Transition from a maternal to external nitrogen source in maize seedlings

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Abstract Maximizing NO3− 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 NO3− uptake capacity in developing seedlings. This study examines the physiological processes involved in root NO3− uptake and metabolism, to gain an understanding of how the NO3− 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 NO3− uptake capacity increases until shoot N% stabilizes. The increase in root NO3− uptake capacity corresponds with a rapid rise in transcript levels of putative NO3− transporters, ZmNRT2.1 and ZmNRT2.2. These processes underlying the increase in root NO3− uptake capacity to meet N demand provide an insight into the processes controlling N uptake.

Intromuction 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
NO3− 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 (NO$_3^-$), as this is the predominant form of N available to plants in most agricultural soils (Wolt 1994; Miller et al. 2007).

Plant 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 NO$_3^-$ concentrations, respectively (Glass and Siddiqi 1995; Glass 2003; Glass and Kotur 2013). In Arabidopsis, the 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 NO$_3^-$ uptake (Huang et al. 1999). However, studies have shown that AtNPF6.3 is a unique NO$_3^-$ transporter, as it has the capacity to mediate both low- and high-affinity 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 NO$_3^-$ uptake (Glass and Kotur 2013). Conversely, AtNRT2.1 and AtNRT2.2 mediate HATS 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 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 NO$_3^-$ uptake, even at NO$_3^-$ concentrations >1 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 NO$_3^-$ starvation period, HATS activity and transcript levels of AtNRT2.1 and AtNRT2.2 increase quickly with NO$_3^-$ resupply, but are later repressed with prolonged exposure to sufficient NO$_3^-$ (Zhuo et al. 1999; Okamoto et al. 2003). Although NO$_3^-$ resupply stimulates NO$_3^-$ uptake capacity, the internal accumulation of NO$_3^-$ and its assimilatory products, such as amino acids, repress NRT2 transcription (Zhuo et al. 1999; Vidmar et al. 2000) and NO$_3^-$ uptake capacity (Muller and Touraine 1992; Tischner 2000). Such observations suggest that a mechanism exists to coordinate 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 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 NO$_3^-$ uptake capacity.

The objective of this study was to improve our understanding of the way that cereals upregulate their 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 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) NO$_3^-$ had produced 18% less shoot dry matter than those in the sufficient NO$_3^-$ treatment (2.5 mM) (Figure 1A). External 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 NO$_3^-$ treatments (Figure 1C). Following this, the root:shoot increased, and this ratio increased further in plants grown with 0.5 mM NO$_3^-$ compared to those in 2.5 mM 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 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 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 NO$_3^-$ treatments, but rapidly diluted to less than 4% by 9 DAI, and stabilizing by 13 DAI (Figure 2B). Unlike
the shoots, root N% was maintained at 4% throughout all sampling days across the 2.5 mM 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 NO$_3^-$ supply, the net N uptake was calculated as described in materials and methods. Values are means ± 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 NO\textsubscript{3}\textsuperscript{−} supply, with seedlings in 0.5 mM NO\textsubscript{3}\textsuperscript{−} capturing 50% less N than those in the 2.5 mM treatment (Figure 2D).

**Tissue NO\textsubscript{3}\textsuperscript{−} concentrations**

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

**Free amino acids**

In both NO\textsubscript{3}\textsuperscript{−} 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 NO\textsubscript{3}\textsuperscript{−}, 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 NO\textsubscript{3}\textsuperscript{−} increased 15–21 DAI. Concentrations of root glycine, glutamine and alanine shared a similar trend with root asparagine and did not differ between NO\textsubscript{3}\textsuperscript{−} treatments across sampling days. Glutamate concentrations in shoot and root tissue dropped until 8 DAI in both NO\textsubscript{3}\textsuperscript{−} treatments, then remained steady; this was not, however, to the same magnitude as the amino acids described above.

