The Responses of Maize Roots to Nitrogen Supply

By

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Abstract

Substantial quantities of costly nitrogen (N) fertilisers are applied to cereal crops each year to maximise yields, but only approximately half of the N is captured by cereals, providing scope to increase root N uptake. However, our understanding of how the nitrate (NO_3^-) uptake system is regulated and how it could be improved is limited. Furthermore, the changes to root morphology in response to NO_3^- supply are not well understood, in this case due to the difficulties associated with phenotyping roots in soil.

To investigate how the NO_3^- uptake system is up-regulated, maize (*Zea mays* var. B73 and Mo17) was grown hydroponically with low or sufficient NO_3^- supply, and a range of physiological parameters associated with NO_3^- uptake were measured across the transition from seed N use, to external N capture. This transition provides an ideal system to clarify how the NO_3^- uptake system up-regulates as this is when plants first rely on increasing root N capture to meet demand. Across both lines and treatments, concentrations of shoot N and free amino acids in roots and shoots rapidly decrease as seed N reserves exhaust. Once free amino acid concentrations decrease to a critical level, root NO_3^- uptake capacity rapidly increased, corresponding with a rise in transcript levels of putative NO_3^- transporter genes *ZmNRT2.1* and *ZmNRT2.2*. As NO_3^- uptake capacity reached maximum levels, shoot N concentrations stabilised. Despite shoot N concentrations stabilising, B73 was unable to maintain net N uptake and shoot growth in low NO_3^-, relative to sufficient NO_3^-. Conversely, Mo17 maintained shoot growth and net N uptake, and increased root mass in low NO_3^- relative to sufficient NO_3^-. The effects of NO_3^- limitation on growth were reflected by an increased root:shoot, which emerged just prior to shoot N concentrations stabilising.

In order to understand how root morphology may reflect the NO_3^- treatments differences observed in growth and net N uptake, morphological root traits were quantified

across seedling development. Analysis showed that although B73 achieved greater absorption area per unit root mass than Mo17, its morphology was unresponsive to NO_3^- supply. Conversely, Mo17 responded to NO_3^- limitation by increasing lateral and axial root length before increasing root mass or volume. Subsequently, 11 putative quantitative trait loci (QTL) associated with morphological root traits corresponding with shoot growth or N uptake were detected across low and sufficient NO_3^-, with one major QTL for lateral root length and surface area being identified in low NO_3^- on chromosome 5.

These results provide insight into the processes involved in up-regulating root NO_3^- uptake capacity and how root morphology can adapt to NO_3^- supply. These findings identify potential control points in the regulation of NO_3^- uptake capacity and root morphology, which may be investigated further via global transcriptional analysis or fine-mapping of identified QTL respectively. Ultimately, this work may lead to identification of candidate regulatory genes that could be either manipulated to generate new lines with enhanced N uptake efficiencies, or allow the identification of germplasm with this trait.

List of Abbreviations

ANOVA	analysis of variance
AvgD	average root diameter
AvgLRL	average lateral root length
AvgLRSA	average lateral root surface area
AvgLRV	average lateral root volume
AvgSM	average seed mass
AxR	axial root
B	boron
BLUPs	best linear unbiased predictors
C	carbon
Ca	calcium
CLC	chloride channel
Cu	copper
d	day
DAI	days after imbibition
DW	dry-weight
EDDHA	ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
EDTA	Ethylenediaminetetraacetic acid
Fe	iron
Fig	figure
FW	fresh-weight
Gln	glutamine
Glu	glutamate
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour
ha	hectares
HATS	high-affinity transport system
IBM	intermated B73 x Mo17
IcM	IBM centimorgans
IRIL	intermated recombinant inbred line

K	potassium
kg	kilograms
kg	kilogram
LATS	low-affinity transport system
LOD	logarithm of odds
LR	lateral root
Mg	magnesium
min	minute
Mn	manganese
Mo	molybdenum
MQ	milli-Q
Mt	megatonne
N	nitrogen
NAOH	sodium hydroxide
NAR	nitrate assimilation related
NH_4^+	ammonium
NiR	nitrite reductase
NO_2^-	nitrite
NO_3^-	nitrate
NPF	nitrate transporter 1/peptide transporter family
NR	nitrate reductase
NRT	nitrate transporter
NRT	nitrate transporter
NUE	nitrogen use efficiency
NUpE	nitrogen uptake efficiency
NUtE	nitrogen utilisation efficiency
P	phosphorous
PTR	peptide transporter
Q-PCR	quantitative polymerase chain reaction

Chapter 1: Literature review

1.1 Cereal crop production

Cereal crops are a major staple food worldwide, directly contributing over 50 % of the total human daily calorie intake (Hawkesford, 2013). Among the cereals, maize (*Zea mays* L.) is one of the most widely cultivated globally (Coudert *et al.*, 2010), having approximately 875 Mt produced globally in 2012; with the United States being the largest producer (FAOSTAT, 2014). However, it is projected that a 50 – 70 % increase in cereal production will be required by the year 2050 to feed an estimated population of 9.3 billion people (Umar and Abrol, 2011). This includes an increased requirement for maize, given the vast use of maize as animal feed (FAO, 2013). Further, as the demand for crop production rises, so does the challenge facing agriculture to continue increasing yields that are both environmentally and economically sustainable (Tilman *et al.*, 2002).

Globally, only approximately 3 billion of the 13.4 billion hectares of land is suitable for crop production (Bruinsma, 2003), with more than half of this already being used for cultivation (Smith *et al.*, 2010). Indeed, it could be suggested to simply grow more crops to alleviate this agricultural pressure, however this would not be sustainable long-term. This is because increasing cropping density per unit land may increase competition between crops for light, water and nutrients, ultimately increasing the effects of any abiotic stresses (Troyer, 1996), whilst using more arable land will generate further competition for land by other human activities (Godfray *et al.*, 2010). Consequently, the majority of the future demand for crops is projected to be met by increasing yields, rather than cultivation on new land, or higher cropping density (Bruinsma, 2003; Gregory and George, 2011).

1.2 Plant nitrogen (N) nutrition

Amongst the panel of mineral nutrients required for plant growth, nitrogen (N) is required in the greatest amounts, accounting for about 1 – 5 % of the total plant dry matter and is an integral component of proteins, nucleic acids, chlorophyll, co-enzymes and phytohormones (Marschner, 2012). Although some plant species, like legumes, have the capacity to utilise atmospheric N_2 via a developed symbiotic relationship with N_2-fixing bacteria (Mylona *et al.*, 1995), most agricultural species (including maize) rely on acquiring other N forms that exist in the soil medium, thus limiting growth when supply to roots is limited.

1.3 N in soils

In most agricultural soils where cereals are grown, the predominant forms of inorganic N are ammonium (NH_4^+) and nitrate (NO_3^-) (Wolt, 1994). Generally, the concentration of NH_4^+ is 10 % of the NO_3^- concentration in these soils (Wolt, 1994), due to the rapid conversion of applied N fertilisers to NO_3^-, which is facilitated by soil microbes (Haynes, 1986). Compared to immobile resources, such a phosphorous (P), N is relatively mobile in the soil medium (Barber, 1995; Tinker and Nye, 2000). However, NO_3^- is much more mobile than NH_4^+ as it moves freely with soil water, whereas NH_4^+ readily binds to negatively charged soil particles (Barber, 1995; Tinker and Nye, 2000). This makes NO_3^- more 'available' to plant roots; however this is highly dependent on the soil water status.

1.4 N fertiliser use

Vast quantities of N fertilisers are applied to agricultural soils each year to maximise N availability, in order to increase harvest quality and yield (Tilman *et al.*, 2002). However, crop producers are facing increasing economic pressure as the cost of N fertilisers rise (Fig. 1A), primarily caused by the rise in the price of fossil fuels required for its production (Rothstein, 2007). Regardless, the global fertiliser consumption continues to rise, and its use over the past

four decades has contributed to a marked increase in food production and decrease in world hunger, despite the global population having doubled (Godfray *et al.*, 2010). Although agricultural food production has increased three-fold over the past four decades, N fertiliser use has increased nearly ten-fold (Fig. 1B) (FAOSTAT, 2014), highlighting inefficiencies in the use of this fertiliser. In addition to the economic consequence associated with excessive N fertiliser use, greenhouse gas emissions are derived from its production (Ahlgren *et al.*, 2008), and eutrophication of water bodies, result from the leaching of residual N in soils, also highlighting its major negative impacts on the environment (Vitousek *et al.*, 1997).

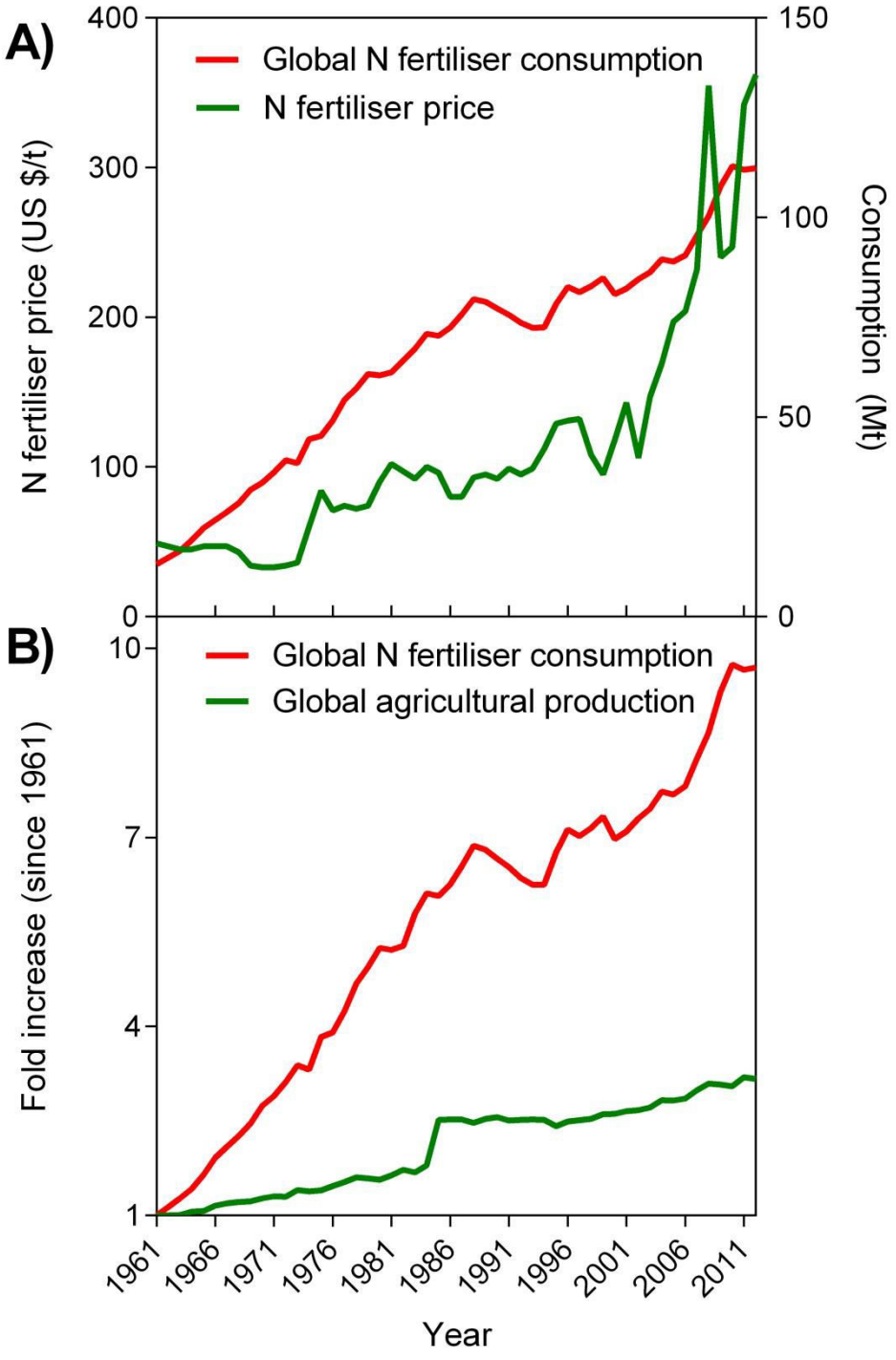

Fig. 1 (A) Nitrogen fertiliser price and global N consumption since 1961. **(B)** Fold-increase in global N fertiliser consumption and agricultural production since 1961. Data for N fertiliser prices sourced from USDA (2014). Data for global N consumption and agricultural production sourced from FAOSTAT (2014).

1.5 Cereal N use efficiency

Nitrogen use efficiency (NUE) can be described as the measure of how applied N is utilised by the crop, and although numerous definitions for NUE exist, here it is defined as the total N in the plant, per unit available N (fertilisation input + residual soil N) (Moll *et al.*, 1982). Although crop yields are responsive to N fertiliser application, this is not a linear relationship and excessive fertiliser use often results in decreased NUE and increased N losses (Fig. 2; Hawkesford, 2013). On a global scale, cereal crops only capture 40 – 50 % of the applied N, highlighting that their poor NUE is greatly contributed by poor N uptake (Peoples *et al.*, 1995; Sylvester-Bradley and Kindred, 2009). This inefficiency of N uptake by cereal crops, combined with the growing demand of N fertilisers provides considerable scope to improve cereal NUE.

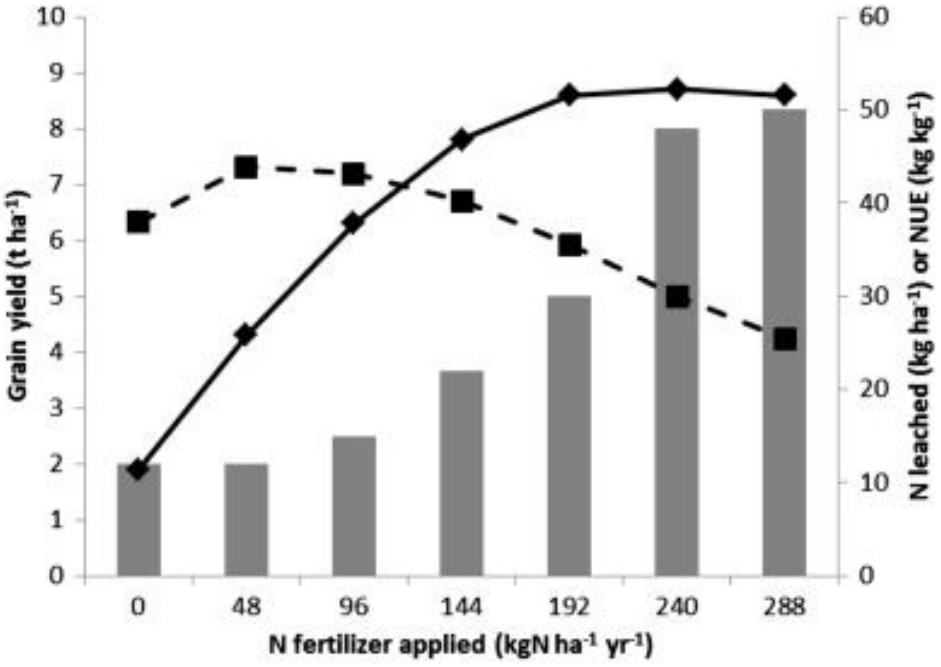

Fig. 2 Impact of increasing N fertilisation on 1990 – 1998 winter wheat yields at Rothamsted Research, UK (solid line, diamonds), N losses directly due to leaching (grey bars) and estimated NUE (dashed line, squares). Figure sourced from Hawkesford (2013).

1.5.1 Improving NUE

Improving NUE has not been a priority for most of the past century as mineral N fertilisers were cheap and the environmental concerns were low. However, given the economic and environmental issues now associated with the excessive N fertiliser use, it has become a more important objective (Legg *et al.*, 2005). Nitrogen use efficiency can be improved by increasing the amount of N captured by the plant (increased uptake efficiency; NUpE), or achieving greater growth per unit of captured N (increased utilisation efficiency; NUtE) (Garnett *et al.*, 2009). However, the impact of improvements to either component of NUE would be sizable, as even a 1 % increase in NUE, resulting from a reduced N fertiliser requirement (same yield, less N), is estimated to globally save approximately $1.1 billion annually on N fertiliser costs (Kant *et al.*, 2011). Although numerous approaches towards improving NUE exist, given the poor NUpE of cereals, this review will focus on those aimed to maximise N recovery.

1.5.1.1 Optimising agronomic practises

Despite the rising cost of N fertilisers, the price per kg is still affordable for farmers in developed countries, often leading to its misuse and over-application, particularly early in the growing season, which often generates greater N losses and lower NUE (Schepers *et al.*, 1991; Raun and Johnson, 1999). However, the European Union has already instigated policies to control N losses and emissions by regulating N use through introducing environmental taxes and regulations aimed at restricting excessive N fertiliser use (Sutton *et al.*, 2011). Although these policies are currently only established in Europe, they may soon be adopted by other nations to manage their N footprint. To improve NUE, agronomic practises can be optimised using split applications, better matching application with plant N demand across development, to maximise N recovery by crops (Cassman *et al.*, 2002). This could also be approached by taking seasonal weather forecasts into consideration when applying N

fertilisers, with respect to soil water status. This is because prolonged periods of rainfall promote N leaching, whereas poor rainfall limits the N availability to roots, ultimately influencing NUE (Powlson *et al.*, 1992; Hayman *et al.*, 2007).

1.5.1.2 Improving N uptake efficiency

1.5.1.2.1 Improving the N uptake system

A number of approaches have been suggested to improve N uptake. Harrison *et al.* (2004) suggests that N uptake could be improved by increasing root N uptake capacity via increased numbers of functional N transporters associated with root N uptake and/or their activity on the plasma membrane. Another approach is through improving N assimilation and protein storage in the leaf, as this is suggested to reduce the concentration of free N assimilates cycling between the shoot and root, which have been proposed to repress N uptake (Imsande and Touraine, 1994). Regardless of the approach taken to increase N uptake, the uptake system is tightly regulated, making its manipulation a difficult task unless our understanding of its regulation is improved. In light of this, genetic variability in N uptake capacity has been documented (Weiland, 1989; Ortiz-Monasterio *et al.*, 1997; Le Gouis *et al.*, 2000), which can be exploited to improve our understanding of the regulation of N uptake capacity.

1.5.1.2.2 Optimising root morphology

For N uptake, root morphology is generally considered to be of lesser importance than the activity of the N uptake system (Burns, 1980; Robinson and Rorison, 1983). However, the level of importance does rise when considering cropping systems with limited N availability (Robinson and Rorison, 1983), or when aiming to minimise N losses due to leaching (Garnett *et al.*, 2009). The ability to quickly develop a root system (early-vigour) enables plants to more quickly explore soil and recover more NO_3^-, reducing leaching potential, particularly in sandy soil environments that are prone to leaching (Liao *et al.*, 2004; Liao *et al.*, 2006).

Likewise, greater root length per unit root volume (greater proportion of finer roots relative to wider diameter roots) can provide a greater root surface area without diverting shoot carbon (C) allocation to increase root growth (Marschner, 2012), potentially enabling greater N uptake rates and reduced leaching (Wiesler and Horst, 1993, 1994). Deep growing roots are also suggested to improve NUE by increasing net N capture as they can access leached NO_3^- and water in subsoil layers, that would otherwise be inaccessible to shallower roots (Lynch, 2013). On the other hand, given that deep roots often comes at a greater C cost to the shoot, this trait may only be worthwhile when water and nutrient availability in the upper soil profile is limited.

Little is known about the genetic basis for the regulation of morphological root traits contributing towards NUE due to the issues associated with phenotyping below-ground root traits. Phenotyping studies in the field are hindered by challenging excavation processes that destructively sample plants and bring great difficulty to sampling whole-roots without destroying their native architecture or introducing artefacts into subsequent analysis (Smit, 2000). Additionally, the heterogeneity across the soil profile markedly influences root morphology under what may be presumed a controlled condition, making it difficult to understand the root system as a whole (Clark *et al.*, 2011). However, Laperche *et al.* (2006) mapped quantitative trait loci (QTL) controlling morphological root traits contributing to N accumulation under N deficient conditions using a wheat mapping population. The mentioned study highlighted that total root length correlated with N accumulation under N deficiency, and mapped 32 QTL for morphological traits such as lateral root frequency along seminal roots, proportion of lateral root length in comparison to total root length, lateral root number and total length of lateral and axial roots. Although this study was conducted using a single N deficient treatment, Liu *et al.* (2008) investigated root traits in maize under low and high N supply and identified N-responsive axial root traits, which contributed to N accumulation.

Subsequently, a total of 17 QTL were detected for these axial root parameters across the two N treatments. However, no QTL were common between the two N treatments, suggesting that a separate genetic basis exists for controlling root growth at low and high N. Although this study found some QTL that coincided with those from other studies using maize, these were for different root traits, highlighting the requirement for further research to generate repeatable QTL controlling root traits, in order to identify candidate genes which regulate root morphology.

1.6 Root N uptake

Nitrogen availability is highly heterogeneous in the soil medium (Robinson, 1994), thus plants (and some lower organisms) have developed several specialised uptake systems to efficiently capture N in a range of concentrations and forms (Harper, 1984; Crawford and Glass, 1998; Von Wiren *et al.*, 2000; Marschner, 2012; Wang *et al.*, 2012). However, this review will focus on NO_3^- uptake, as it is generally the predominant form of inorganic N available and taken up by crops in agricultural soils (Wolt, 1994).

1.6.1 Root NO_3^- uptake

The acquisition of NO_3^- relies on two co-ordinately functioning uptake systems that capture NO_3^- from the soil, namely, the low- and high-affinity transport systems (LATS and HATS respectively), which are defined by the external NO_3^- concentrations at which they predominately operate (Glass, 2003). The HATS have shown to predominantly operate when external NO_3^- concentrations are low (< 250 µM), whereas the LATS operate predominantly when the HATS system is saturated (Siddiqi *et al.*, 1990; Glass *et al.*, 1992; Kronzucker *et al.*, 1995). Additionally, each of these uptake systems consist of constitutive and NO_3^- inducible components (Glass and Siddiqi, 1995). The NO_3^- transporters (NRTs) responsible for this

uptake capacity belong to the *NRT1* and *NRT2* gene families, which are associated with the LATS and HATS respectively (Crawford and Glass, 1998; Forde, 2000; Tsay *et al.*, 2007).

