGCCATCAACCT, reverse primer: 5'-ACGGCAATAGACTCCTCGTC); and two *NADH:NR* genes, *NR1* (GRMZM2G568636: forward primer: 5'-GAGGACCACACGGAGATG, reverse primer: 5'-CCAACGCTGTACTTCCAC) and *NR2* (GRMZM2G428027: forward primer: 5'-GCTTTGGCTAACGAATGT C, reverse primer: 5'-GCTCGCTACTATTACAACAAG) (Long et al. 1992).

### Statistical analyses

Seedlings were grown and selected randomly from four separate hydroponic systems corresponding to NO<sub>3</sub><sup>-</sup> treatment, which constituted blocks. There was no significant difference between blocks. Statistical analysis for calculated LATS NO<sub>3</sub><sup>-</sup> uptake capacity was carried out using a student t-test for two independent means. All other statistical analyses within this study were carried out using two-way analysis of variance (ANOVA).

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## AUTHOR CONTRIBUTIONS

K.S., D.P., T.G. designed and conceived the research. K.S., L.H., J.G. performed the experiments; B.B. and U.R. performed the quantification of the free amino acids; M.T., S.H. contributed to the discussion and editing; K.S., D.P., T.G. wrote the manuscript.

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## SUPPORTING INFORMATION

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### Figure S1. Root morphology

Root morphological parameters of *Zea mays* var. B73 seedlings grown in 0.5 mM (red circles) and 2.5 mM (blue squares)  $\text{NO}_3^-$ . Total (A) number, (B) length, (C) surface area, (D) volume, and (E) average length of axial roots, as well as total (F) number (G) density, (H) length, (I) surface area, (J) volume, and (K) average length of lateral roots were measured over a time course. Values are means  $\pm$  SEM ( $n = 6$ ). \*Points significantly different between the two growth conditions ( $P < 0.05$ ).

### Figure S2. Individual free amino acid concentrations

Individual free amino acid concentrations in fresh shoot and root tissue of maize (*Zea mays* var. B73) grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . Values are means  $\pm$  SEM ( $n = 4$ ). \*Points significantly

different between the two growth conditions ( $P < 0.05$ ).

### Figure S3. Maize (*Zea mays* var. B73) HATS $\text{NO}_3^-$ uptake capacity, relative to root surface area

Seedlings were grown in 0.5 mM (red circles) or 2.5 mM (blue squares)  $\text{NO}_3^-$ . Values are means  $\pm$  SEM ( $n = 4$ ).

\*Points significantly different between the two growth conditions ( $P < 0.05$ ). Dotted line at 8 DAI represents the time point at which concentrations of free amino acids began to stabilize.

### Figure S4. Identification of lateral (LR) and axial roots (Ax) of maize seedlings (*Zea mays* L.)

### Figure S5. Root fresh weight and surface area correlation

Correlations between root fresh weight and root surface area of maize (*Zea mays* var. B73) grown in (A) 0.5 mM  $\text{NO}_3^-$  and (B) 2.5 mM  $\text{NO}_3^-$ . All plants were sampled 7–17 DAI. Each data point represents a value for an individual plant and linear trend lines were fitted to determine each correlation.



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