![Figure 3. NO\textsubscript{3}\textsuperscript{−} and free amino acid concentrations](image)

**Figure 3. NO\textsubscript{3}\textsuperscript{−} and free amino acid concentrations**

Concentrations of (A) shoot NO\textsubscript{3}\textsuperscript{−}, (B) root NO\textsubscript{3}\textsuperscript{−}, 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) NO\textsubscript{3}\textsuperscript{−}. Values are means ± SEM (n = 4). *Points significantly different between the two growth conditions (P < 0.05).
Root NO$_3^-$ uptake capacity
In 2.5 mM NO$_3^-$, HATS 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 NO$_3^-$; however, HATS NO$_3^-$ uptake capacity peaked at 13 DAI, at levels higher than those in the 2.5 mM treatment, before stabilizing at 15 DAI. Similar trends were observed for HATS NO$_3^-$ uptake capacity relative to root surface area for both NO$_3^-$ treatments (Figure S3). Mean LATS NO$_3^-$ uptake capacity began increasing after 8 DAI (Figure 4B), reaching maximum capacity, double compared to baseline activity, at 12 DAI regardless of NO$_3^-$ supply, with peak NO$_3^-$ uptake rates being lower than those of the HATS. In 0.5 mM 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 NO$_3^-$ transporter (NRT) gene expression
Transcript levels of genes encoding putative high-affinity NO$_3^-$ transporters ZmNRT2.1 (Figure 5A), ZmNRT2.2 (Figure 5B), and ZmNRT3.1A (Figure 5C) began increasing 10 DAI in both 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 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 NO$_3^-$ supply. ZmNRT2.5 transcript levels remained close to zero until 15 DAI, in both NO$_3^-$ treatments. Subsequently, transcript levels in 0.5 mM 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 NO$_3^-$ treatment (Figure 5E). Conversely, ZmNPF6.3A transcript levels in seedlings grown in 2.5 mM 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 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 NO$_3^-$ (Figure 5F). Transcript levels of ZmNPF7.3A decreased by more than half 8–9 DAI across both NO$_3^-$ treatments, and then remained steady through the remaining sampling days (Figure 5G).

Figure 4. Root NO$_3^-$ uptake capacity in *Zea mays* var. B73 tissue
(A) HATS and (B) calculated LATS NO$_3^-$ uptake capacity of *Zea mays* var. B73 grown in 0.5 mM (red circles) or 2.5 mM (blue squares) NO$_3^-$ (as). HATS values are means ± SEM (n = 4), whereas those of LATS are calculated means ± SE. 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 α = 0.05).
Root nitrate reductase (NR) gene expression

ZmNR1 transcript levels were similar in both NO$_3^-$ treatments and slowly increased from 13 DAI (Figure 5H). In seedlings grown in 0.5 mM 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 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 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 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 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 NO$_3^-$ supply. This is in agreement with Srivastava et al. (1976), and shows that external 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 NO$_3^-$ uptake capacity increased, reaching maximum rates by 12–13 DAI, when shoot N% stabilised. The rise in HATS NO$_3^-$ uptake capacity corresponded with ZmNRT2.1 and ZmNRT2.2 transcript levels, suggesting that these NRTs facilitate root HATS NO$_3^-$ uptake in maize. Numerous studies have characterized root 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 NO$_3^-$ inducible NRT2 genes and NO$_3^-$ uptake capacity itself had not risen substantially in the developing seedling by this time. The rise in 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 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 NO$_3^-$ treatment, may also be related to reduced ZmNRT3.1A transcription, given the role of NRT3.1 to regulate NRT2 NO$_3^-$ transport activity (Okamoto et al. 2006; Yan et al. 2011; Kotur et al. 2012). Prior to the increase in root NO$_3^-$ capture, there was an abundance of root ZmNPF6.3 transcripts. We hypothesize that this may provide baseline root NO$_3^-$ uptake whilst N demand is low; then, as seedling N demand rises and is not met by NPF-mediated NO$_3^-$ uptake, NRT2 transcription and HATS uptake is upregulated.