In *Arabidopsis*, four NO_3^- transporters (NRT1.1, NRT1.2, NRT2.1 and NRT2.2) have been linked to root NO_3^- uptake (Tsay *et al.*, 2007). *AtNRT1.1* (*CHL1)* is a member of the NRT1/peptide transporter (NRT1/PTR) family, which has recently been renamed NPF family (NRT1/PTR family) (Léran *et al.*, 2014). In *Arabidopsis*, *NRT1.1* is predominantly expressed on the epidermis of young roots (tips), enabling it to be in direct contact with newly explored soil (Huang *et al.*, 1996). Its transcription is NO_3^- inducible and its function has been shown to contribute to LATS and HATS uptake, depending its phosphorylation state, which depends on the external concentration of NO_3^- (Liu *et al.*, 1999; Ho *et al.*, 2009; Parker and Newstead, 2014; Sun *et al.*, 2014). Although the function of NRT1.1 has been widely characterised in *Arabidopsis*, four co-orthologues of *NRT1.1* have been identified in maize, however their functional roles are yet to be defined (Plett *et al.*, 2010). Unlike *AtNRT1.1*, which is NO_3^- inducible, *AtNRT1.2* is constitutively expressed in root hairs and the epidermis of root tips and mature root regions and participates in LATS uptake (Huang *et al.*, 1999).

The functional role of AtNRT1.1 and AtNRT1.2 is not solely NO_3^- transport. In addition to NO_3^-, AtNRT1.2 has shown to also transport the plant hormone abscisic acid and was suggested to be involved in the regulation of stomatal aperture (Kanno *et al.*, 2012). AtNRT1.1 has also shown to transport the plant hormone auxin (Krouk *et al.*, 2010), and is suggested to play a role regulating lateral root growth, given that *NRT1.1* mutants have diminished capacity to elongate lateral roots towards NO_3^- rich patches, compared to the wild-type (Zhang and Forde, 1998; Remans *et al.*, 2006). *Arabidopsis* mutants defective in *AtNRT1.1* also display an impaired capacity to down-regulate *AtNRT2.1* expression (involved in HATS uptake) and HATS activity, compared to the wild-type, in conditions that would normally repress NO_3^- uptake (Munos *et al.*, 2004; Krouk *et al.*, 2006). Thus, AtNRT1.1 is

proposed to play a direct or indirect role in NO_3^- sensing and NO_3^- signalling pathways (Ho *et al.*, 2009; Gojon *et al.*, 2011).

NRT2.1 and *NRT2.2* are part of the *NRT2* family and in *Arabidopsis*, have shown to participate in HATS NO_3^- uptake (Wang *et al.*, 2012). Unlike *NRT1.1* and *NRT1.2*, which are predominately expressed in younger parts of the *Arabidopsis* root, *NRT2.1* is expressed in more distal, mature parts (Nazoa *et al.*, 2003; Wirth *et al.*, 2007). In *Arabidopsis*, NRT2.1 is responsible for the majority of HATS activity (Filleur *et al.*, 2001), whereas NRT2.2 only provides a minor contribution to HATS activity under normal conditions, except when *NRT2.1* is knocked-out, the contribution of NRT2.2 then increases, resulting in a partial compensation of HATS activity (Li *et al.*, 2007). Although the NRT2s are widely characterised in *Arabidopsis*, it is worthwhile to note that marked phylogenetic separation of the *NRT2*s exists between *Arabidopsis* and grass species, indicating that their function cannot be assumed to be similar across these species (Plett *et al.*, 2010). Currently, the maize orthologues of NRT2.1 and NRT2.2 are yet to be functionally characterised. However, their putative roles in high-affinity NO_3^- transport and HATS activity is proposed, given the strong correlation of their transcript levels with NO_3^- uptake capacity (Quaggiotti *et al.*, 2003; Garnett *et al.*, 2013).

The function of NRT2.1 has been shown to require a second protein, NAR2.1 (NRT3.1), in *Arabidopsis* (Okamoto *et al.*, 2006; Orsel *et al.*, 2006) and barley (Tong *et al.*, 2005). However, Kotur *et al.* (2012) later showed using a *Xenopus* oocytes expression system that the NO_3^- transport capacity of all the *Arabidopsis* NRT2s, except NRT2.7, which has a physiological role in seed NO_3^- accumulation (Chopin *et al.*, 2007), is directly or indirectly regulated by NRT3.1. In rice, NRT3.1 has shown to interact with NRT2.1, NRT2.2 and NRT2.3A, suggesting the broad interaction of NRT3.1 with NRT2s observed in *Arabidopsis* may also occur in cereals (Yan *et al.*, 2011). Although the interaction of NRT3.1 and the

NRT2s has not been shown in maize to date, the transcript profile of *ZmNRT3.1A* shows considerable overlap with *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT2.5* across the maize lifecycle, suggesting that ZmNRT3.1A may be essential for the function of these NRT2s (Garnett *et al.*, 2013). In *Arabidopsis* roots, the expression of NRT2.5 is localised to the epidermis and cortex of roots at the root hair zone of N-starved plants, and facilitate HATS NO_3^- uptake (Lezhneva *et al.*, 2014). Conversely, the rice NRT2.5 orthologue (NRT2.3A) is mainly expressed in xylem parenchyma cells of the root stele and suggested to play a key role in root to shoot NO_3^- transport in low NO_3^- (Tang *et al.*, 2012). This discrepancy in the localisation and proposed function of NRT2.5 between *Arabidopsis* and cereals highlights the requirement to clarify the role of NRT2.5, at least in cereals.

Given the evidence for the predominate localisation and physiological function of these NRTs, it is postulated that the LATS is responsible for the majority of NO_3^- uptake from the soil solution (Glass, 2003). This is because *AtNRT1.1* is predominately expressed in root tips (Huang *et al.*, 1996) and are first exposed to NO_3^- in newly explored soil, which in agricultural systems is generally in the millimolar range (Wolt, 1994), above the saturation level of the HATS (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995). However, *AtNRT2.1* is expressed in more mature roots (Nazoa *et al.*, 2003), where external NO_3^- concentrations may be reduced due to prior uptake by the root tip. Contrary to this, Malagoli *et al.* (2004) measured HATS and LATS activity in oilseed rape over time, as well as their responses to various factors and used this information in conjunction with modelling of field data to suggest that HATS could solely satisfy most of the plant N requirements. Likewise, Garnett *et al.* (2013) showed that it was the HATS that responded to N-demand and N treatment across the maize lifecycle, supporting Malagoli *et al.* (2004). This suggests that the HATS are more important towards net NO_3^- uptake, highlighting the requirement to re-examine the relative roles of these two NO_3^- transport systems, with respect to net NO_3^- uptake.

1.6.2 Regulation of NO_3^- uptake

Nitrate uptake capacity reflects the plant's demand for N and is tightly regulated (Morgan and Jackson, 1988b; Henriksen *et al.*, 1992; Jackson and Volk, 1992; Aslam *et al.*, 1993). Physiological studies using *Arabidopsis* have shown that external NO_3^- can regulate HATS NO_3^- uptake capacity, as a strong increase in HATS activity is observed within minutes after its resupply to plants, following a starvation period, correlating with the rapid transcription of *AtNRT2.1* and *AtNRT2.2* (Jackson *et al.*, 1973; Aslam *et al.*, 1993; Zhuo *et al.*, 1999; Okamoto *et al.*, 2003; Hu *et al.*, 2009). However, its repression following the accumulation of NO_3^- and its assimilatory products, such as amino acids is also observed with prolonged exposure to sufficient NO_3^-, suggesting a feedback regulation mechanism (Aslam *et al.*, 1993; Henriksen and Spanswick, 1993; Glass *et al.*, 2002). Feedback regulation by the plant's N status is a means of coordinating NO_3^- uptake with N demand (Forde, 2002). Indeed, studies suggest that NH_4^+ and free amino acids, in particular glutamine, are responsible for the repression of *NRT2* transcription (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000) and NO_3^- uptake (Muller and Touraine, 1992; Sivasankar *et al.*, 1997; Tischner, 2000), however a particular N-containing metabolite is yet to be confirmed. This may be because these studies are generally performed whilst supplementing the N-metabolite of interest into the external medium. The subsequent observations may consequently reflect the plant's greater uptake capacity for the supplemented product compared to NO_3^-, evidenced by the higher uptake capacity of NH_4^+ compared to NO_3^- in maize roots when supplied in equimolar concentrations (Gu *et al.*, 2013). Ultimately, these supplementation experiments may not provide an ideal reflection of what plant roots are exposed to in agricultural conditions, highlighting that in order to examine the role of these metabolites, in what is anticipated to be an agriculturally relevant environment, alternate experimental approaches are required.

Diurnal regulation of NO_3^- uptake as also been documented (Clement *et al.*, 1978; Macduff *et al.*, 1997; Peuke and Jeschke, 1998). Having measured NO_3^- uptake rates across day-to-day periods over 12 d, Clement *et al.* (1978) showed that peak uptake rates occur during the day period, whereas the minimum occur whilst dark. Similar to NO_3^- uptake rates, transcript levels of *NRT*s involved in root NO_3^- uptake have shown to be diurnally regulated with peak observed during light period (Ono *et al.*, 2000; Lejay *et al.*, 2003; Feng *et al.*, 2011). Although a mechanism for the diurnal control NO_3^- uptake is yet to be defined, a role for transpiration has been suggested as its rates are lowest during the dark period, resulting in peak concentrations of free N-compounds, which have been suggested to repress NO_3^- uptake, and vice versa (Delhon *et al.*, 1995; Schurr, 1999; Herdel *et al.*, 2001).

Nitrogen and C metabolism are closely linked as both are fundamental to plant growth and development (Miyashita and Good, 2008). Nitrogen and C metabolism are hypothesised to be coordinated together by sensing cellular C/N balance and regulating transcription of genes involved in photosynthesis, respiration and N assimilation accordingly (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Plaxton and Podestá, 2006). Thus, the metabolites generated from N metabolism or photosynthesis influence the metabolism of one another, evidenced by a study showing that the decline in NO_3^- uptake rates in dark, after a light period, could be delayed by the addition of sucrose in external medium (Lejay *et al.*, 1999). However, a study showed that plants grown with low light intensities, and with shorter light periods, contain very high NO_3^- concentrations within leaf tissue, suggesting that N uptake is less sensitive to changes to C metabolites, compared to N metabolites (Matt *et al.*, 1998).

1.6.3 NO_3^- storage and assimilation

Once NO_3^- has been captured by the root, it is either stored in vacuoles or assimilated into NH_4^+ for amino acid synthesis (Marschner, 2012). Transport of NO_3^- into vacuoles is

achieved via chloride channels, CLCa and CLCb, with both having higher specificities for NO_3^- than chloride (De Angeli *et al.*, 2006; Von Der Fecht-Bartenbach *et al.*, 2010).

In higher plants, NO_3^- assimilation can occur in the roots and shoots, however the balance between the two varies greatly between and within species, and also on soil NO_3^- availability (Smirnoff and Stewart, 1985). Generally speaking, NO_3^- is primarily assimilated in the root when external NO_3^- concentrations are low and as external NO_3^- concentrations increase, so does the level of shoot NO_3^- assimilation (Andrews, 1986; Andrews *et al.*, 2004). For NO_3^- to be translocated from the root to the shoot, it must first be loaded into the xylem via non-specific anion channels (Gilliham and Tester, 2002; Kohler *et al.*, 2002) and LATS associated NO_3^- specific transporters. In *Arabidopsis*, NRT1.5 has been proposed to be the LATS component involved in xylem loading (Lin *et al.*, 2008), and a study rice proposed that OsNRT2.3A (the orthologue of AtNRT2.5) may be the HATS involved, given it is predominately expressed in xylem parenchyma cells of the root stele, and demonstrates that it plays a key role in root to shoot NO_3^- transport in low NO_3^- (Tang *et al.*, 2012).

For its assimilation, NO_3^- is first reduced into NH_4^+ by two consecutive enzymatic steps (Andrews *et al.*, 2004). Nitrate is first converted to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR), where it is then translocated to the chloroplast or plastid, depending on whether assimilation is occurring in the shoots or roots respectively (Maathuis, 2009). Nitrite is then reduced to NH_4^+ by nitrite reductase (NiR) (Meyer and Stitt, 2001). Ammonium can however be toxic to plant function (Hodges, 2002), thus it rapidly is assimilated into glutamine and glutamate, the precursor for other amino acids, by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Oaks, 1994) (Fig. 3). The generated amino acids are subsequently incorporated into newly synthesised proteins with or without enzymatic capacity (enzymes vs. storage proteins) (Heldt and Piechulla, 2010), and this can occur in the organ

where the amino acids were assimilated, or in target organs via xylem and phloem transport (Crawford and Glass, 1998).

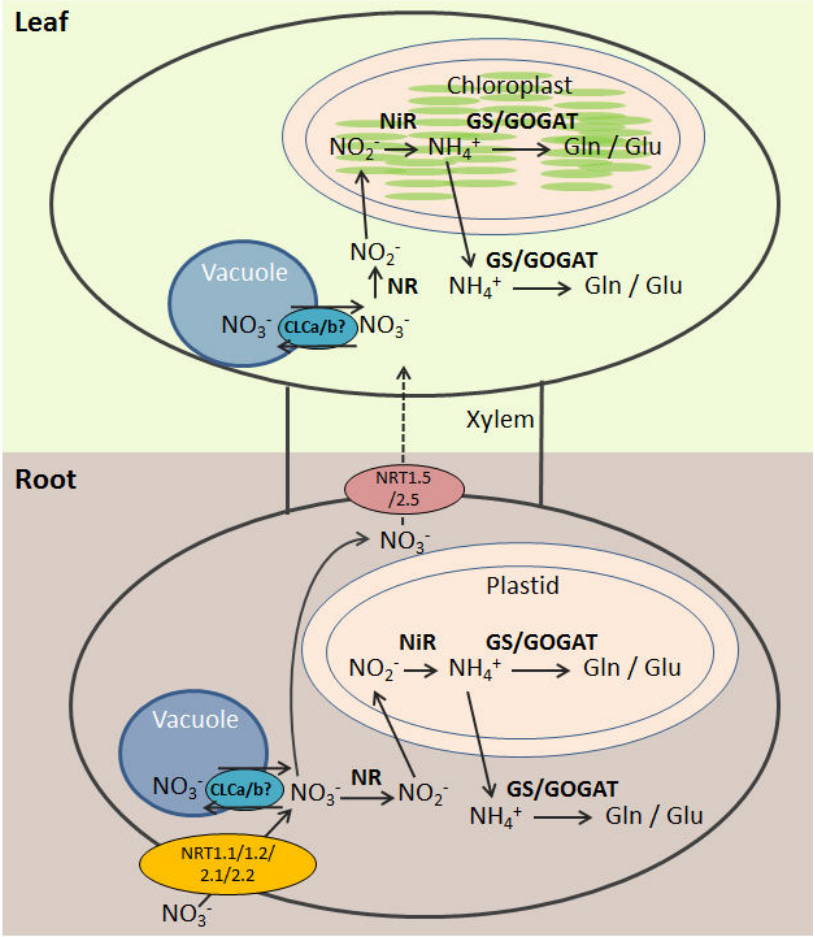

Fig. 3 Schematic outlining uptake, storage and assimilation processes of NO_3^- within plants (adapted from Marschner (2012)).

1.7 Adapting to N limitation

In order to maintain maximum growth, plants must meet their N demand. However, as N availability may not always be optimal, plants have developed strategies to adapt to supply, in order to survive and achieve maximum growth (Marschner, 2012). Achieving maximum growth is important when considering agricultural situations, as early growth differences can greatly influence final crop harvest (Claassen and Shaw, 1970; Koutroubas *et al.*, 1998; Grant *et al.*, 2001).

1.7.1 Increasing NO_3^- uptake capacity

Early studies have shown that a reduction in NO_3^- supply to plant roots for several days results in a transient heightened increase in NO_3^- uptake capacity (and NH_4^+), in comparison to those with sufficient NO_3^- supply (Lee and Rudge, 1986; Morgan and Jackson, 1988a). This is consistent with Garnett *et al.* (2013) who reported that a reduction in NO_3^- supply resulted in a transient heightened increase in both HATS NO_3^- uptake capacity and transcription of putative high-affinity NO_3^- transporters *ZmNRT2.1* and *ZmNRT2.2*, relative to plants with sufficient NO_3^-. Moreover, this was observed in maize grown with steady-state reduced NO_3^- supply together suggesting that up-regulating HATS activity may be one strategy employed by some species to meet N demand when supply is low.

1.7.2 N Remobilisation

During short-term N limitation, vacuole-stored NO_3^- can act as a buffer to maintain cytosolic NO_3^- concentrations (Glass *et al.*, 2002), and can also be remobilised to young leaves (Fan *et al.*, 2009). However, these reserves are finite and only maintain cytosolic NO_3^- concentrations of N-starved barley roots and shoots for 24 and 5 h respectively (Vanderleij *et al.*, 1998). During prolonged N limitation, free amino acids can be transported to developing sinks to maintain growth, however amino acids can also be generated from the catabolism of proteins (Masclaux-Daubresse *et al.*, 2008). Within the leaf, approximately 80 % of the total N is located within chloroplasts in the form of protein (Adam *et al.*, 2001). RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) appears to be the primary protein subject to degradation for N remobilisation (Mae *et al.*, 1993). However, RuBisCO is essential for C fixation by photosynthesis, thus its degradation consequently decreases maximum photosynthetic capacity, ultimately reducing plant growth (Rogers and Humphries, 2000).

1.7.3 Modifying root morphology

Plant roots have two major functions; water and nutrient acquisition from the surrounding soil, and plant anchorage (Hochholdinger *et al.*, 2004). The growth and development of the plant root system is highly sensitive to modifications by internal factors, including the plant N status (Song *et al.*, 2011), and external factors, such as localised N-rich patches within soil medium (Forde and Walch-Liu, 2009). When N supply is limited, plants may respond by increasing lateral root number and length towards N-rich patches (Drew *et al.*, 1973; Granato and Raper, 1989; Laine *et al.*, 1995; Zhang and Forde, 1998). This focussed root proliferation may be an evolutionary adaptive response ensuring that biomass is efficiently allocated to roots exposed to areas with high N, rather than roots exposed to no N (Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Garnett *et al.*, 2009). Although these studies detail changes to root morphology in heterogeneous N environments, Linkohr *et al.* (2002) observed that the growth of both the *Arabidopsis* lateral and primary root was stimulated with uniform low NO_3^- availability, relative to high NO_3^-, highlighting that plants can adapt to uniform low N environments by increasing the effective absorptive area of the root.

1.7.4 Changing biomass allocation

It has long been observed that plant nutrition influences plant biomass allocation between roots and shoots (Brouwer, 1962; Bloom *et al.*, 1985; Müller *et al.*, 2000; Poorter and Nagel, 2000). Plants possess the capacity to respond to nutrient supply, by allocating biomass to the organ required for its acquisition (Chapin *et al.*, 1987). With increasing nutrient availability, plants invest less biomass in their roots, relative to the shoot, allowing for greater leaf area development and net photosynthesis (Gulmon and Chu, 1981; Agren and Franklin, 2003); whereas with low nutrient availability, more biomass is allocated to the root, relative to the shoot, increasing the absorptive area of the root system and nutrient capture (Ingestad and Agren, 1991) (Fig. 4). Increasing root biomass allocation consequently reduces shoot C

supply, reducing shoot growth rate and ultimately above-ground yield potential, reflected in an increase in root:shoot (Davidson, 1969; Ericsson, 1995; Bonifas *et al.*, 2005; Lambers *et al.*, 2008).

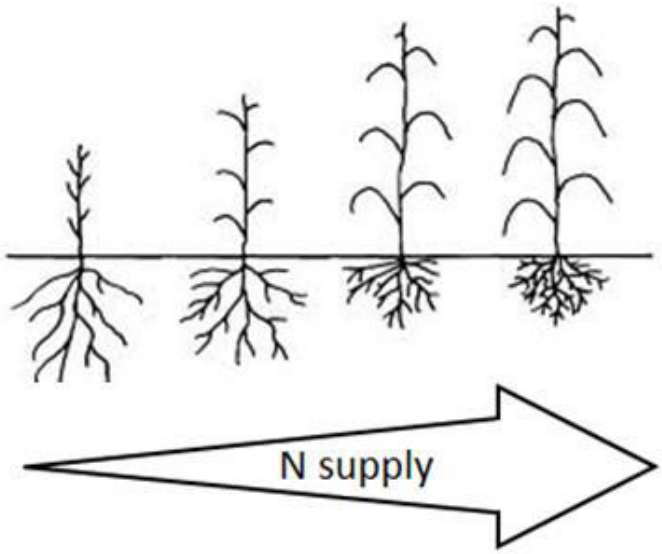

Fig. 4 Schematic representation of root and shoot growth of cereal crops with increasing N provision. Figure sourced from Marschner (2012).

Numerous models have been developed to explain the relationship between N nutrition and biomass allocation (Johnson and Thornley, 1987; Wilson, 1988; Kachi and Rorison, 1989; Levin *et al.*, 1989; Hilbert, 1990; Gleeson, 1993), yet although the mechanism underlying this biomass allocation remains to be defined, several models support the concept that the physiological regulation of biomass allocation hinges on maintaining cellular C/N balance (Brouwer, 1962; Thornley, 1972; Dewar, 1993). Brouwer (1962) and Thornley (1972) proposed that growth is dependent on both N and C supply from roots and shoots respectively. Shoot N supply is driven by root to shoot N fluxes (xylem transport), whereas root C supply is driven by shoot to root C fluxes (phloem transport) (Ericsson, 1995), and these flux rates are determined by the concentration gradient between the where the nutrient was captured and its destination. This suggests that conditions leading to increased cellular C

concentrations, relative to N, increase cellular C/N, favouring growth of the organ required for N capture, and vice versa. Although the molecular coordination of biomass allocation is not known, N limitation has shown to lead to the transcriptional repression of genes involved in photosynthesis, chlorophyll synthesis, plastid protein synthesis and C assimilation (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Scheible *et al.*, 2004), suggesting that it may be partly coordinated by changing transcription levels of genes involved in the optimal function and growth of the target organ required for nutrient capture, and those involved in the assimilation of the particular nutrient.

1.8 What is required to improve cereal root N uptake?

One possible avenue to improve cereal net N uptake is increasing root NO_3^- uptake capacity, however how it is not well understood how the NO_3^- uptake system is regulated, hindering the development of approaches towards improving this system. Indeed, studies have investigated the regulation of the NO_3^- uptake system by starving and resupplying NO_3^-, or by supplementing the substrate of interest. However, these experimental systems perturb N supply and may not provide a good reflection of what cereal roots are exposed to in most agricultural soils given that NO_3^- is predominately available to roots. Understanding how NO_3^- uptake capacity is up-regulated in a system that may better reflect agriculturally relevant NO_3^- availability, such as steady-state NO_3^- supply, may identify targets for manipulation to increase NO_3^- uptake capacity. In addition, the majority of studies investigating root NO_3^- uptake and the associated *NRT*s are conducted in *Arabidopsis*. Given the dichotomy between *Arabidopsis* and cereals (Plett *et al.*, 2010), a requirement exists to clarify which cereal *NRT* orthologues are associated with root NO_3^- uptake, in order to begin determining the molecular regulation of the cereal NO_3^- uptake system.

A number of studies have now proposed that it may also be possible to improve cereal N uptake by optimising root morphology (Robinson and Rorison, 1983; Garnett *et al.*, 2009; Lynch, 2013). However, the role of morphological changes of roots in response to NO_3^- supply is also not well understood, in this case, due to the difficulties associated with phenotyping roots in soil. A greater understanding the role of any adaptive morphological root responses to N supply would provide insight towards how root morphology may change to meet N demand, relative to supply.

1.9 Aims and objectives

Soon after imbibition, amino acids and amides are derived from the hydrolysis of finite seed N reserves (storage proteins) (Oaks and Beevers, 1964), and translocated into the developing seedling. These reserves can adequately supply the developing seedling for the first 7 d of germination (Watt and Cresswell, 1987), and external NO_3^- has little effect on the seedlings N content whilst growth is supported by the seed (Srivastava *et al.*, 1976). It is generally believed that when seed N reserves exhaust, seedlings transition to capturing inorganic N from external sources, providing the ideal period to begin investigating the regulation of N uptake capacity and root morphology without perturbing external N supply. Likewise, if any adaptive responses to N supply do occur, they may be observed during this growth period, and then the molecular and physiological regulation of these changes can be subsequently dissected. Recent work without our laboratory has shown that NO_3^- treatment differences in maize root NO_3^- uptake capacity and putative NO_3^- transporter gene expression already existed 16 d after imbibition, suggesting that adaptive responses to NO_3^- supply may begin occurring even earlier in development (Garnett *et al.*, 2013).

The research objectives of this thesis are:

i) to understand how maize (*Zea mays* L.) seedlings manage the transition from seed N use to external N capture;
ii) to determine whether seedlings adapt to NO_3^- supply by changing root morphology;
iii) to identify QTL associated with morphological root traits contributing to shoot dry matter accumulation and N uptake, relative to NO_3^- supply.

Chapter 2 investigates the processes leading to, and involved in, the up-regulation of the NO_3^- uptake system across the transition of seed N use to external N capture using two maize inbred lines (*Zea mays* var. B73 and Mo17) grown with low and sufficient N supply.

Chapter 3 examines how maize seedlings change root morphology in response to NO_3^- supply, and how early in development any morphological responses occur, using the same lines and N treatments as Chapter 2. Additionally, genotypic differences in root morphological traits are highlighted and associated with their suitability for high- and low-input agricultural systems.

Chapter 4 maps QTL for morphological root traits correlating with shoot dry matter accumulation and/or have been previously associated with N uptake, using the intermated B73 × Mo17 mapping population.

Chapter 5 gives a broad overview of the outcomes from this thesis, along with the discussion points of interest and proposals for future directions.

Chapter 2: The transition from maternal to external nitrogen sources in maize seedlings

TITLE

The transition from maternal to external nitrogen sources in maize seedlings

Running title

Up-regulation seedling nitrate uptake capacity

Authors

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ABSTRACT

Cereal crops have poor nitrogen (N) uptake efficiency, providing scope for its improvement by increasing root nitrate (NO_3^-) uptake. Maximising NO_3^- uptake during seedling development is important as it often influences subsequent growth and yield. However, little is known about the processes leading to, and involved in, the initiation of root NO_3^- uptake capacity in developing seedlings. This study examines the physiological processes involved in root NO_3^- uptake and metabolism, to understand how the NO_3^- uptake system up-regulates to meet demand as seedlings transition from seed N use to external N capture. Results show that concentrations of seed-derived free amino acids within root and shoot tissues are initially high, but rapidly dilute until 8 d after imbibition (DAI). Similarly, shoot N % dilutes, but does not stabilise until 12 - 13 DAI. After free amino acid concentrations decrease, root N capture increases until shoot N % stabilises. The increase in root N capture corresponds with a rapid rise in root NO_3^- uptake capacity and transcript levels of putative NO_3^- transporters *ZmNRT2.1* and *ZmNRT2.2*. The way NO_3^- uptake capacity increases to meet demand provides insight into the processes controlling N uptake during seedling development, which may also be applicable to later growth stages.

Keyword index:

Amino acid, NRT, nitrate, uptake, seed, Zea mays

Abbreviations

N, nitrogen; NO_3^-, nitrate; DAI, days after imbibition; NRT, nitrate transporter; Mt, megatonne; LATS, low-affinity nitrate transport system; HATS, high-affinity nitrate transport system; IBM, intermated B73 × Mo17; RN, root nitrogen; SN, seed nitrogen; ShN, shoot nitrogen; S.E.M, standard error of mean; S.E.D, standard error of difference; ANOVA, analysis of variance; LSD, least significant difference; DW, dry-weight; NR, nitrate reductase.

INTRODUCTION

Currently over 100 Mt of costly nitrogen (N) fertilisers are applied to crops each year to maximise growth and ultimately yield, with around 60 % applied to cereals (Heffer, 2013). However on a global scale, cereal crops only capture 40 – 50 % of the applied N (Peoples *et al.*, 1995; Sylvester-Bradley and Kindred, 2009), allowing for the remaining N to be lost by leaching into groundwater, surface run-off and volatilisation into the atmosphere, which all considerably impact the environment (Vitousek *et al.*, 1997). Improving this low N uptake efficiency could greatly reduce the economic and environmental impacts derived from N losses. The key to improving N uptake is increasing the uptake of nitrate (NO_3^-), as it is the predominant form of N available to plants in most agricultural soils (Miller *et al.*, 2007; Wolt, 1994).

Plant NO_3^- uptake is mediated by low-affinity (LATS) and high-affinity (HATS) transport systems, which are thought to predominately operate at high or low external NO_3^- concentrations respectively (Glass and Siddiqi, 1995; Glass, 2003). In *Arabidopsis*, NO_3^- transporters (NRTs, now named NPFs (Léran *et al.*, 2014)) AtNRT1.1 and AtNRT1.2 have been associated with LATS NO_3^- uptake (Huang *et al.*, 1999). However, studies have shown that AtNRT1.1 is a unique NO_3^- transporter, having the capacity to mediate both low- and high-affinity NO_3^- uptake, subject to its phosphorylation state (Ho *et al.*, 2009; Liu *et al.*,

1999; Parker and Newstead, 2014; Sun *et al.*, 2014). Conversely, AtNRT2.1 and AtNRT2.2 mediate HATS NO_3^- uptake, and one study revealed that AtNRT2.1 is responsible for the majority of HATS activity (72 %), whilst AtNRT2.2 has a smaller contribution (19 %) (Li *et al.*, 2007). Although the role of NRT2.5 remains to be clarified, it is suggested to facilitate root HATS NO_3^- uptake and remobilisation in N-starved *Arabidopsis* (Lezhneva *et al.*, 2014). Studies in oilseed rape and maize suggest that in comparison to the LATS, it is the HATS that is responsible for much of the NO_3^- uptake, even at reasonably high NO_3^- concentrations, and it is the HATS that respond to N supply and demand (Garnett *et al.*, 2013; Malagoli *et al.*, 2004); together this suggests that it is the HATS that is more important towards net NO_3^- uptake, in comparison to the LATS.

Studies in *Arabidopsis* have shown that HATS activity, and *AtNRT2.1* and *AtNRT2.2* transcript levels strongly increase with NO_3^- resupply following a NO_3^- starvation period, and are later repressed with prolonged exposure to sufficient NO_3^- (Aslam *et al.*, 1993; Okamoto *et al.*, 2003; Zhuo *et al.*, 1999). Although external NO_3^- can stimulate NO_3^- uptake capacity, the internal accumulation of NO_3^- and its assimilatory products, such as amino acids, repress *NRT2* transcription (Vidmar *et al.*, 2000; Zhuo *et al.*, 1999) and NO_3^- uptake capacity (Muller and Touraine, 1992; Sivasankar *et al.*, 1997; Tischner, 2000). This feedback regulation by internal concentrations of NO_3^- and its assimilates suggests a mechanism exists to coordinate NO_3^- uptake with plant N demand (Forde, 2002). However, most of these observations were made in systems where the free N-metabolite of interest was exogenously applied, making it difficult to distinguish between the internal and external effects of these substrates. This highlights a requirement to employ a different experimental system to examine the regulation of NO_3^- uptake capacity.

This study exploits the transition of seedlings from seed N use to external N capture, to investigate the processes leading to, and involved in, the up-regulation of the NO_3^- uptake

system. During early growth, the N requirements of developing seedlings are met by the hydrolysis of finite seed protein reserves that begin to hydrolyse and be transported into developing sinks soon after imbibition (Guardiola and Sutcliffe, 1971; Harvey and Oaks, 1974; Watt and Cresswell, 1987). When this reserve exhausts, seedlings must transition to external N capture to meet N demand and maintain growth. This provides an ideal system to dissect the regulation of root NO_3^- uptake capacity. Managing this transition is vital, as growth differences early in development are often maintained until final crop harvest (Claassen and Shaw, 1970; Grant *et al.*, 2001; Koutroubas *et al.*, 1998).

The objective of this study was to improve our understanding of how cereals up-regulate their NO_3^- uptake system to meet N demand, by investigating the processes involved in NO_3^- uptake and metabolism with high-temporal resolution, across the transition from seed N use to external N capture. Maize seedlings were grown in steady-state N conditions and supplied with either low or sufficient NO_3^- to determine the effects of NO_3^- supply on the processes involved in NO_3^- uptake and metabolism. Additionally, the parents of the widely-studied intermated B73 × Mo17 (IBM) mapping population were used to explore if these processes may differ between varieties.

MATERIALS AND METHODS

Plant growth

Maize seeds (*Zea mays* L. var. B73 and Mo17) of similar size were imbibed in aerated RO-H_2O 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 mm × 50 mm). This allowed the roots to remain separate from adjacent seedlings whilst allowing free access to solution. The hydroponic system was

situated in a controlled environment room with a day:night cycle of 14 h:10 h, 26 °C:20 °C, with a flux density at canopy level of *c.* 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 NO_3^- N, 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P for the 0.5 mM NO_3^- treatment, and 2.5 NO_3^- N, 1.8 K, 0.6 Ca, 0.5 Mg, 0.5 S, 0.5 P for the 2.5 mM NO_3^- treatment. The choice of NO_3^- concentrations was based on those used by Garnett *et al.* (2013), as *c.* 0.5 mM was suggested to be the threshold concentration eliciting a major response of the maize NO_3^- uptake system. Both treatment solutions also contained (in µM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and ethylendiamine-*N*,*N'*-bis(2-hydroxyphenylacetic acid) (FeEDDHA)). Iron was supplemented twice weekly with the addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (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 NO_3^- were monitored using a NO_3^- 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.

Determination of tissue N and cumulative net N uptake

To determine total tissue N content, sampled plants were blotted and the root, shoot and seed were separated then 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_0 is the point before imbibition. Shoot and root N (ShN and RN respectively) were added to derive total plant N. Subsequently, the cumulative

amount of seed N (SN) calculated to be translocated into the seedling ($T_0 - T_n$) was subtracted from total plant N:

$$\text{Net N uptake}_{Tn} = (\text{ShN}_{Tn} + \text{RN}_{Tn}) - \text{SN}_{T0\text{-}Tn}$$

Amino acid determination

Concentrations of free amino acid in root and shoot tissue were determined using liquid chromatography electrospray ionization-mass spectrometry as described by Boughton *et al.* (2011), once the samples had been derivatized following the method of Cohen & Michaud (1993).

Tissue NO_3^- determination

To extract NO_3^- from plant tissue, 20 mg of homogenous finely-ground frozen plant tissue was added to 1 ml MQ-H_2O then 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 H_2SO_4 and incubated (20 min, RT). Then, 125 µL of the mixture containing the sample and 5 % w/v salicylic acid in H_2SO_4 was added to NaOH (2.375 ml, 2 N) and incubated (20 min, RT). Samples ready for analysis were then loaded into 96-flat well plates (200 µL /well) (Greiner Bio-One, Vic, Australia), read at an absorbance of 410 nm (POLARstar Optima, BMG Labtech, Germany) and compared against KNO_3 standards.

NO_3^- uptake capacity measurement

On sampling days, between 11:00 and 13:00 h, plants were transferred to nutrient solutions that matched growth solutions within the same controlled environment room. Roots were then given a 5 min rinse with the same nutrient solution, but with either 100 or 1000 µM NO_3^-, followed by 10 min of exposure to the same solution, but with ^{15}N-labelled NO_3^- (^{15}N 10 %). The concentration of 100 µM NO_3^- was used as it is thought to be close to saturation of the

HATS and 1000 µM would include both HATS and some LATS uptake (Crawford and Glass, 1998; Kronzucker *et al.*, 1995a; Siddiqi *et al.*, 1990). At the end of the 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 sec rinse to remove unlabelled solution adhering to the root surface. The flux timing was based on that used by Kronzucker *et al*. (1995b) and chosen to minimise 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 ^{15}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 ^{15}N content in the plant. Mean LATS uptake capacity values for a given time-point were calculated by subtracting the mean 100 µM NO_3^- uptake capacity value from that of 1000 µM NO_3^- at the same time-point and NO_3^- treatment (Okamoto *et al.*, 2003). Error bars for calculated LATS NO_3^- uptake capacity plots (1000 µM – 100 µM) represent the standard error of difference (S.E.D) between NO_3^- treatments (0.5 mM and 2.5 mM NO_3^-), which was calculated using the equation below:

$$\text{S.E.D}_{(1000\,\mu\text{M} - 100\,\mu\text{M})} = \sqrt{\sigma^2\,(1000\,\mu\text{M}) + \sigma^2(100\,\mu\text{M})}$$

σ represents the standard error of a mean (S.E.M) of 100 µM or 1000 µM NO_3^- uptake capacity measurement, and is calculated using the below equation:

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

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_2 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_2O. Extraction of RNA was performed using the method of Chomczynski (1993). Extracted RNA was then DNase treated (Ambion, USA), according to the manufacturer's instructions. The integrity of the RNA was then checked on a 1.2 % w/v agarose gel and then cDNA synthesis was performed on 1 µg of total, using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Q-PCR was then 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 *ZmNRT1.1B* (GRMZM2G161459: forward primer: *5'*- GTC ATC AGC GCC ATC AAC CT, reverse primer: *5'*- ACG GCA ATA GAC TCC TCG TC); and two *NADH:NR* genes, *NR1* (GRMZM2G568636: forward primer: *5'*- GAG GAC CAC ACG GAG ATG, reverse primer: *5'*- CCA ACG CTG TAC TTC CAC) and *NR2* (GRMZM2G428027: forward primer: *5'*- GCT TTG GCT AAC GAA TGT C, reverse primer: *5'*- GCT CGC TAC TAT TAC AAC AAG) (Long *et al.*, 1992). Based on melt-curve analysis of Q-PCR products, no allelic differences were observed for any of the tested genes between the two lines.

Statistical analyses

Seedlings were grown and selected randomly from four separate hydroponic systems corresponding to its NO_3^- treatment, which constituted blocks. There was no significant difference between blocks. Statistical analysis for calculated LATS NO_3^- 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).

RESULTS

Tissue growth

On the final sampling day (21 d after imbibition (DAI)), B73 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) (Fig. 1A), whereas Mo17 maintained shoot growth in 0.5 mM NO_3^-, compared to the 2.5 mM treatment (Fig. 1B). External NO_3^- supply had no effect on root DW of B73 (Fig. 1C). However, the root dry-weight (DW) of Mo17 increased by 41 % in 0.5 mM NO_3^-, compared to the 2.5 mM treatment (Fig. 1D). During early growth (6 - 8 DAI), root:shoot decreased by half for both lines and NO_3^- treatments (Fig. 1E, 1F). After 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 emerged 12 DAI in B73, compared to 11 DAI in Mo17 and carried through to the final harvest.

Tissue N

Seed N analysis, prior to imbibition (0 DAI), showed that initial N concentrations were significantly lower in B73 seeds compared to Mo17 (1.65 % ± 0.035 % S.E.M and 2.22 % ± 0.082 % S.E.M, respectively ($P < 0.05$, $n = 8$)). Across early growth, the majority of B73 seed N reserves depleted by 8 DAI (Fig. 2A), and this was similarly observed in Mo17 (Fig. 2B). Shoot N % was initially high (8 %) across both NO_3^- treatments and lines, but rapidly diluted to less than 4 % by 9 DAI (Fig. 2C, 2D). This dilution then slowly stabilised by 12 DAI in plants grown at 2.5 mM NO_3^-, whilst those in 0.5 mM NO_3^- stabilised 13 DAI. Unlike the shoots, root N % was maintained at 4 % throughout all sampling days across both lines grown in 2.5 mM NO_3^-, whereas those in the 0.5 mM treatment decreased 15 – 21 DAI (Fig. 2E, 2F).

As with B73 shoot DW, the net N uptake per plant was affected by NO_3^- supply 21 DAI. B73 seedlings in 0.5 mM NO_3^- captured 50 % less N, 21 DAI, than those in the 2.5 mM treatment (Fig. 3A). B73 captured more N than Mo17 when supplied with 2.5 mM NO_3^-,

however Mo17 maintained net N uptake at 0.5 mM NO_3^-, compared to those in the 2.5 mM treatment (Fig. 3B).

Tissue NO_3^-

Shoot NO_3^- concentrations were similar across both lines in 0.5 mM NO_3^-, remaining steady through to the final sampling day (Fig. 4A, 4B). Shoot NO_3^- concentrations in B73 seedlings in 2.5 mM NO_3^- matched those in 0.5 mM until the final sampling day, where concentrations were higher in 2.5 mM NO_3^-, compared to the 0.5 mM treatment. In Mo17, these treatment differences in shoot NO_3^- concentrations emerged from 12 DAI. Across both lines and NO_3^- treatments, root NO_3^- concentrations generally decreased until 11 DAI (Fig. 4C, 4D). However, root NO_3^- concentrations were generally lower in seedlings grown in 0.5 mM NO_3^-, compared to those in the 2.5 mM treatment.

Free amino acids

Across both lines and NO_3^- treatments, concentrations of total free amino acids in both shoot (Fig. 5A, 5B) and root (Fig. 5C, 5D) tissue rapidly decreased until 8 DAI. Similarly, concentrations of most individual free amino acids rapidly decreased until 8 DAI, in both shoots and roots tissue of B73 (Fig. S1) and Mo17 (Fig. 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 B73 seedlings grown in 0.5 mM NO_3^-, concentrations of shoot glycine, glutamine and alanine dropped until 8 DAI, then remained steady through to the final sampling day, whereas those in 2.5 mM NO_3^- increased 15 - 21 DAI. Similarly in Mo17, concentrations of shoot glycine, glutamine and alanine increased 15 – 21 DAI. Across both lines, concentrations of root glycine, glutamine and alanine shared a similar trend to root asparagine and did not differ between NO_3^- treatments across sampling days. Across both lines and NO_3^- treatments, glutamate concentrations in shoot and root tissue dropped until 8

DAI, then remained steady. However, this was not to the same magnitude as the previously described amino acids.

Root NO_3^- uptake capacity

In B73 seedlings grown in 2.5 mM NO_3^-, HATS NO_3^- uptake capacity rapidly increased from 10 DAI, then plateaued from 15 DAI (Fig. 6A). This was similarly observed in B73 seedlings grown in 0.5 mM NO_3^-, however HATS NO_3^- uptake capacity peaked 13 DAI, at levels higher those in the 2.5 mM treatment before stabilising at 15 DAI. Similarly to B73, HATS NO_3^- uptake capacity in Mo17 rapidly increased 10 - 15 DAI across both NO_3^- treatments (Fig. 6B). However, this decreased 15 - 21 DAI in seedlings grown in 0.5 mM NO_3^-, resulting in a HATS NO_3^- uptake capacity 41 % lower than those in the 2.5 mM treatment. Mean LATS NO_3^- uptake capacity began increasing after 8 DAI (Fig. 6C, 6D), reaching maximum capacity 12 DAI regardless of NO_3^- supply, with peak NO_3^- uptake rates being lower than those of the HATS. In B73 grown in 0.5 mM NO_3^-, LATS uptake capacity decreased 15 – 21 DAI, to half the capacity of those in the 2.5 mM treatment, whereas this decrease was not observed in Mo17.

Root NO_3^- transporter (NRT) gene expression

Transcript levels of genes encoding putative high-affinity NO_3^- transporters ZmNRT2.1 (Fig. 7A, 7B), ZmNRT2.2 (Fig. 7C, 7D) and NO_3^- uptake related protein ZmNRT3.1A (Fig. 7E, 7F) began increasing 10 DAI across both lines and NO_3^- treatments. In B73, transcript levels of *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT3.1A* increased until 15 DAI, where they then stabilised. Between lines, no NO_3^- treatment differences were observed in *ZmNRT2.1* transcript levels, however treatment differences in *ZmNRT2.2* transcript levels were observed. Across both lines, *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 21 and 15 DAI in B73 and Mo17, respectively. *ZmNRT3.1A* transcript levels in B73 increased from 10 DAI and

plateaued from 15 DAI regardless of NO_3^- supply, similar to *ZmNRT2.1*. Although this was also observed in Mo17 seedlings grown in 2.5 mM NO_3^-, *ZmNRT3.1A* transcripts in 0.5 mM NO_3^- decreased 13 - 15 DAI, resulting in levels lower than those in the 2.5 mM treatment, which carried through to the final sampling day. Across both lines and NO_3^- treatments, *ZmNRT2.5* transcript levels remained near-zero until 15 DAI. After this, transcript levels increased in seedlings grown in 0.5 mM NO_3^-, to levels three times higher than those in the 2.5 mM treatment (Fig. 7G). Although a similar trend was observed in Mo17, the NO_3^- treatment difference was markedly smaller than B73 (Fig. 7H).