Given transcript levels of ZmNRT2.1, ZmNRT2.2, ZmNRT3.1A, ZmNR1, ZmNR2 and 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 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 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 NO$_3^-$ concentrations also decreased prior to the increase in NO$_3^-$ uptake capacity; however, it is not believed that the observed decrease contributes to the physiological cue, as treatment differences in root NO$_3^-$ concentrations were not reflected in the NO$_3^-$ uptake capacity. Additionally, shoot NO$_3^-$ concentrations remained constant across the transition from seed N use to external N capture, which excludes the involvement of shoot 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 NO$_3^-$ uptake capacity, to meet N demand. The first response to meet N demand is to utilize any latent NO$_3^-$ uptake capacity (existing root 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 NO$_3^-$ uptake capacity to meet demand, thus not possessing any latent 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 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 NO$_3^-$ uptake capacity peaked at 13 DAI in 0.5 mM 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 NO$_3^-$ required greater NO$_3^-$ uptake capacity to meet their N demand, and transcript levels of putative NO$_3^-$ transporters involved in root 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 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 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 NO$_3^-$ transport in low NO$_3^-$, suggesting that it may be the inducible HATS component involved in 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 NO$_3^-$ uptake capacity. ZmNPF6.3A transcript levels in plants grown in 2.5 mM NO$_3^-$ are greater than those in 0.5 mM NO$_3^-$ at 15 DAI, whilst for ZmNPF6.3B, these 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 NO$_3^-$ concentrations in root tissue in 2.5 mM NO$_3^-$, relative to 0.5 mM, a greater requirement for root to shoot 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 NO$_3^-$ in the roots, there was little difference in free amino acid or NO$_3^-$ tissue concentrations between NO$_3^-$ treatments. The root NO$_3^-$ concentration being higher in the 2.5 mM NO$_3^-$ treatment, but not the shoots, is thought to reflect that higher NO$_3^-$ availability, but suggest root to shoot transport of NO$_3^-$ is limiting and supports the hypothesis regarding NPF6.3 above. This hypothesis supports that both the shoot-free amino acid and NO$_3^-$ concentrations were higher in the 2.5 mM 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 NO$_3^-$ treatments in net N uptake and growth, the processes leading to and involved in the upregulation of the 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 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 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 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 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 NO$_3^-$ grown seedlings, changes to root:shoot, relative to those in the 2.5 mM treatment, occurred 12 DAI, when root 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 NO$_3^-$ uptake capacity to meet N demand (Garnett et al. 2013). This means that studies investigating the upregulation of 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 NO$_3^-$ uptake capacity increases to meet demand, keeping in mind that any latent 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 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 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-H$_2$O for 24 h at room temperature, after which they were transferred onto filter paper moistened with 0.5 mM 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 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

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level of 650 μmol m⁻² 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 NO₃⁻ N, 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P for the 0.5 mM NO₃⁻ treatment, and 2.5 NO₃⁻ N, 1.8 K, 0.6 Ca, 0.5 Mg, 0.5 S, 0.5 P for the 2.5 mM NO₃⁻ treatment. The choice of NO₃⁻ 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 NO₃⁻ uptake system. Both treatment solutions also contained (in μmol/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 Fe(NH₄)₂(SO₄)₂.6H₂O (8 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 NO₃⁻ were monitored using a NO₃⁻ electrode (TPS, Springwood, Qld, Australia) and maintained at the target concentration ±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 NO₃⁻ 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 Tₘ denotes the day of sampling and T₀ 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 (T₀–Tₘ) was subtracted from total plant N:

$$\text{Net N uptake}_{T_M} = (\text{ShN}_{T_M} + \text{RN}_{T_M}) - \text{SN}_{T_0-T_M}$$