Across both lines grown in 0.5 mM NO_3^-, *ZmNRT1.1A* transcript levels were highest early in growth and generally decreased until 21 DAI (Fig. 8A, 8B). Conversely, *ZmNRT1.1A* transcript levels in B73 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 15 - 21 DAI. Unlike B73, *ZmNRT1.1A* transcript levels in Mo17 seedlings grown in 2.5 mM NO_3^- matched those in the 0.5 mM treatment until 7 DAI, where they then increased at 8 DAI, then steadily decreased from 11 DAI through to the final sampling day. Transcript levels of *ZmNRT1.1B* in B73 seedlings grown in 0.5 mM NO_3^- remained similar 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^- (Fig. 8C). A similar trend was observed in Mo17 (Fig. 8D), however NO_3^- treatment differences emerged from 12 DAI. *ZmNRT1.5A* transcript levels in B73 seedlings, decreased by more than half 8 – 9 DAI, across both NO_3^- treatments where it then remained steady through the remaining sampling days (Fig. 8E). In Mo17 seedlings grown in 2.5 mM NO_3^-, *ZmNRT1.5A* transcript levels progressively declined 6 - 21 DAI (Fig. 8F), whereas those in the 0.5 mM treatment increased 9 – 10 DAI, however, decreased from 12 DAI.

Root nitrate reductase (NR) gene expression

In B73, *ZmNR1* transcript levels were similar across both NO_3^- treatments and slowly increased from 13 DAI (Fig. 9A). In seedlings grown in 0.5 mM NO_3^-, *ZmNR1* transcripts reached maximum levels 15 DAI, whilst those in the 2.5 mM treatment continued rising, to levels twice those grown in 0.5 mM NO_3^- 21 DAI, similar to *ZmNRT2.2* transcript levels. *ZmNR1* transcript levels in Mo17 also shared a similar trend to *ZmNRT2.2*, having NO_3^- treatment differences emerge from 15 DAI, as transcript levels in seedlings grown in 0.5 mM NO_3^- decreased to levels lower than those grown in the 2.5 mM treatment (Fig. 9B).

ZmNR2 transcript levels followed a different trend to *ZmNR1*. In B73 seedlings grown in 0.5 mM NO_3^-, *ZmNR2* transcript levels began increasing 10 DAI, reaching peak levels at 15 DAI, before decreasing back to baseline at 21 DAI (Fig. 9C). This was similarly observed in B73 seedlings grown in 2.5 mM NO_3^-; however, *ZmNR2* transcript levels began increasing earlier and at higher levels than those in the 0.5 mM treatment, before returning to baseline 15 – 21 DAI. In Mo17 seedlings grown in 0.5 mM NO_3^-, *ZmNR2* transcript levels remained low across sampling days, whereas those in the 2.5 mM treatment increased until 15 DAI, then returned to baseline 21 DAI (Fig. 9D).

DISCUSSION

Within this study B73 had a greater response to increasing N supply than Mo17, achieving greater net N uptake and shoot growth in 2.5 mM N. We hypothesise that B73 may achieve this by having a greater shoot mass, relative to its root (evident by its lower root:shoot compared to Mo17). This may be due to lower allocation of C to root growth, which would favour shoot growth and greater net photosynthesis, potentially increasing root N capture (Bonifas *et al.*, 2005; Davidson, 1969; Ericsson, 1995; Lambers *et al.*, 2008). Conversely, Mo17 has a higher root:shoot, allocating more C to root growth, relative to its shoots, possibly reducing its shoot growth potential. Although the above N response is observed in seedlings

within this study, Balko and Russell (1980) showed that the final grain yield of B73 was more responsive to N supply than Mo17 across four field environments, suggesting that the N-responsiveness in seedling stage may impact growth at maturity and yield. Seedling vigour often determines competitiveness and resource capture and often positively correlates with seed mass (Richards and Lukacs, 2002) and protein content (Evans and Bhatt, 1977; Ries and Everson, 1973). However here, seed mass was kept similar between lines, and although seed protein was not quantified, the initial N content in B73 seeds was lower than Mo17. This suggests that the differences in N-responsiveness between these lines may likely be due to other traits associated with these lines, rather than differences in seed size and seed N content.

During early growth, root:shoot, shoot N % and concentrations of shoot and root free amino acids all rapidly decreased until 8 DAI, independent of external NO_3^- supply. This is in agreement with Srivastava *et al.* (1976), showing that external NO_3^- supply had little effect on maize seedling N content whilst growth was supported by the seed. Although the decreasing shoot N % is due to dilution as a consequence of shoot emergence, we hypothesise that free amino acid concentrations rapidly decrease due to a combination of dilution and incorporation into newly-synthesized proteins.

Following seed N exhaustion, 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 strongly corresponded with *ZmNRT2.1* and *ZmNRT2.2* transcript levels, suggesting these NRTs facilitate root HATS NO_3^- uptake in maize. Numerous studies have characterised root NO_3^- uptake in <5 d old maize seedlings (Colmer and Bloom, 1998; Jackson *et al.*, 1973; Neyra and Hageman, 1975; Taylor and Bloom, 1998). We suggest that the results obtained from these studies should be interpreted keeping in mind that in our study, transcription of NO_3^- inducible *NRT2* genes and NO_3^- uptake capacity itself had not substantially risen by this time. The rise in NO_3^- uptake

capacity and *ZmNRT2.1* and *ZmNRT2.2* transcripts corresponded with a rise in *ZmNRT3.1A* transcript levels, 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 the NO_3^- treatment differences in HATS activity profiles may also be related to reduced *ZmNRT3.1A* transcription, given the capacity of NRT3.1 to influence NRT2 NO_3^- transport activity (Kotur *et al.*, 2012; Okamoto *et al.*, 2006; Yan *et al.*, 2011). Prior to the rise in root NO_3^- capture, there was an abundance of root *ZmNRT1.1* transcripts. We hypothesise this may provide base-level root NO_3^- uptake whilst N demand is low; then as seedling N demand rises and is not met by NRT1 mediated NO_3^- uptake, *NRT2* transcription and HATS uptake is up-regulated.

Given that transcript levels of *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A, ZmNR1, ZmNR2* and NO_3^- uptake capacity increase shortly after free amino acid concentrations decrease, we propose that this may be the physiological cue triggering the up-regulation of the NO_3^- uptake system. This supports earlier studies showing that NR activity rises as the products of seed protein hydrolysis deplete (Sivasankar and Oaks, 1995), and studies suggesting the repression of *NRT2* transcription and root NO_3^- uptake may be the result of free amino acid accumulation in tissues, in particular glutamine, resulting from exogenous application of amino acids (Nazoa *et al.*, 2003; Vidmar *et al.*, 2000; Zhuo *et al.*, 1999). Indeed, root NO_3^- concentrations also decreased prior to NO_3^- uptake capacity increasing within this study, however we believe this did not contribute to this 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 this transition, excluding the involvement of shoot NO_3^- concentrations to this cue.

It was anticipated that seedlings would respond to low N by increasing transcript levels of *ZmNRT2.1*, *ZmNRT2.2* and ultimately HATS activity to beyond those in sufficient N

to meet demand, as shown in dwarf maize (Garnett *et al.*, 2013), however this was not observed here. In fact in Mo17, *ZmNRT2.2* transcript levels and HATS activity decreased in low N, yet shoot growth and net N uptake was maintained, with respect to sufficient N. As the decreased HATS activity in low N was only observed on the final sampling day, the maintenance of net N uptake may be contributed by increasing root DW, as the percentage increase in root DW was equivalent to the percentage decrease in HATS activity (per gram root DW). This suggests that when N demand is not being met by up-regulating the NO_3^- uptake system in low N, Mo17 may down-regulate this system and resort to increasing root biomass, possibly to increase the absorptive area of the root. The increase in Mo17 root DW at low N was reflected in a higher root:shoot compared to seedlings in sufficient N, possibly reflecting an adaptive response to low N enabling Mo17 to continue meeting N demand. The root:shoot of both lines increased in low N, relative to sufficient N, just prior to shoot N % stabilising. However, NO_3^- treatment differences in B73 root:shoot were characterised by differences in shoot growth, rather than root growth, possibly due to its greater shoot growth in increased N supply, or conversely due to reduced shoot growth in low N, resulting from decreased shoot N supply from the root. *ZmNRT2.5* was the only *NRT2* that was up-regulated in low N, supporting its possible involvement in responses to low N limitation (Garnett *et al.*, 2013; Lezhneva *et al.*, 2014), however its transcript profile does not correspond with NO_3^- uptake capacity. In *Arabidopsis* roots, the expression of NRT2.5 is localised to the epidermis and cortex of roots at the root hair zone, and facilitates HATS NO_3^- uptake in N-starved plants (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 it may be the inducible HATS component involved in NO_3^- loading into the xylem (Tang *et al.*, 2012). Given the discrepancy in the localisation and proposed function of

NRT2.5 between *Arabidopsis* and cereals, further investigation is required to further clarify its role, at least in cereals.

Despite the differences between lines and NO_3^- treatments in net N uptake and growth, the processes leading to, and involved in the up-regulation of the NO_3^- uptake system were similar. Seed N content and free amino acid concentrations rapidly decreased 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 impacted growth, reflected in an increase in root:shoot, to values beyond seedlings in sufficient N. This may be because during early growth, low N supply may satisfy the low N demand of seedlings, whereas later N demand may exceed supply, suggesting that small differences in N uptake and growth are being magnified over time as consequence of increasing N demand. Given the order of the physiological processes involved in NO_3^- uptake and assimilation observed, we propose a model outlining the key processes managing the transition from seed N use to external N capture (Fig. 10). We believe that this model captures how seedlings manage this transition, to maintain control of plant N status during early vegetative growth, and how this can be influenced by NO_3^- supply. This model may also be applicable to other stages of the plant lifecycle, where root NO_3^- uptake capacity increases to meet demand. Future work will focus on the global gene expression during this key developmental stage of plant growth, to begin elucidating the molecular regulation of NO_3^- uptake capacity. This knowledge may ultimately help develop cereal crops with enhanced NO_3^- uptake capacities.

SUPPLEMENTARY MATERIAL

Additional supporting information may be found in the online version of this article.

Fig. S1 Individual free amino acid concentrations in fresh B73 shoot and root tissue.

Fig. S2 Individual free amino acid concentrations in fresh Mo17 shoot and root tissue.

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FIGURES

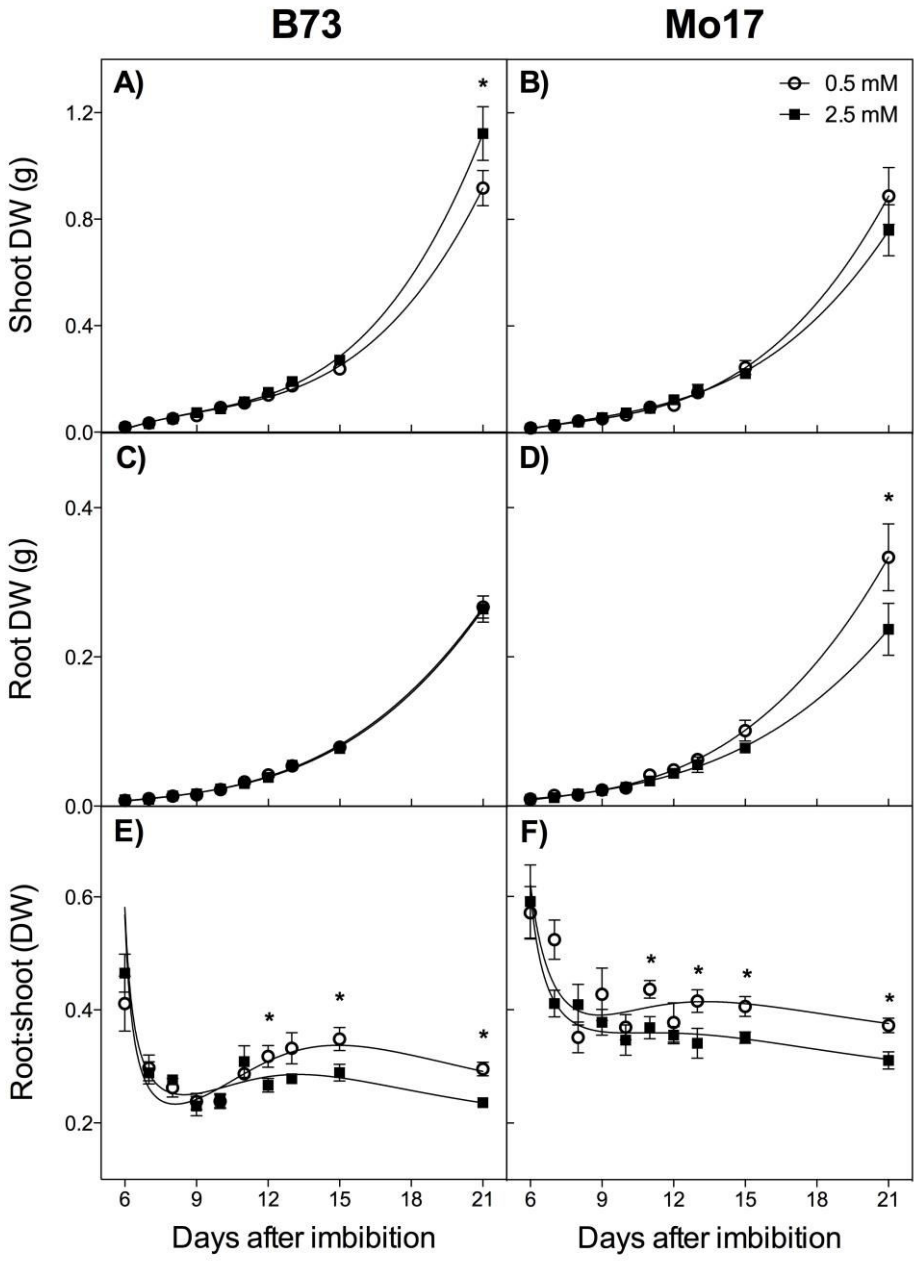

Fig. 1 Growth parameters of maize (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. **(A, B)** shoot dry weight (DW), **(C, D)** root DW, and **(E, F)** root:shoot were measured until 21 d after imbibition. A cubic polynomial function was fitted to tissue DWs. Values generated from cubic functions were plotted along with root:shoot (solid lines). Values are means ± S.E.M (n = 8). *Points significantly different between the two growth conditions ($P < 0.05$).

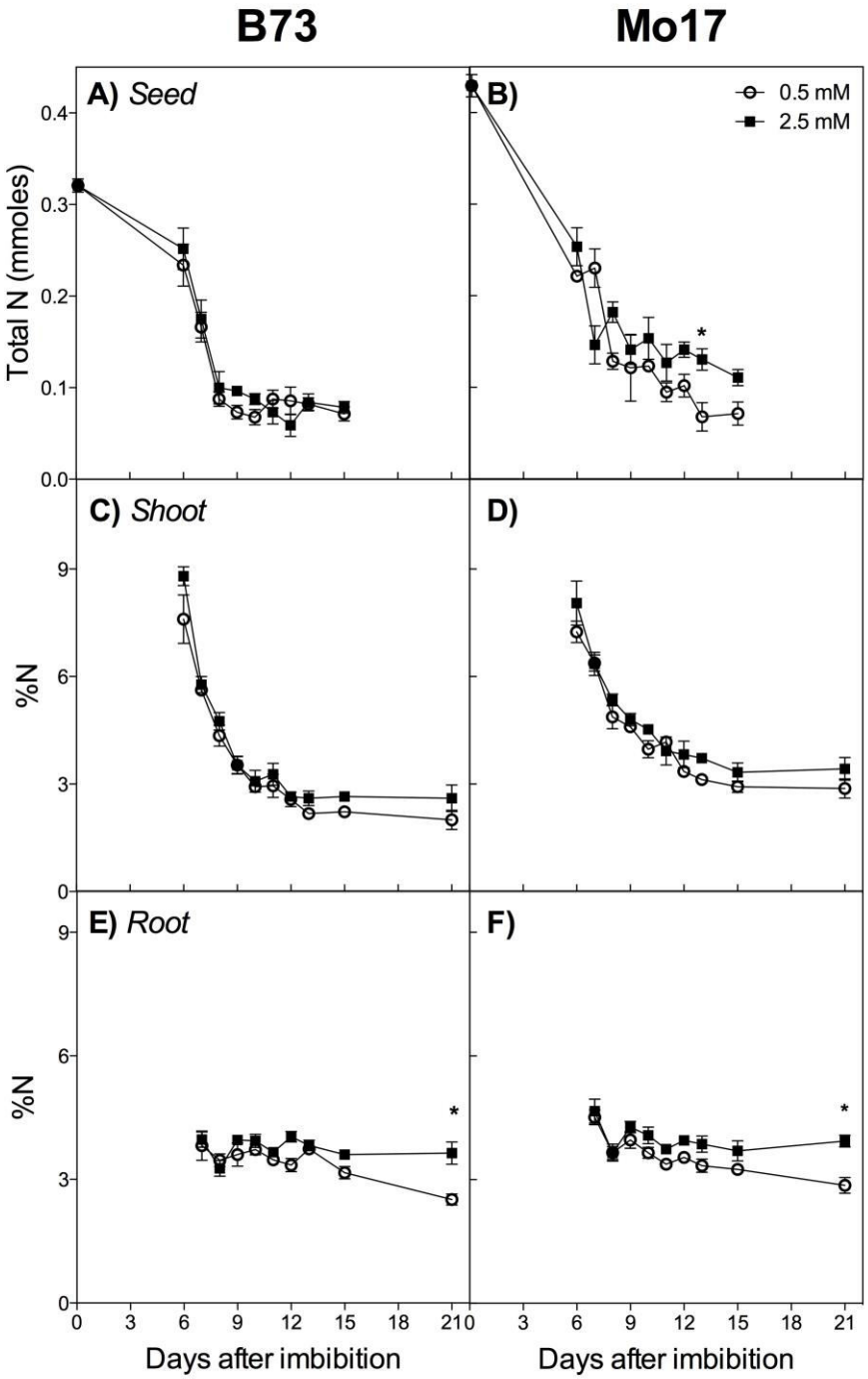

Fig. 2 Nitrogen content in maize (*Zea mays* var. B73 and Mo17) tissue, sampled up to 21 d after imbibition. Plants were grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^- and **(A, B)** total seed N, as well as N% in **(C, D)** shoot, and **(E, F)** root tissue was measured from dried samples. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions ($P < 0.05$).

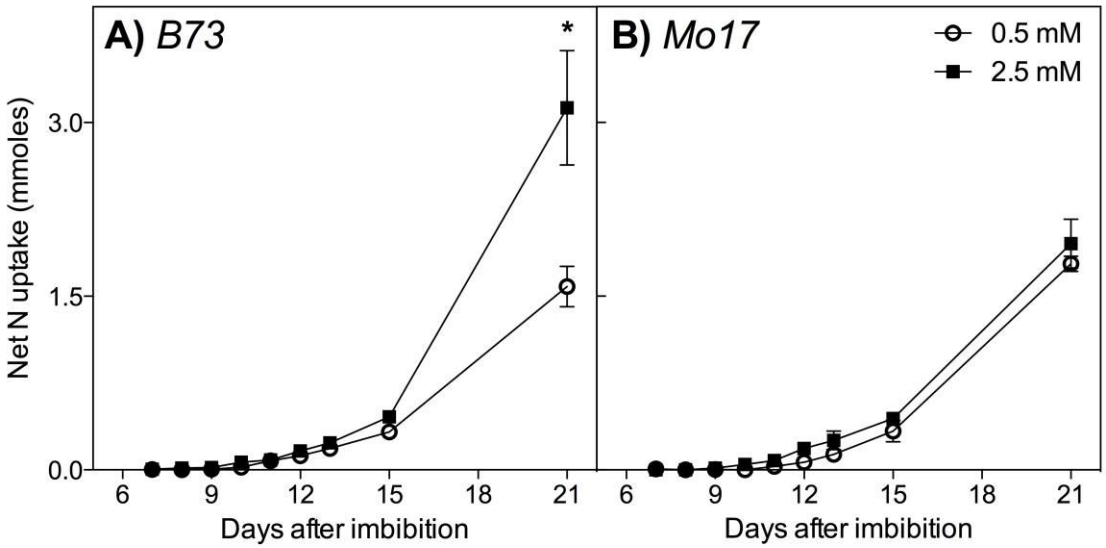

Fig. 3 Net N uptake in **(A)** B73 and **(B)** Mo17 grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^- . Values were calculated as described in materials and methods. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions (P < 0.05).

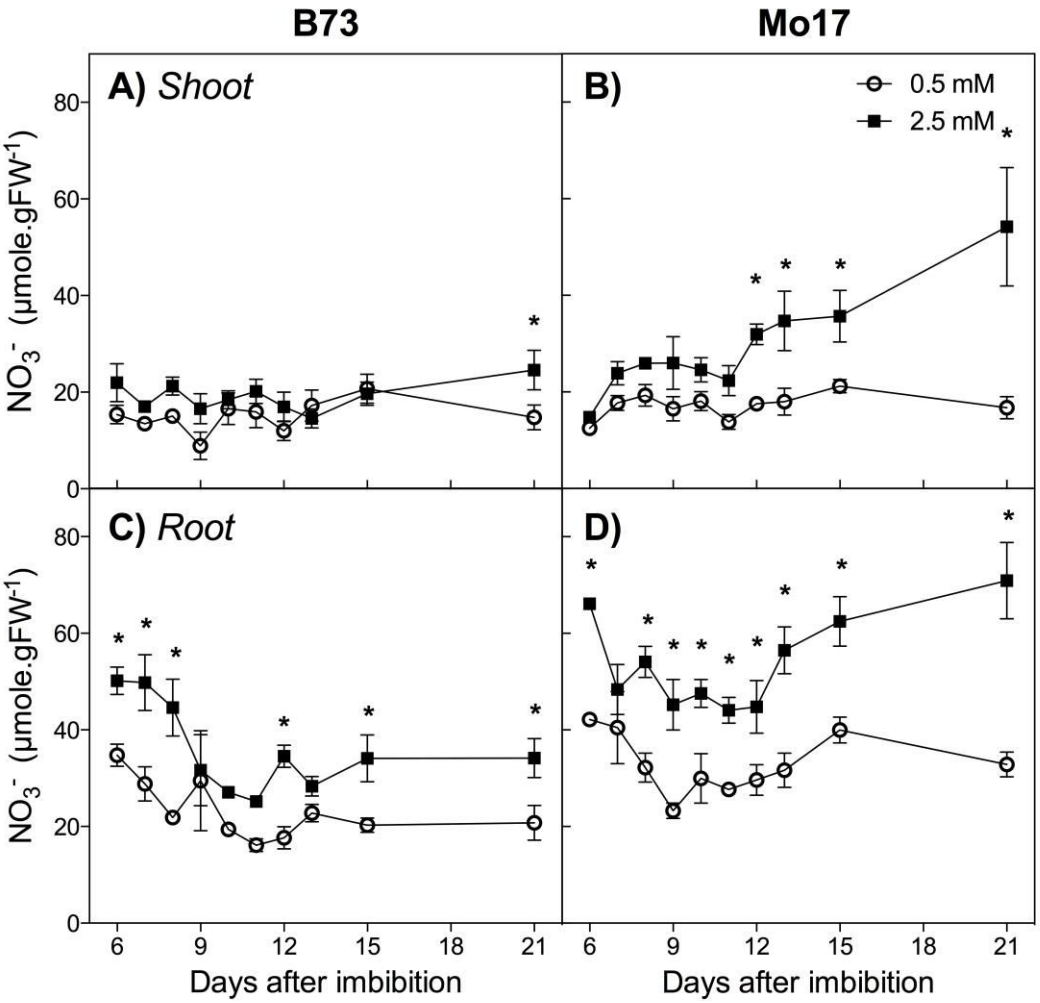

Fig. 4 Nitrate concentration in fresh maize (*Zea mays* var. B73 and Mo17) **(A, B)** shoot and **(C, D)** root tissue. Plants were grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions ($P < 0.05$).

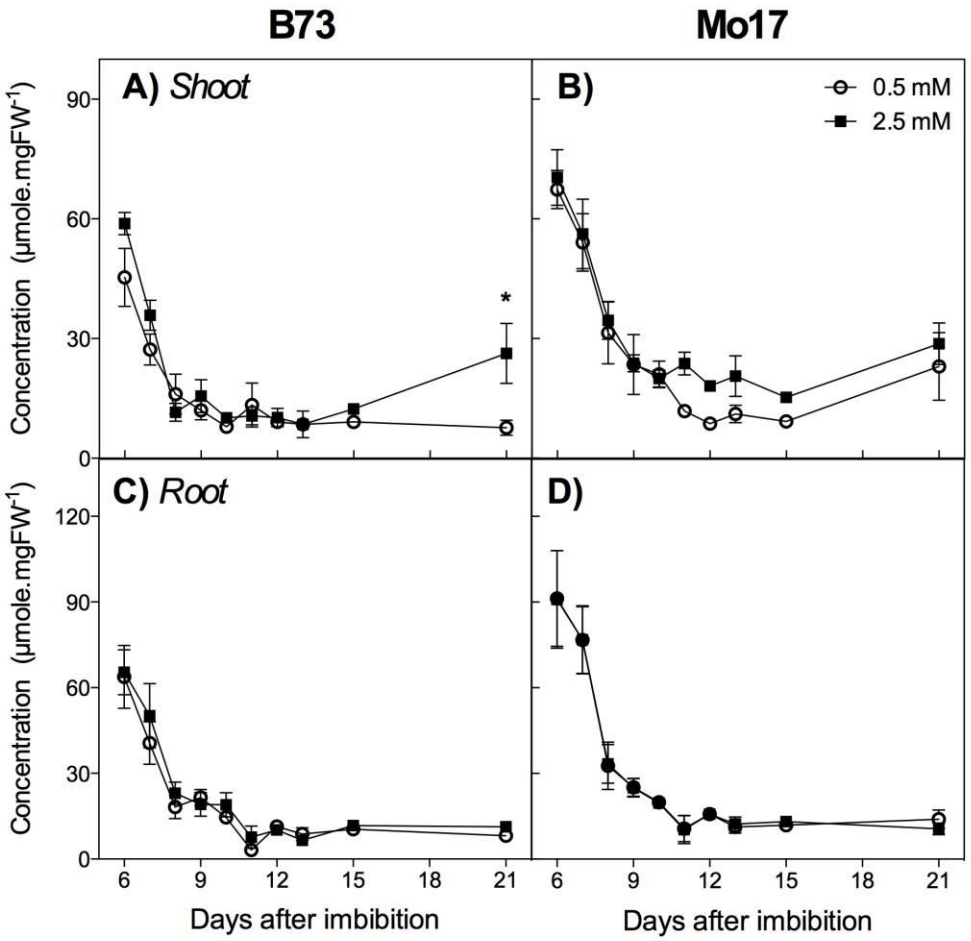

Fig. 5 Concentrations of total free amino acids in **(A, B)** shoot and **(C, D)** root tissue of maize (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Values are means ± S.E.M (n = 4). *Points significantly different between the two growth conditions ($P < 0.05$).

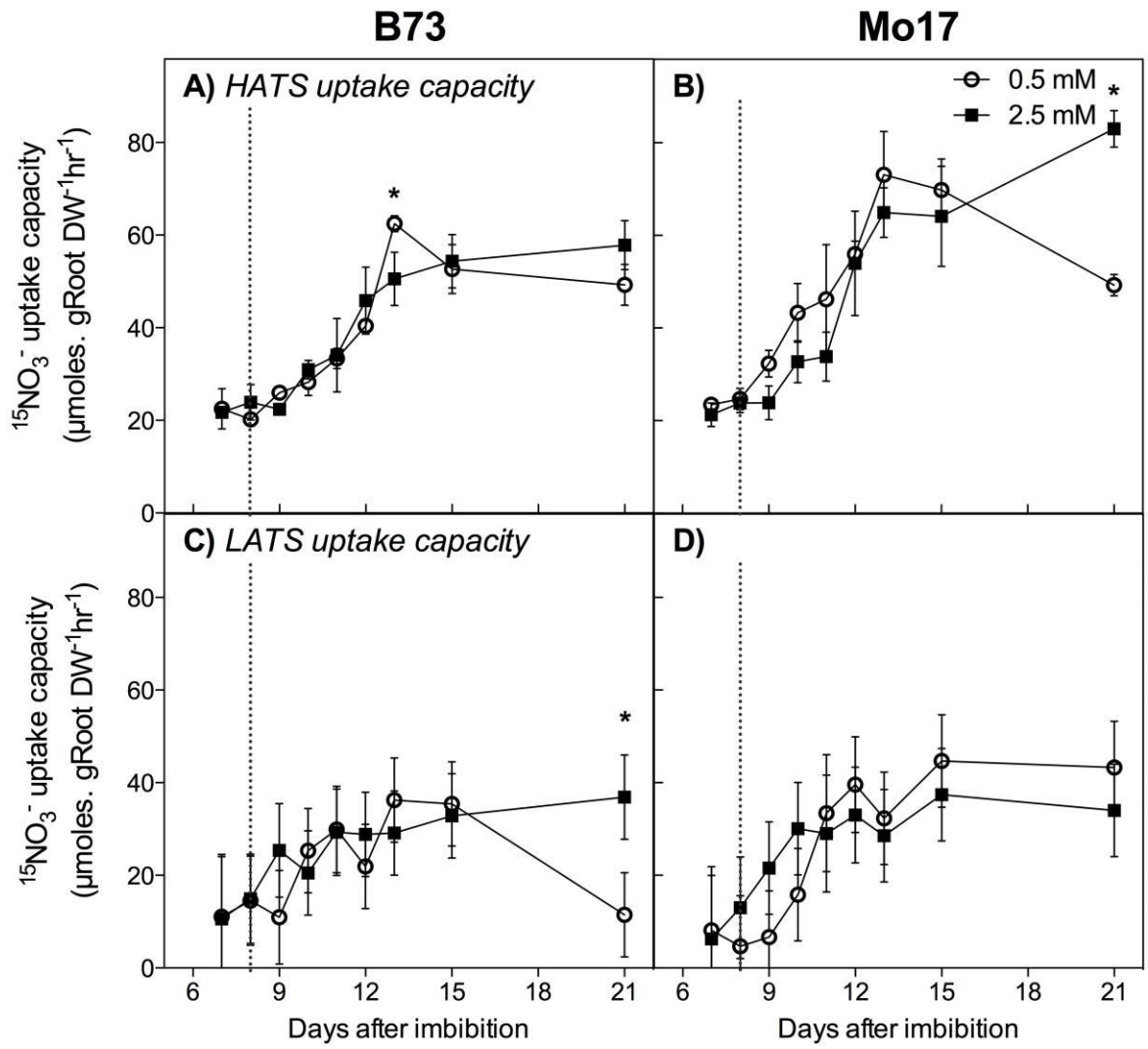

Fig. 6 (A, B) HATS and **(C, D)** calculated LATS NO_3^- uptake capacity of maize (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. HATS values are means ± S.E.M (n = 4), whereas those of LATS are calculated means ± S.E.D. Dotted line at 8 DAI represents the time-point when free amino acids concentrations began to stabilise. *Points significantly different between the two growth conditions (HATS $P < 0.05$; LATS $\alpha = 0.05$).

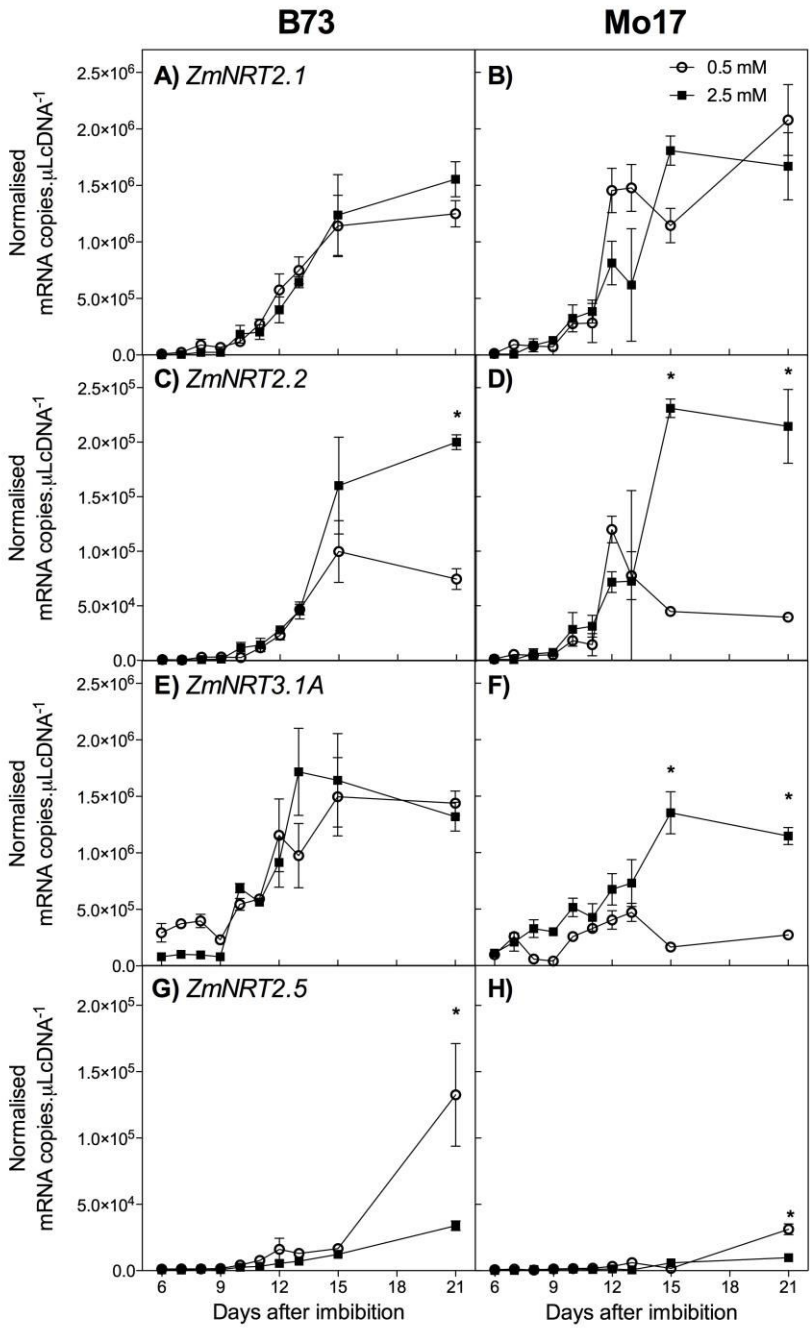

Fig. 7 Transcript levels of **(A, B)** *ZmNRT2.1*, **(C, D)** *ZmNRT2.2,* **(E, F)** *ZmNRT3.1A,* and **(G, H)** *ZmNRT2.5* in maize roots (*Zea mays* var. B73 and Mo17). Plants were grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Each data point is normalised to control genes as described in materials and methods. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions ($P < 0.05$).

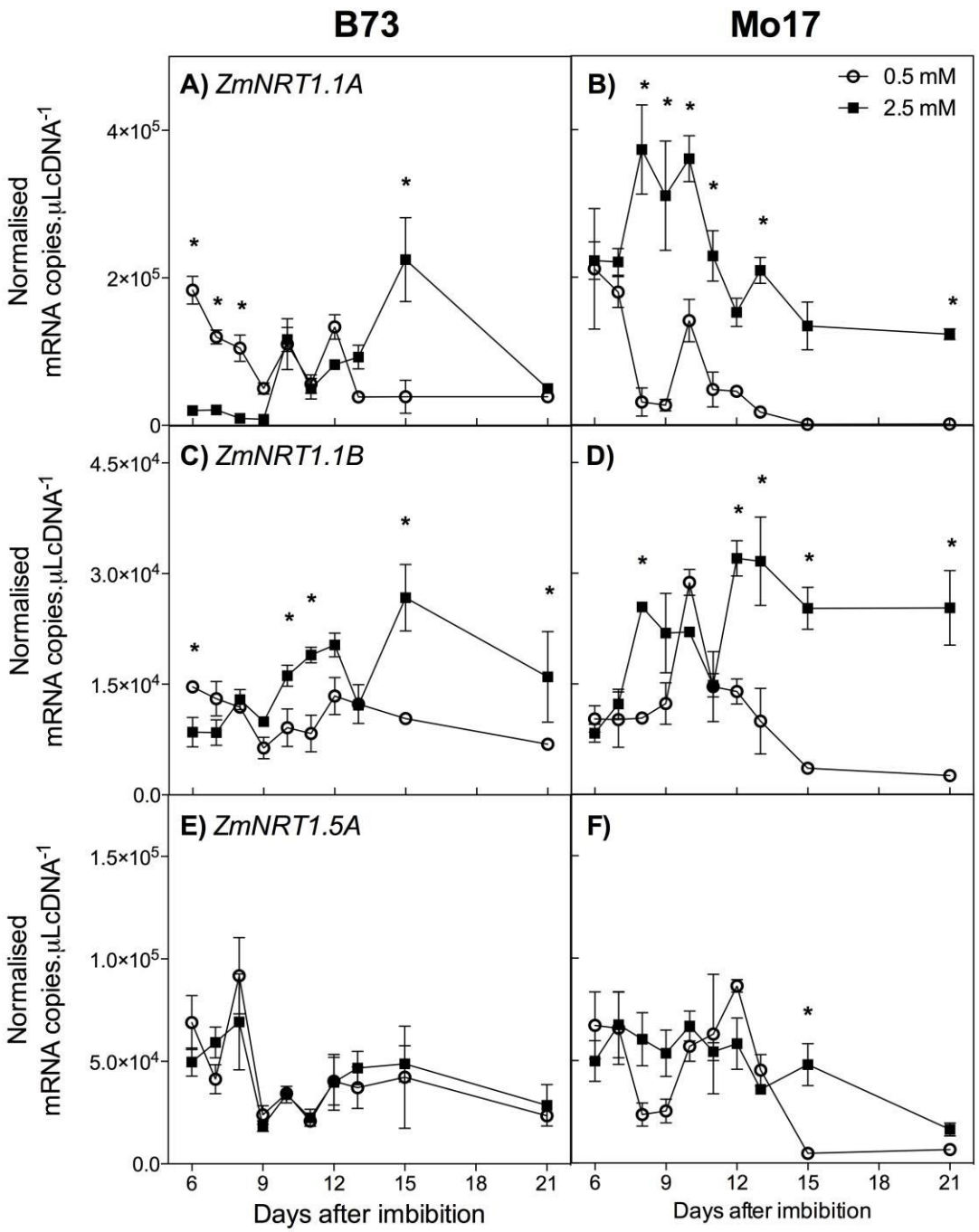

Fig. 8 Transcript levels of *NRT1* genes **(A, B)** *ZmNRT1.1A* **(C, D)** *ZmNRT1.1B,* and **(E, F)** *ZmNRT1.5A* in maize roots (*Zea mays* var. B73 and Mo17). Plants were grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Each data point is normalised to control genes as described in materials and methods. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions ($P < 0.05$).

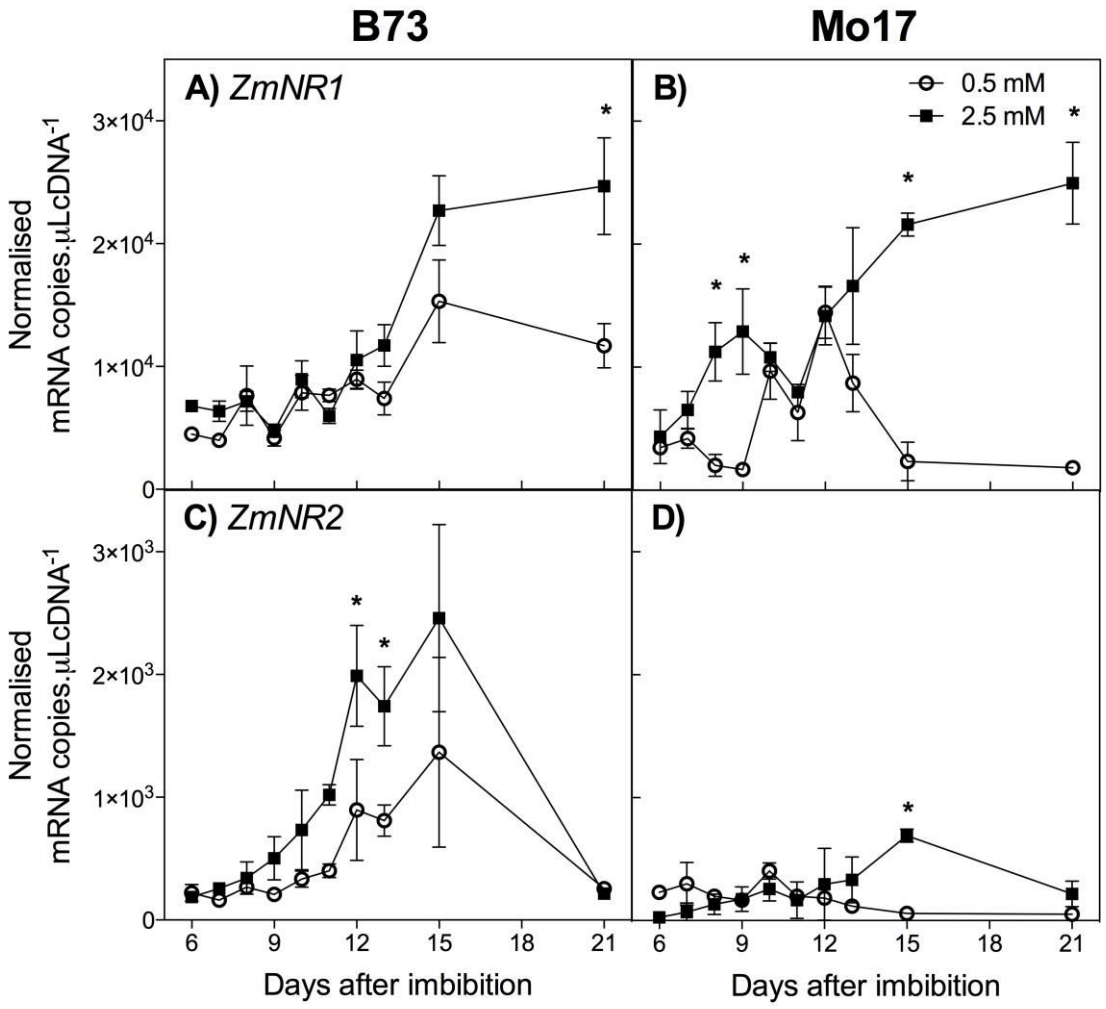

Fig. 9 Transcript levels two *NADH:NR* genes; **(A, B)** *ZmNR1* and **(C, D)** *ZmNR2*, in maize roots (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Each data point is normalised to control genes as described in materials and methods. Values are means ± S.E.M (n = 4). *Points significantly different between two growth conditions ($P < 0.05$).