**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 NO₃⁻ determination**

To extract NO₃⁻ from plant tissue, 20 mg of homogenous finely ground frozen plant tissue was added to 1 mL MQ-H₂O 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 μL supernatant was added to 200 μL 5% w/v salicylic acid in H₂SO₄ and incubated (20 min, RT). Then, 125 μL of the mixture containing the sample and 5% w/v salicylic acid in H₂SO₄ was added to NaOH (2.375 mL, 2 N) and incubated (20 min, RT). Processed samples were then loaded into 96-flat well plates (Greiner Bio-One, Vic, Australia), read at an absorbance of 410 nm (POLARStar Optima, BMG Labtech, Germany), and compared against KNO₃ standards.
NO₃⁻ 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 NO₃⁻, followed by 10 min of exposure to the same solution, but with ¹⁵N-labelled NO₃⁻ (¹⁵N 10%). The concentration of 100 μM NO₃⁻ 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 ¹⁵N 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 timing was based on that used by Kronzucker et al. (1995b) and chosen to minimize ¹⁵N 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 ¹⁵N in the plant samples were determined using an isotope mass spectrometer (Sercon, Crewe, Cheshire, UK). Nitrate uptake capacity was calculated on the basis of ¹⁵N content in the plant. Mean LATS uptake capacity for a given time-point was calculated by subtracting the mean 100 μM NO₃⁻ uptake capacity value from that of 1,000 μM NO₃⁻ uptake capacity, at the same time-point and NO₃⁻ treatment (Okamoto et al. 2003). Error bars for calculated LATS NO₃⁻ uptake capacity plots (1,000 μM – 100 μM) represent the standard error of difference (SED) between NO₃⁻ treatments (0.5 mM and 2.5 mM NO₃⁻), which was calculated using the equation below:

\[
\text{SED}_{1000 \mu M-10 \mu M} = \sqrt{\sigma^2(1000 \mu M) + \sigma^2(100 \mu M)}
\]

σ represents the standard error of a mean (SEM) of 100 μM or 1,000 μM NO₃⁻ uptake capacity measurement, and is calculated using the below equation:

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

HATS NO₃⁻ uptake capacity was also calculated relative to the root surface area. Root surface area (cm²) of dried roots exposed to the ¹⁵N 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 NO₃⁻ 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 N₂ 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-H₂O. 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 ZmElf1) were used to calculate the normalisation factor. Primer sequences were the same as those used by Garnett et al. (2013), except for ZmNP6f.3B (GRMZM2G161459: forward primer: 5’-GTCATCAGGGCCATCAAAT, reverse primer: 5’-ACGGCAATAAGACTCTCCGC); and two NADH:NR genes, NR1 (GRMZM2G568636: forward primer: 5’-GAGGACCA-CACGGAGATG, reverse primer: 5’-CCAAGCTGATTTCCAG) and NR2 (GRMZM2G428027: forward primer: 5’-GCTTTGGCTAAGGAATGC, reverse primer: 5’-GCTCGCCTACTATTACAACAG) (Long et al. 1992).

Statistical analyses

Seedlings were grown and selected randomly from four separate hydroponic systems corresponding to NO₃⁻ treatment, which constituted blocks. There was no significant difference between blocks. Statistical analysis for calculated LATS NO₃⁻ 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.

REFERENCES

Johnson CM, Stout PR, Brewer TC, Carlton AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8: 337–353
Upregulation of seedling nitrate uptake capacity


Yan M, Fan X, Feng H, Miller AJ, Shen Q, Xu G (2011) Rice OsSNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. Plant Cell Environ 34: 1360–1372

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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) 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 ± 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) NO$_3^-$. Values are means ± SEM (n = 4). *Points significantly different between the two growth conditions (P < 0.05).

**Figure S3. Maize (*Zea mays var. B73*) HATS NO$_3^-$ uptake capacity, relative to root surface area**
Seedlings were grown in 0.5 mM (red circles) or 2.5 mM (blue squares) NO$_3^-$. Values are means ± 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 NO$_3^-$ and (B) 2.5 mM 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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