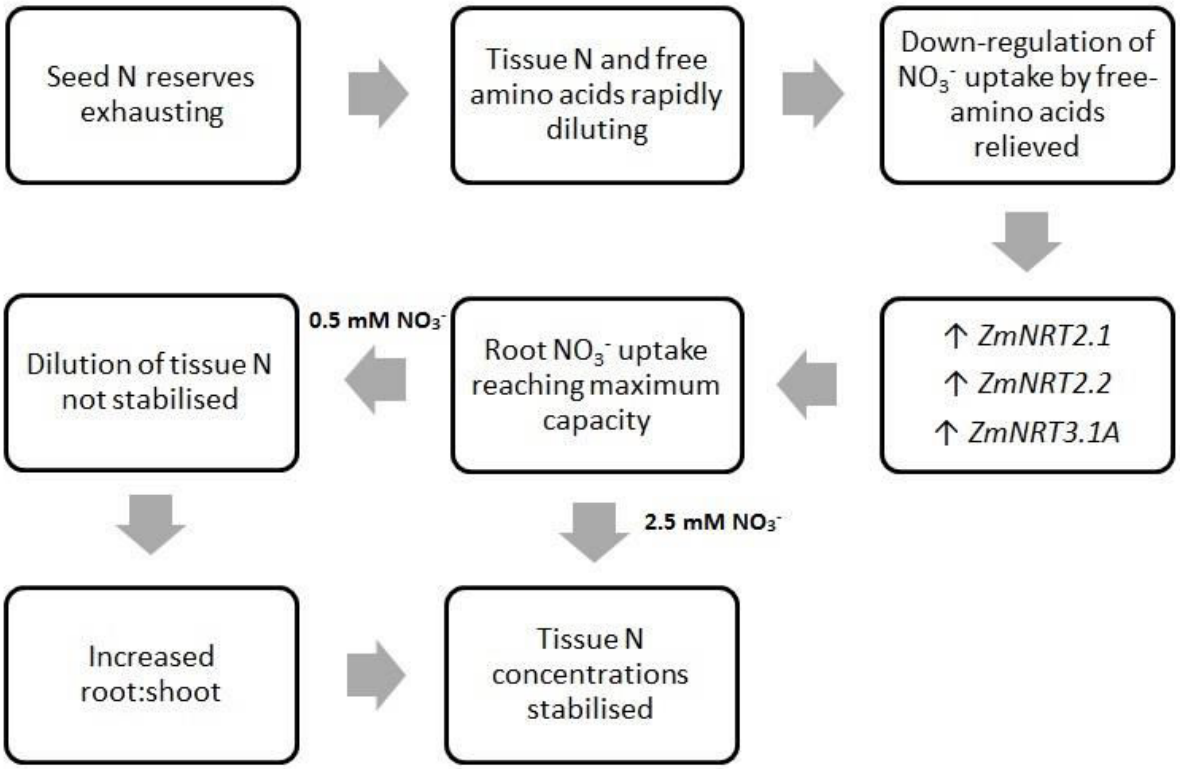

Fig. 10 Proposed model detailing the how maize (*Zea mays* L.) seedlings manage the transition from seed N use to external NO_3^- capture to maintain plant N status.

SUPPLEMENTARY MATERIAL

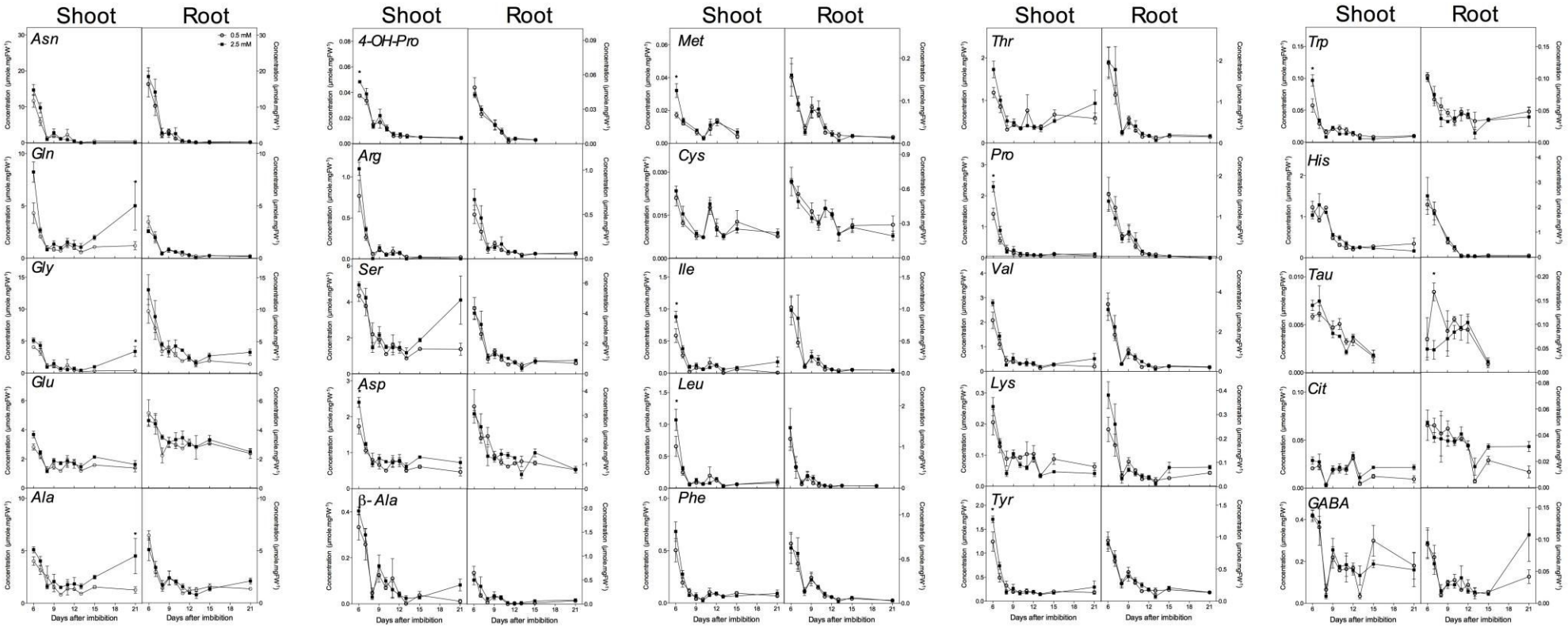

Fig. S1 Individual free amino acid concentrations in fresh shoot and root tissue of maize (*Zea mays* var. B73) grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Values are means ± S.E.M (n = 4). *Points significantly different between the two growth conditions ($P < 0.05$).

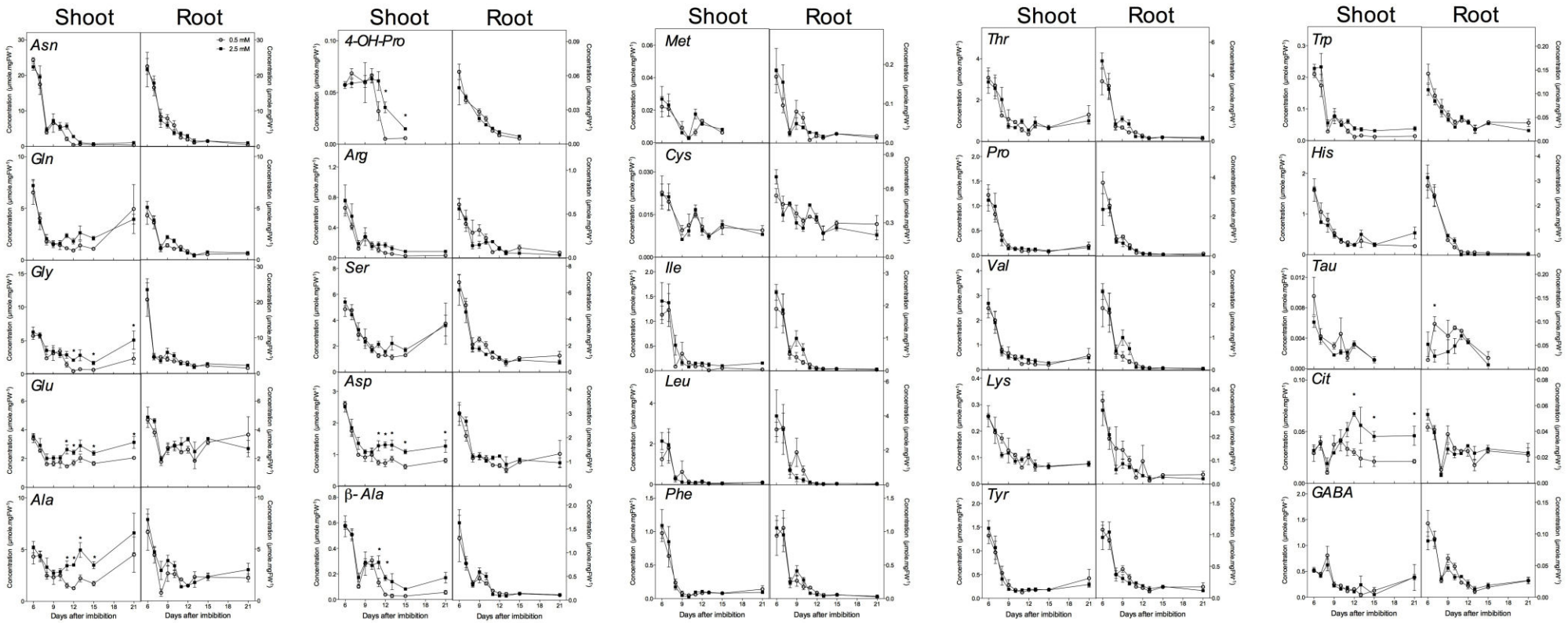

Fig. S2 Individual free amino acid concentrations in fresh shoot and root tissue of maize (*Zea mays* var. Mo17), grown in 0.5 mM (open circles) or 2.5 mM (closed squares) NO_3^-. Values are means ± S.E.M ($n = 4$). *Points significantly different between the two growth conditions ($P < 0.05$).

Chapter 3: Responses of maize seedling root morphology to nitrate supply

TITLE

Responses of maize seedling root morphology to nitrate supply

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ABSTRACT

The poor nitrogen (N) uptake efficiency of cereals leads to substantial losses of costly N fertilisers. One possible avenue for improvement is through optimising root morphology for N uptake, however our understanding of root morphology and its adaptation to N supply is limited due to the difficulties associated with phenotyping them in soil. This study investigates how maize root morphology adapts to nitrate (NO_3^-) supply in hydroponics, how early in growth this occurs, and whether this differs between maize inbred lines (*Zea mays* var. B73 and Mo17). Time-course analyses of roots show that NO_3^- limitation leads to changes in morphology by 17 d after imbibition. Although B73 achieve greater absorption area per unit root mass than Mo17, its root morphology does not change in response to NO_3^- limitation. Conversely, Mo17 roots respond by increasing axial and lateral root length, before an increase in root mass or volume is observed. Additionally, the axial roots of Mo17 are thick, with a broad distribution of lateral roots along them, relative to B73, each of which may be traits desirable for low-input agricultural systems. This highlights that morphological root responses to NO_3^- supply differ within maize and can occur early in seedling development.

Keyword index

Axial root, development, early growth, lateral root, Zea mays

INTRODUCTION

Currently, over 100 Mt of nitrogen (N) fertilisers are applied to crops each year to maximise growth and ultimately yield, with around 60 % applied to cereals (Heffer, 2013). Given that a 50 – 70 % increase in cereal crop production is projected to be required by the year 2050 to feed an estimated population of 9.3 billion people (Umar and Abrol, 2011), this will be linked to a substantial increase in N fertiliser use. However currently, cereal crops only capture 40 – 50 % of the applied N (Peoples *et al.*, 1995; Sylvester-Bradley and Kindred, 2009), allowing for much of the remaining N to be lost by leaching into groundwater; surface run-off;

volatilisation into the atmosphere; and bacterial denitrification (Vitousek *et al.*, 1997). This low N uptake efficiency is partly due to current agronomic practice (Schepers *et al.*, 1991), but also due to the inefficiency of the crops themselves in capturing N. Nitrogen use efficiency (NUE) is defined as the yield obtained per unit available N (Hirel *et al.*, 2011), and may be improved by increasing N capture per crop, relative to available N (increased N uptake efficiency; NUpE), or improving the use of the N they capture (increased N utilisation efficiency; NUtE) (Garnett *et al.*, 2009). However, given the inefficiency of cereals to capture N, there is scope to improve cereal NUpE by increasing root N capture, to reduce N losses. Given that nitrate (NO_3^-) is the predominant form of N available to crops in most agricultural soils, this will be the focus of this study (Wolt, 1994).

Compared to immobile nutrients like phosphorus, root morphology is thought to be of lesser importance than uptake capacity for the acquisition of soil mobile nutrients like NO_3^- (Barber, 1995; Tinker and Nye, 2000). However, several studies highlight the significance of morphological root traits, such as rooting depth and root length per unit soil volume (root length density) in maximising NO_3^- recovery in subsoil layers (Kristensen and Thorup-Kristensen, 2004) and reducing NO_3^- leaching (Habib *et al.*, 1991; Wiesler and Horst, 1993, 1994; Liao *et al.*, 2004; Liao *et al.*, 2006). Moreover, a study using turf-grass observed correlations between root length, surface area and root fineness with NO_3^- uptake rates (Sullivan *et al.*, 2000).

Simply increasing root size, relative to the shoot, should increase the effective absorptive area of the root, and ultimately resource capture. However, this comes at a greater carbon (C) cost to the shoot, reducing shoot growth potential, and hence C capture (Davidson, 1969; Ericsson, 1995; Bonifas *et al.*, 2005; Lambers *et al.*, 2008). A more efficient strategy is to optimise root morphology to maximise N uptake with minimal extra C input. However, the

role of root morphology in NO_3^- uptake remains poorly understood, chiefly due to the difficulties associated with phenotyping roots in soil, as it requires extensive excavation processes that introduce artefacts when quantifying root architecture (Smit, 2000). Furthermore, the heterogeneity found across most soil profiles drastically influences root morphology which makes data interpretation challenging (Clark *et al.*, 2011). Despite this, a number of morphological root traits have been suggested to contribute to NUpE. The ability to quickly develop a root system (early-vigour) enables plants to quickly explore soil and recover more NO_3^-, reducing leaching potential, particularly in sandy soil environments (Liao *et al.*, 2004; Liao *et al.*, 2006). Likewise, greater root length per unit root volume (greater proportion of finer roots relative to wider diameter roots) can provide a greater root surface area whilst minimising C cost (Marschner, 2012), potentially enabling greater N uptake rates and reduced leaching (Wiesler and Horst, 1993, 1994). Deep growing roots are also suggested to improve N capture, as they can access leached NO_3^- and water in subsoil layers that may be inaccessible to shallower roots (Lynch, 2013). On the other hand, deep roots often come with a greater C cost; consequently, this trait may only be worthwhile when water and nutrient availability in the upper soil profile is limited.

Root morphology can adapt to N supply. Classically, roots have shown to focus lateral root proliferation towards localised N-rich patches of soil (Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Laine *et al.*, 1995). This ensures that C allocated to root growth is not wasted towards regions where N is limited, which may be important in environments where soil N heterogeneity is high (Garnett *et al.*, 2009). Wang *et al.* (2005) highlighted that the mass and effective absorptive area of 32 d old maize seedling roots increased with decreasing N supply. Also, genotypic comparison of root morphology showed that in very low N, the effective absorptive area of the root, which was mainly contributed by long axial roots (primary and seminal roots), was important for tissue N accumulation. Maizlish *et al.* (1980)

quantified the responses of maize roots to N supply across three time-points during seedling development, using five different N levels, highlighting that root length increased with increasing N supply as early as 10 d after emergence. However, these measurements were taken with low temporal resolution (7 d intervals) and using a single maize line, hindering the opportunity to investigate how these responses may differ within maize.

The aforementioned studies were generally conducted using a single N treatment, time-point or genotype, limiting the opportunity understand when roots adapt to N availability and whether genetic variation exists for any adaptive response. This study investigates the influence of NO_3^- supply on maize root morphology (*Zea mays* var. B73 and Mo17), by quantifying the morphological root parameters in seedlings grown using low or sufficient NO_3^- supply, with high temporal resolution. These lines were selected as they are the parents of the intermated B73 × Mo17 (IBM) mapping population, providing future opportunity to dissect the genetic basis for the regulation of root responses to N using a forward genetics approach. A time-course study in hydroponics has already shown that B73 root growth was not responsive to N supply by 21 d after imbibition (DAI), whereas Mo17 increased root mass in low N, relative to the sufficient N treatment, suggesting Mo17 may adapt to N supply by modulating root growth and morphology (Sabermanesh, 2014). Although the N treatment differences in growth were evident 21 DAI within the mentioned study, changes to root:shoot occurred as early as 12 DAI, suggesting that growth changes due to N supply may occur much earlier in development. This provides the scope to not only clarify when changes to root morphology occur as a result of N supply, but also how it may differ within maize.

MATERIALS AND METHODS

Plant growth

Maize seeds (*Zea mays* L. var. B73 and Mo17) of similar size were imbibed in aerated RO-H_2O 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 six 120 L ebb and flow hydroponic systems, with a complete fill/drain cycle of 30 min (three separate systems for each NO_3^- treatment). Individual seedlings were grown on mesh collars within tubes (300 mm × 50 mm). This allowed the roots to remain separate from adjacent seedlings, whilst allowing free access to solution. The hydroponic system was situated in a controlled environment room with a day:night cycle of 14 h:10 h, 26 °C:20 °C, with a flux density at canopy level of *c.* 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 NO_3^- N, 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P for the 0.5 mM NO_3^- treatment, and 2.5 NO_3^- N, 1.8 K, 0.6 Ca, 0.5 Mg, 0.5 S, 0.5 P for the 2.5 mM NO_3^- treatment. The choice of NO_3^- concentrations was based on those used by Garnett *et al.* (2013), as *c.* 0.5 mM was suggested to be the threshold concentration eliciting a major response of the maize N uptake system. Both treatment solutions also contained (in µM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and ethylendiamine-*N*,*N'*-bis(2-hydroxyphenylacetic acid) (FeEDDHA)). Iron was supplemented twice weekly with the addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (8 mg L^{-1}) to avoid Fe deficiency (Cramer *et al.*, 1994). Solutions were maintained within 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 NO_3^- were monitored using a NO_3^- 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 seedlings were sampled, and roots were separated from the remainder of the plant, blotted and weighed. 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 are comprised of both primary and seminal roots, whereas lateral roots are defined as the roots developing from axial roots (Fig. S1). Lateral roots were differentiated from the axial roots on WinRHIZO using a distinguishing diameter of 0.677 mm (verified for both inbred lines and NO_3^- treatments). The number of axial roots was manually counted 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 minimal (< 2 %). The average lateral and axial root length was calculated by dividing the total length of the root type by the total number of the root type. Lateral root density was calculated by dividing the total number of root tips by total axial root length.

Statistical analyses

Seedlings were selected randomly from three separate hydroponic systems (each NO_3^- treatment), which constituted blocks. Each replicate was a single plant. There was no significant difference between blocks. All statistical analyses within this study were carried out using two-way analysis of variance (ANOVA).

RESULTS

Plant biomass

B73 seedlings had lower shoot fresh weight (FW) in 0.5 mM NO_3^-, relative to the 2.5 mM treatment from 14 - 17 DAI (Fig. 1A), whereas no NO_3^- treatment differences in Mo17 shoot FW were observed (Fig. 1B). No NO_3^- treatment differences were observed in the root FW of either line across sampling days (Fig. 1C, 1D). However, Mo17 roots were larger than B73, as the root FW of B73 at 17 DAI was 55 % of Mo17. Root:shoot (FW) did not differ between NO_3^- treatments, however compared to B73, the root:shoot of Mo17 was initially higher (Fig. 1E, 1F).

Whole root morphology

The total length (Fig. 2A, 2B) and surface area (Fig. 2C, 2D) of B73 roots in both NO_3^- treatments did not differ from Mo17 roots in 2.5 mM NO_3^-. However at 17 DAI, the total length and surface area of Mo17 roots grown in 0.5 mM NO_3^- were 22 % and 31 % greater than those in the 2.5 mM treatment, respectively. Conversely, no NO_3^- treatment differences in total root volume were observed in B73 (Fig. 2E) and Mo17 (Fig. 2F) roots by 17 DAI. Differences in the root morphology between lines were measured in morphological root parameters relative to root FW. Although B73 roots were smaller in terms of mass, they achieved greater length (Fig. 3A, 3B) and surface area (Fig. 3C, 3D) per unit root FW at 17 DAI, compared to Mo17. Unlike the length and surface area per unit root FW, root volume per unit root FW was similar between lines and treatments (Fig. 3E, 3F).

Axial roots

Axial roots provide the scaffold from which lateral roots develop. Between both lines and NO_3^- treatments, the number of axial roots increased until 12 DAI, establishing approximately six axial roots (Fig. 4A, 4B). The total length (Fig. 4C), surface area (Fig. 4E) and volume

(Fig. 4G) of B73 axial roots did not differ between NO_3^- treatments. Conversely, the axial root length (Fig. 4D) and surface area (Fig. 4F) of Mo17 seedlings grown were greater in 0.5 mM NO_3^-, compared to the 2.5 mM treatment, 14 – 17 DAI, whilst no NO_3^- treatment differences in axial root volume were observed (Fig. 4H). Similar to total axial root length, the average axial root length was similar between B73 and Mo17 seedlings grown in 2.5 mM NO_3^- (Fig. 4I, 4J). However, the average axial root length of Mo17 increased in 0.5 mM NO_3^-, relative to the 2.5 mM treatment.

In order to determine the average diameter of axial roots alone, measurements were taken at 7 DAI, before lateral roots began developing. At the point of measurement, the average diameter of Mo17 axial roots were larger than B73 (1.4 mm compared to 1.25 mm respectively), though no NO_3^- treatment differences were observed for either line (Fig. 5).

Lateral roots

The numbers of lateral roots did not differ between NO_3^- treatments, however at 17 DAI, the number of laterals on B73 roots appear to be greater than Mo17 (Fig. 6A, 6B). The frequency of laterals along axial roots describes the lateral root density, and although no differences were observed between NO_3^- treatments, the lateral root density of B73 (Fig. 6C) was almost double of Mo17 (Fig. 6D). Similar to whole roots, the total lateral root length (Fig. 6E), surface area (Fig. 6G) and volume (Fig. 6I) of B73 did not differ between NO_3^- treatments. Conversely, the total lateral root length and surface area of Mo17 seedlings grown in 0.5 mM NO_3^- increased by 28 % and 24 % respectively 14 - 17 DAI, in 0.5 mM NO_3^-, relative to the 2.5 mM treatment (Fig. 6F, 6H), whereas the total lateral root volume did not differ between NO_3^- treatments (Fig. 6J). For both lines and both NO_3^- treatments, the average lateral root length began increasing after 7 DAI (Fig. 6K, 6L), coinciding with emergence of lateral roots (Fig. S2). The average lateral root length rapidly increased until 14 DAI. At 17 DAI, the

average lateral root length of Mo17 seedlings grown in 0.5 mM NO_3^- was 13 % higher than the 2.5 mM treatment, whereas no NO_3^- treatment difference was observed in B73.

DISCUSSION

Quantification of maize seedling root development under two NO_3^- treatments revealed differences in morphological responses between lines. Mo17 roots were much larger than B73 and responded to low NO_3^-, increasing the absorptive area of the root from 14 – 17 DAI, whereas B73 roots were unresponsive. This may explain why Mo17 could maintain shoot growth with low N provision whilst B73 had reduced shoot growth.

The ideal ideotype for root morphology in maize is dictated by the N and water available within the agricultural system it is to be grown (Fig. 7; Lynch, 2013), and both lines used within this study possess morphological roots traits that may be beneficial for N capture in either high- or low-input agricultural systems. B73 roots were almost half the mass of Mo17 yet achieved similar absorptive area, suggesting that B73 roots are 'cheaper' than Mo17 regarding C cost. Cheap roots benefit plants by reducing the C cost associated with constructing and maintaining the root system, which could favour shoot growth (Eissenstat, 1992). Thus, cheap roots may be more desirable for low-input systems as foraging could be achieved with minimal C cost. Cheap roots are chiefly the result of finer roots, thus may not possess the same capacity to penetrate harder soils as thicker roots (Clark *et al.*, 2008; Bengough *et al.*, 2011). One could consequently speculate that the thicker roots of Mo17 may be more desirable for low-input agricultural systems, given their greater potential to explore harder soils when water is limited (Lynch, 2013). Within this study, the cheap roots of B73 were unresponsive to NO_3^- supply and did not benefit growth in low NO_3^-, evident by the reduced shoot FW, relative to plants in sufficient NO_3^-. We suggest this may be because like root growth, the NO_3^- uptake capacity of B73 seedlings does not increase in low NO_3^-,

consequently reducing net NO_3^- uptake and ultimately shoot growth, as observed by Sabermanesh (2014).

Although the number of lateral roots were similar between lines, the distance between laterals along axial roots (lateral root density) were greater in Mo17. Lateral root density is important for soil nutrient acquisition, particularly when considering mobile nutrients like NO_3^-, which generate large depletion zones in soil in comparison to more immobile nutrients (Casper and Jackson, 1997). Neighboring lateral roots can compete for NO_3^- sources (Nye and Tinker, 1977) generating overlapping depletion zones around the same plant, which are inefficient (Ge *et al.*, 2000). However when soil water is limiting, NO_3^- mobility reduces, decreasing the size of the resulting depletion zone. Given this, the higher lateral root density of B73 axial roots may be a desirable trait for low-input systems to promote fine-scale foraging for water and N by increasing the frequency of roots per unit soil, whereas the lower lateral root density of Mo17 would be desirable for higher input systems, minimising overlapping depletion zones around the root. Unlike B73 roots which were unresponsive to N supply, Mo17 roots increased specific lateral and axial root length in low NO_3^-, providing a greater absorptive area for N capture. Conversely, the capacity of Mo17 to increase axial root length may also be desirable for low-input systems where water and N availability is limited, enabling access to subsoil water and leached N which may be otherwise inaccessible to shallower roots (Yoshida and Hasegawa, 1982; Mambani and Lal, 1983; Gowda *et al.*, 2011).

Mo17 changed root morphology in response to low N 14 – 17 DAI, before a change in root mass was observed. This suggests that optimisation of root morphological traits may occur before plants resort to increasing root biomass allocation to maintain sufficient N uptake for growth. This study was conducted using the same lines and growth conditions as Sabermanesh (2014). Further, the period where Mo17 roots alter morphology in this study (14

– 17 DAI) closely aligns with when Mo17 root reaches maximum NO_3^- uptake capacity in the mentioned study. Given this, we hypothesise that morphological root responses may occur in low NO_3^- if first increasing NO_3^- transporter expression and uptake capacity on the current root surface area does not meet the seedling's growing N demand. This morphological plasticity could be an important physiological and developmental trait enabling the maintenance of net N uptake in low NO_3^-. However, further investigation is required to clarify this putative relationship between root NO_3^- uptake capacity and root surface area.

The elongation of lateral and axial roots in Mo17 may be a response to NO_3^- limitation, however studies in *Arabidopsis* and tobacco indicate that lateral root growth is negatively correlated with both shoot and external NO_3^- concentrations (> 1 mM) (Scheible *et al.*, 1997; Zhang *et al.*, 1999). Similarly, increasing the external NO_3^- concentration reduced axial root length of *Arabidopsis* (Linkohr *et al.*, 2002), suggesting that the longer roots of Mo17 in 0.5 mM NO_3^- may be due to repressed root growth at 2.5 mM NO_3^-, rather than stimulation at 0.5 mM. This perspective is evidenced by previous work showing Mo17 seedlings accumulated more root and shoot NO_3^- when grown in 2.5 mM NO_3^-, relative to the 0.5 mM treatment, whereas minimal treatment differences were observed in B73 (Sabermanesh, 2014).

The adaptive root responses within this study were observed in hydroponics, a system where plant roots are supplied with constant uniform nutrient availability. This must be kept in mind when interpreting these results, as this is not soil where N availability can be heterogeneous. Indeed, the NO_3^- treatment differences in morphological root traits were not large. We suspect one cause may be because the measurements were taken early in development. If measurements were taken later in development, we believe these differences would be more profound as plant N demand would be greater. The small NO_3^- treatment differences may also be due to the choice of NO_3^- concentrations. These concentrations were

selected as they have previously shown to elicit a major response of the maize NO_3^- uptake system to low NO_3^- (Garnett *et al.*, 2013). However, these concentrations did not affect root or shoot growth in the mentioned study, suggesting a greater separation in NO_3^- concentrations between the low and sufficient NO_3^- treatment may be required to better exploit the effects of NO_3^- supply on growth and/or root morphology. If a greater concentration was used for our sufficient NO_3^- treatment (5 mM), we predict B73 root morphology still may not differ, as it is already small and cheap regarding C cost, favouring shoot growth. Conversely, Mo17 may deploy a smaller root system with less surface area to minimise its root C allocation, as less absorbing area will be required to capture enough N to meet demand.

Changing root morphology 14 - 17 DAI prior to increasing root mass highlights the plasticity of the maize root system and identifies how early these responses may occur in development. Identification of this critical developmental event provides scope towards investigating the molecular occurrences leading to the change in root morphology, and to identify candidate genes regulating root growth. Moreover, the differences in root morphology and responses to N supply between these parental lines of the IBM mapping population provide the basis to dissect the genetic regulation underlying these traits by mapping quantitative trait loci. Future identification of any loci or genes regulating the root responses measured in this study may help develop new germplasm with optimised root morphology for efficient N capture.

SUPPLEMENTARY MATERIAL

Additional supporting information may be found in the online version of this article.

Fig. S1 Identification of lateral roots (LR) and axial roots (AxR) of maize seedlings (*Zea mays.* L).

Fig. S2 Digital images of maize seedling roots (*Zea mays*. L) 7 and 8 DAI.

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FIGURES

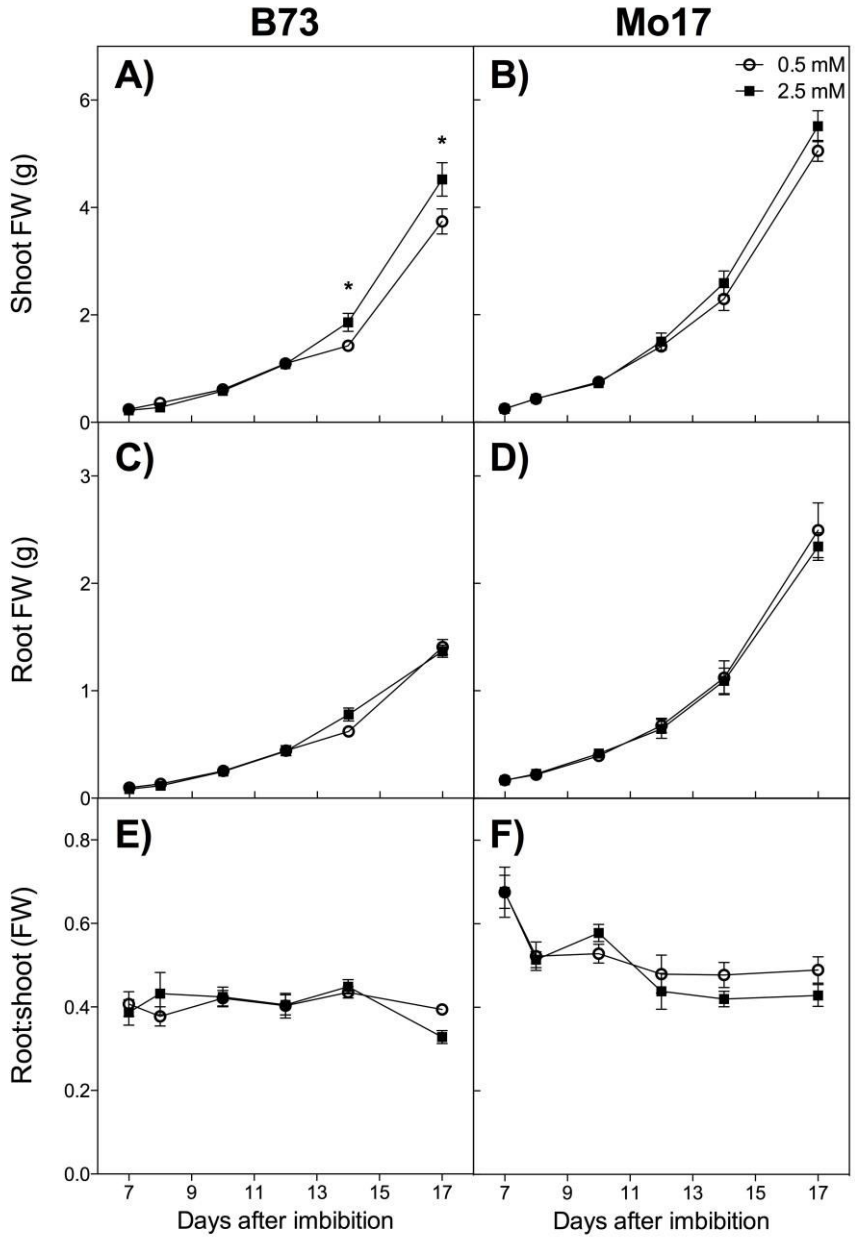

Fig. 1 Tissue fresh-weights (FW) of **(A, B)** shoots, **(C, D)** roots, and the resulting **(E, F)** root:shoot of maize seedlings (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) and 2.5 mM (closed squares) NO_3^-. Seedlings were sampled across a time-course. Values are means ± S.E.M (n = 6). *Points significantly different between two growth conditions (P < 0.05).

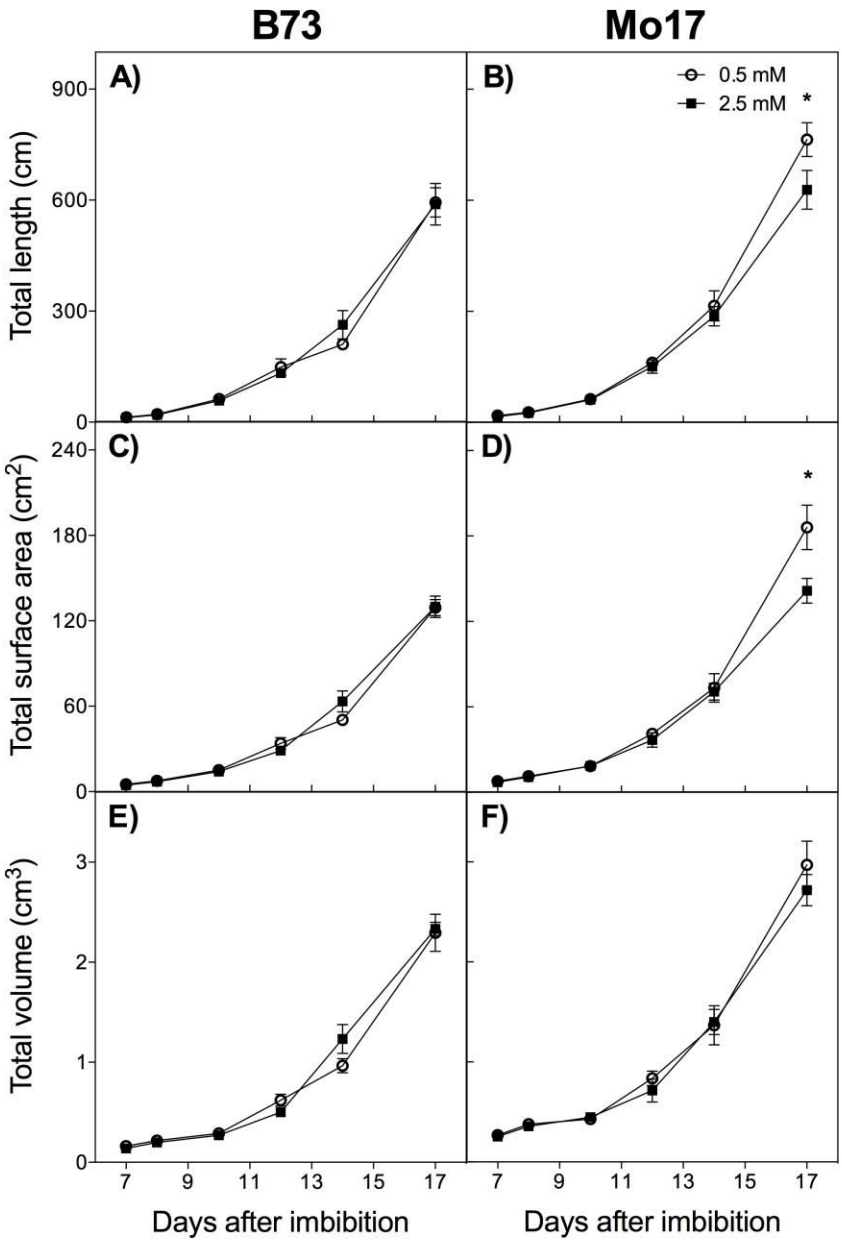

Fig. 2 Morphological parameters of the maize root system (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (open circles) and 2.5 mM (closed squares) NO_3^-. Total root **(A, B)** length, **(C, D)** surface area, and **(E, F)** volume were measured over a time-course. Values are means ± S.E.M ($n = 6$). *Points significantly different between two growth conditions ($P < 0.05$).

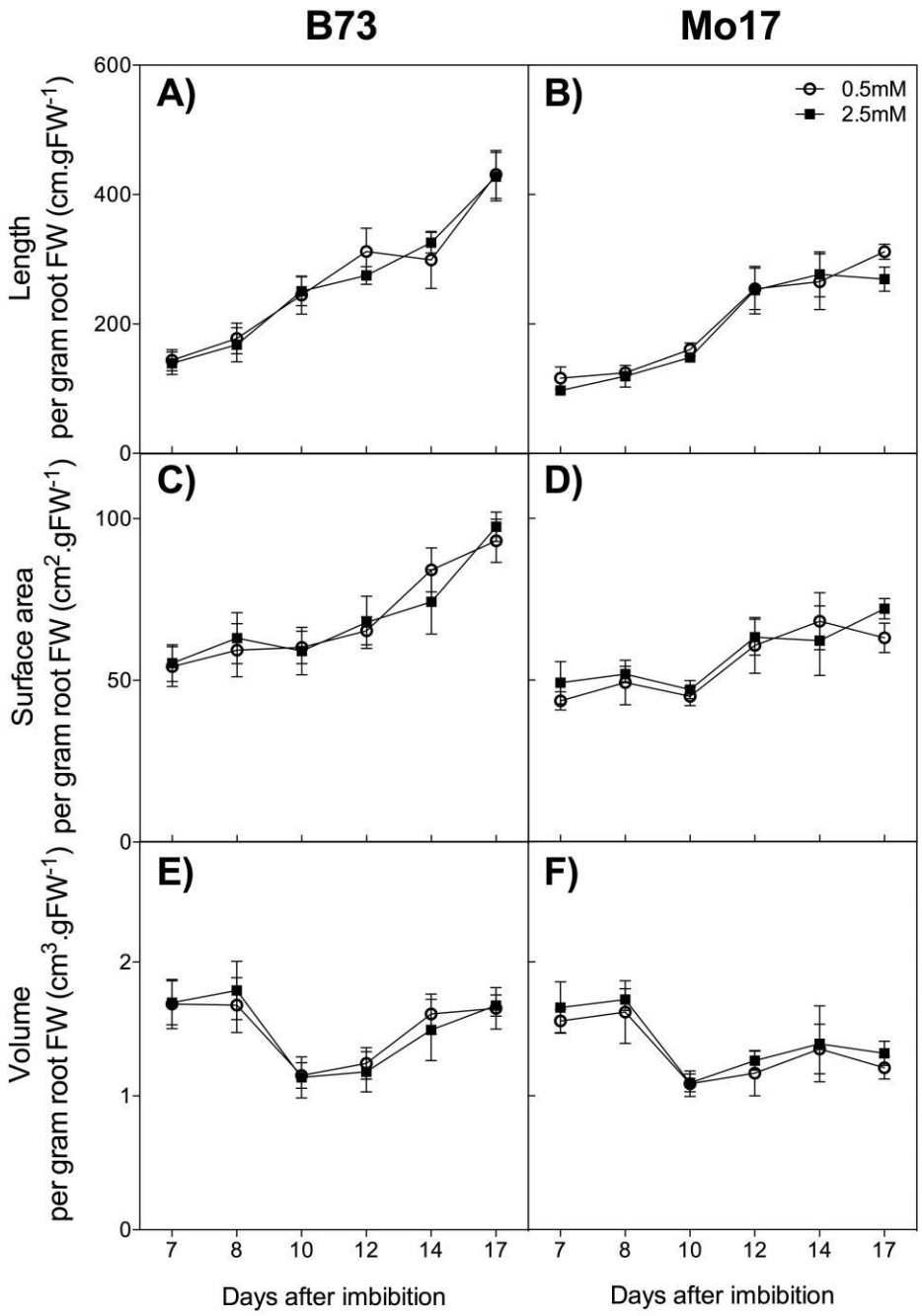

Fig. 3 Total root **(A, B)** length, **(C, D)** surface area and **(E, F)** volume per gram root fresh-weight (FW) of maize roots (*Zea mays* var. B73 and Mo17) over a time-course. Plants grown in 0.5 mM (open circles) and 2.5 mM (closed squares) NO_3^-. Values are means ± S.E.M (*n* = 6).

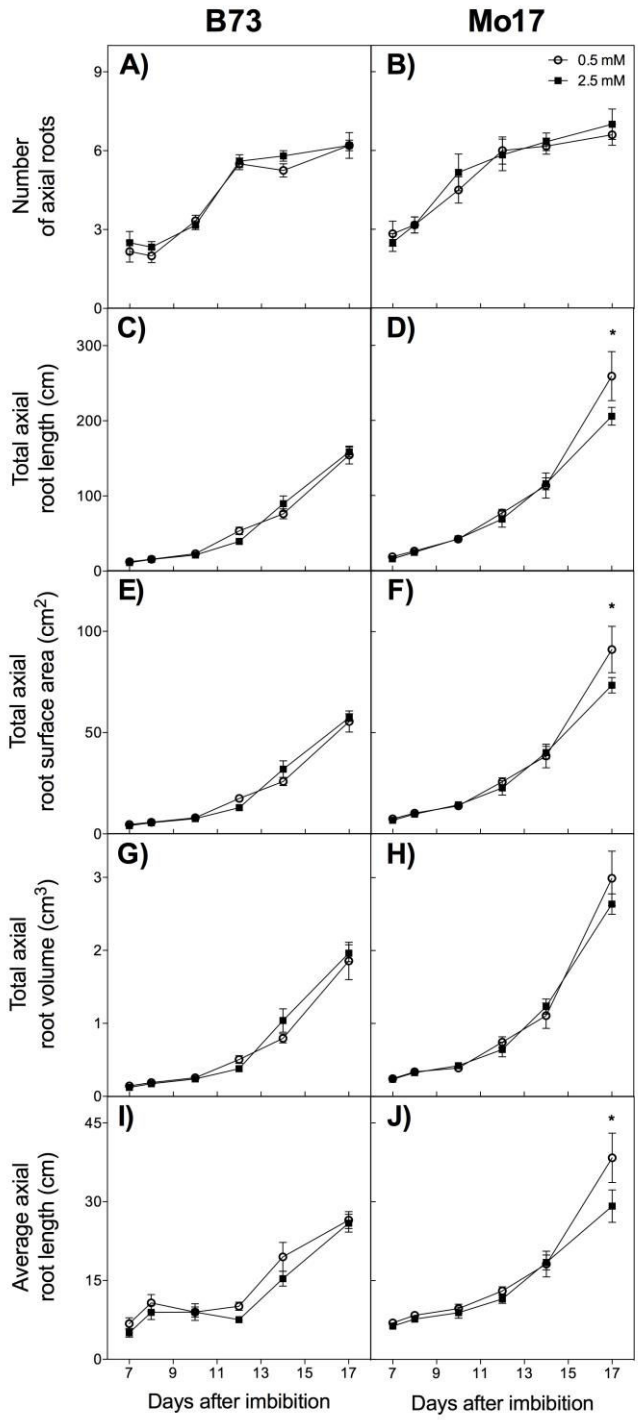

Fig. 4 Parameters of maize axial roots (*Zea mays* var. Mo17 and B73) grown in 0.5 mM (open circles) and 2.5 mM (closed squares) NO_3^-. Total **(A, B)** number, **(C, D)** length, **(E, F)** surface area, **(G, H)** volume, and **(I, J)** average length were measured over a time-course. Values are means ± S.E.M (n = 6). *Points significantly different between two growth conditions ($P < 0.05$).

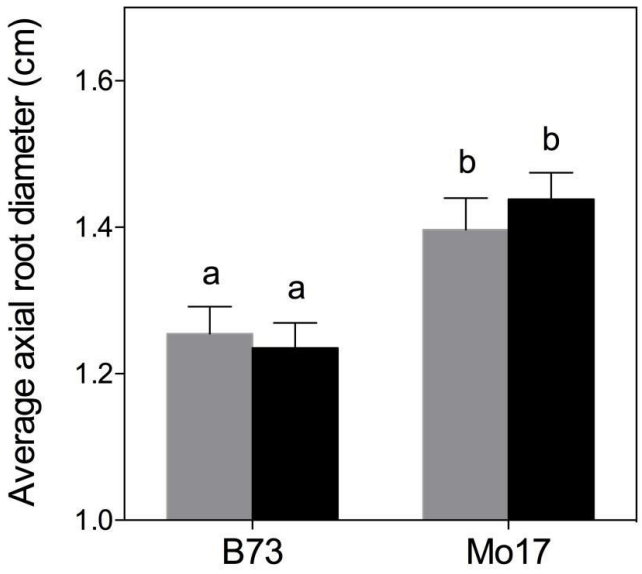

Fig. 5 Comparison of average axial root diameter of maize seedlings (*Zea mays* var. B73 and Mo17) grown in 0.5 mM (grey columns) and 2.5 mM (black columns) NO_3^-, 7 DAI. Values are means ± S.E.M (n = 6). Different letters above columns represents significantly different means ($P < 0.05$).

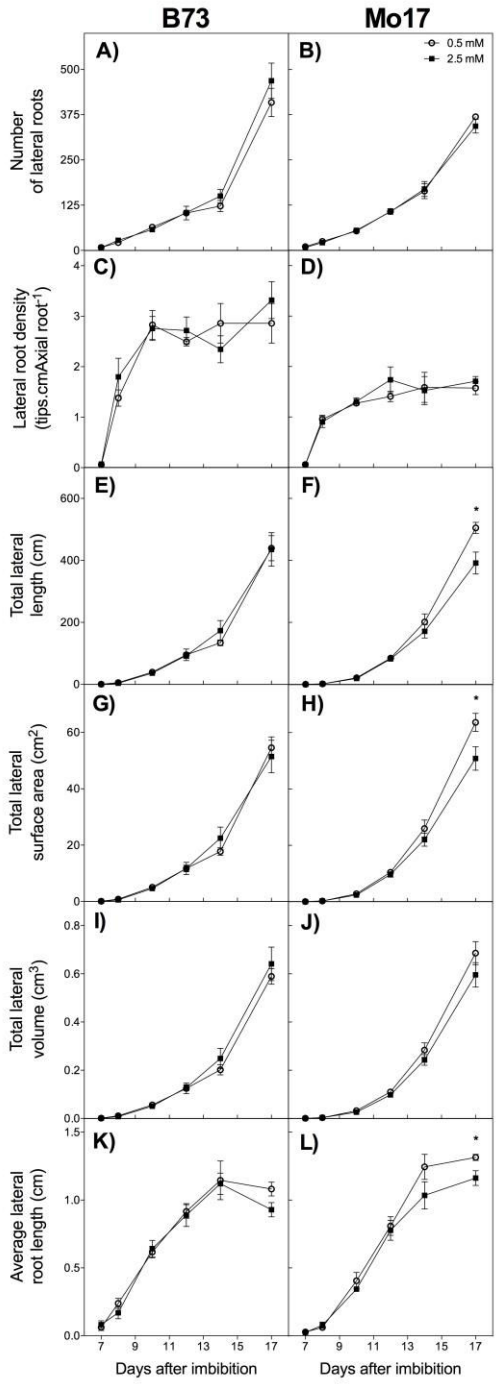

Fig. 6 Lateral root parameters of maize (*Zea mays* var. B73 and Mo17) seedlings grown in 0.5 mM (open circles) and 2.5 mM (closed squares) NO_3^-. Total **(A, B)** number **(C, D)** density, **(E, F)** length, **(G, H)** surface area, **(I, J)** volume, and **(K, L)** average length of lateral roots were measured over a time-course. Values are means ± S.E.M (n = 6). *Points significantly different between two growth conditions ($P < 0.05$).

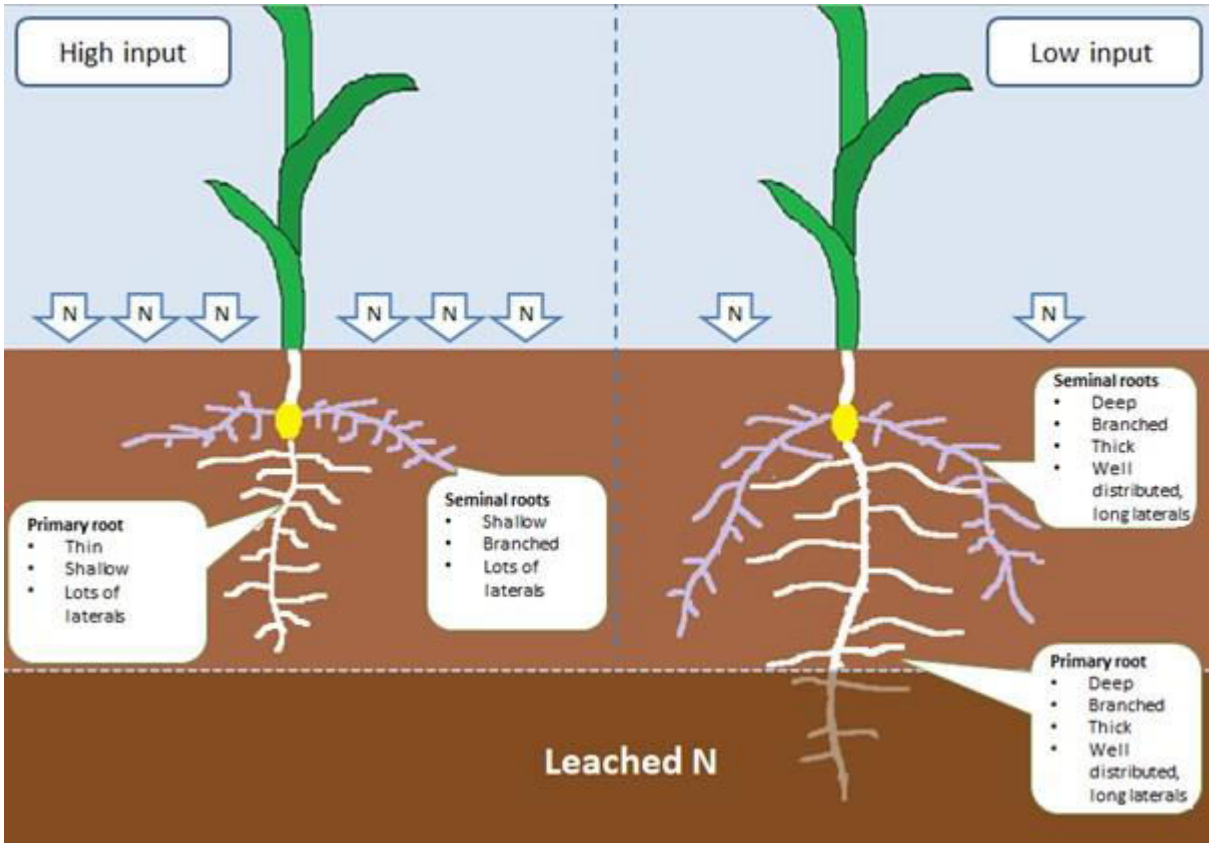

Fig. 7 Schematic of the ideotype for maize (*Zea mays*. L) seedling root systems ideal for high- or low-input agricultural systems. Adapted from Lynch (2013).

SUPPLEMENTARY MATERIAL

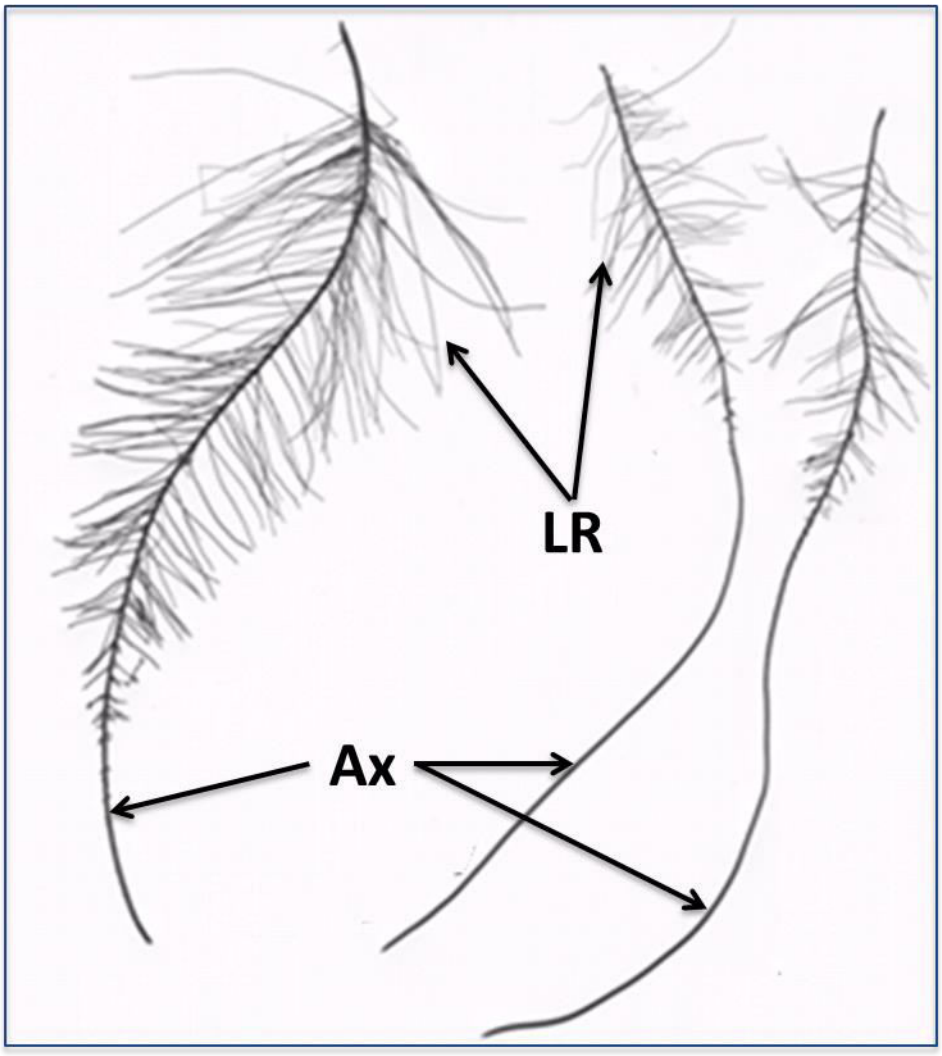

Fig. S1 Identification of lateral (LR) and axial roots (AxR) of maize seedlings (*Zea mays* L.).

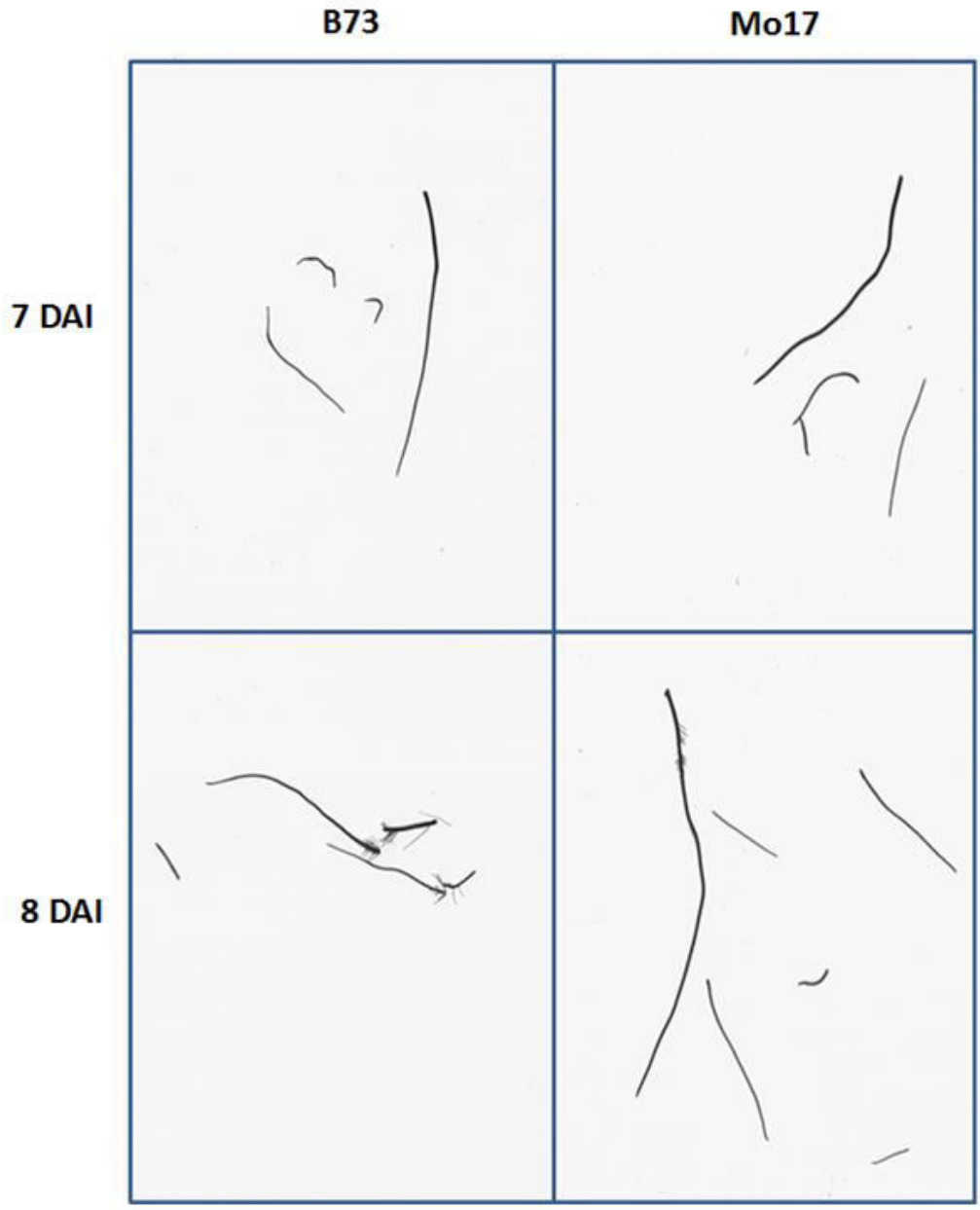

Fig. S2 Digital images of maize seedling roots (*Zea mays* var. B73 and Mo17) 7 and 8 DAI.

Chapter 4: Mapping quantitative trait loci for morphological root traits in maize seedlings, relative to nitrate supply

TITLE

Mapping quantitative trait loci for morphological root traits in maize seedlings, relative to nitrate supply

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ABSTRACT

Increasing nitrogen (N) uptake by optimising root morphology could be one approach towards improving cereal N use efficiency. However, little is known about the genetic regulation of root morphology, partly due to it being a complex trait that is likely regulated by numerous genes. This study uses a subset of the intermated B73 × Mo17 (IBM) mapping population to identify quantitative trait loci (QTL) under low and sufficient nitrate (NO_3^-) supply for morphological root traits correlating with shoot growth, or previously associated with N uptake. Nine putative QTL associated with lateral root (LR) traits correlating with shoot growth were detected in low NO_3^-. In sufficient NO_3^-, two putative QTL for morphological traits associated with root volume were detected. Three QTL for LR traits co-located with published QTL for different morphological root traits; including one on chromosome 5 explaining 20.3 % of the total LR length variation within the population. These results suggest the QTL on chromosome 5 may be important for producing longer LR, which could lead to increased shoot growth in low NO_3^- environments.

Keyword index

IBM, nitrogen, growth, lateral root, axial root, QTL

Abbreviations

IBM, intermated B73 × Mo17; QTL, quantitative trait loci; NUE, nitrogen use efficiency; IRILs, intermated recombinant inbred lines; DAI, days after imbibition; LR, lateral root; LOD, logarithm of odds; AvgSM, average seed mass; DW, dry-weight; R:S, root:shoot; TRL, total root length; TRSA, total root surface area; TRV, total root volume; AvgD, average diameter; tips, number of root tips; TAxL, total axial root length; TAxSA, total axial root surface area; TAxV, total axial root volume; TLRL, total lateral root length; TLRSA, total lateral root surface area; TLRV, total lateral root volume; AvgLRL, average lateral root length; AvgLRSA, average lateral root surface area; AvgLRV, total lateral root volume; RLE,

root length efficiency; RSAE, root surface area efficiency; IcM, intermated B73 × Mo17 centimorgan.

INTRODUCTION

Substantial quantities of costly nitrogen (N) fertilisers are applied to crops each year to maximise growth and ultimately yield. However, although agricultural production has doubled over the past five decades, N fertiliser consumption has increased more than 9-fold (FAOSTAT, 2014). Further, cereal crops only capture 40 – 50 % of the applied N (Peoples *et al.*, 1995; Sylvester-Bradley and Kindred, 2009), allowing for much of the remaining N to be lost by leaching into groundwater; surface run-off and volatilisation into the atmosphere, each considerably impacting the environment (Vitousek *et al.*, 1997). This low N use efficiency (NUE) could be improved by increasing N capture, relative to N supply (N uptake efficiency) by improving root-specific phenotypes (Garnett *et al.*, 2009).

In comparison to immobile nutrients like phosphorous, root morphology is often deemed of low importance for the uptake of more mobile nutrients, such as N (Barber, 1995; Tinker and Nye, 2000). However, several studies provide evidence to the contrary. Rooting depth (Kristensen and Thorup-Kristensen, 2004; Liao *et al.*, 2004) and more specifically, axial root length (consisting of primary and seminal roots) (Liu *et al.*, 2009) have shown to positively correlate with N recovery and reduced nitrate (NO_3^-) leaching (Habib *et al.*, 1991; Wiesler and Horst, 1993, 1994), as deep-growing roots have access to leached N in subsoil layers that may otherwise be inaccessible to shallower roots. Cereal roots have shown to adapt to N supply by changing morphology early in development (Maizlish *et al.*, 1980; Liu *et al.*, 2008; Sabermanesh, 2014b). Nitrogen responsive morphological root traits, such as increased root size are suggested to help maintain net N uptake when N availability is low by increasing the absorbing area of the root (Ingestad and Agren, 1991). However, this generally comes at a

greater carbon (C) cost to the shoot, resulting in increased root:shoot and reduced shoot growth potential (Davidson, 1969; Ericsson, 1995; Bonifas *et al.*, 2005; Lambers *et al.*, 2008).

The above studies associate some specific morphological root traits with N uptake, however Lynch (2013) proposed a theoretical ideotype for maize root morphology for the efficient acquisition of N and water. This ideotype highlights that efficient N and water acquisition could be achieved with a 'cheap' root system; a root system with a high proportion of fine roots, which minimises the C cost involved in constructing and maintaining the root whilst achieving the same absorptive area (Eissenstat, 1992), ultimately favouring shoot growth. Moreover, LR phenes for efficient N acquisition would be long and exist in low numbers along axial roots to minimise overlapping depletion zones around the same plant, which are generated as a consequence of root N uptake.

Studies identifying putative QTL for a number the aforementioned morphological root traits, relative to water or nutrient supply have mostly been conducted under differing water or phosphorous (P) availabilities (Kamoshita *et al.*, 2002; Tuberosa *et al.*, 2002; Zhu *et al.*, 2005; Zhu *et al.*, 2006; Ruta *et al.*, 2010). These studies highlight that putative QTL for morphological root traits of interest are not necessarily similar between water/nutrient limited and non-limited conditions, suggesting that the genetic basis for their regulation may be environmentally influenced. Given this, the genetic regulation of morphological root traits needs to be dissected under differing N supply. This is evidenced by a study that found no common QTL between two N treatments among the 11 putative QTL identified for morphological root traits correlating with N accumulation (Liu *et al.*, 2008). Although some of putative high N QTL identified by Liu *et al.* (2008) did coincide with published QTL for similar traits in different environments (e.g. mean axial root length in high N and primary root length in high P), many of the coinciding QTL were associated with contrasting

morphological traits (e.g. LR length with primary root diameter). This highlights the requirement to identify and confirm more or repeatable QTL for morphological root traits in differing N supply, to determine the genetic basis for its regulation.

This study aims to identify putative QTL for morphological root traits correlating with shoot growth, or previously associated with N uptake (Lynch, 2013). Bai *et al.* (2013) used the cigar-roll culture system to identify putative QTL for morphological root traits coincident with plant height using a wheat doubled-haploid mapping population, as this system was both high-throughput and effective. Here, a subset of the widely-studied maize (*Zea mays* L.) intermated B73 × Mo17 mapping population (IBM-94) was used, as the parental lines of this population have recently shown to have differing root morphologies and responses to N availability in hydroponics during early development (Sabermanesh, 2014b), providing the opportunity to work with seedlings with root systems that are not too complex. This is conducted under low and sufficient NO_3^- supply to examine how it can influence both morphological root traits and their associated QTL.

MATERIALS AND METHODS

Plant growth

A subset of 94 intermated recombinant inbred lines (IRILs) of maize derived from the B73 × Mo17 cross (IBM-94; www.maizegdb.org), along with the parental lines (*Zea mays* var. B73 and Mo17) were used for QTL mapping. The IRILs were derived from four rounds of intermating following the F_2 stage (Lee *et al.*, 2002). Seeds were imbibed in aerated RO-H_2O 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). Two germinated seedlings for a selected IRIL were then equally distributed along the top of a brown germination paper (Anchor Paper, St. Paul, MN, USA) that had been soaked in nutrient solution, and then wrapped as a cigar-roll.

Measurements of the germination paper were 41 cm × 28 cm (height × width). Cigar-rolls were vertically aligned in one of ten plastic trays (five trays per N treatment) with measurements 47 cm × 35 cm × 3.8 cm (length × width × height) containing 1.6 L nutrient solution in a controlled environment cabinet with a day:night cycle of 14 h:10 h, 26 °C:21 °C, with a flux density at canopy level of *c.* 450 µmol m^{-2} s^{-1}. A relative humidity of 75 % was used to maintain moisture in the cigar-rolls. The nutrient solution was a modified Johnson's solution (Johnson *et al.*, 1957) containing (in mM), 0.1 NO_3^- N, 2.85 K, 1.15 Ca, 0.5 Mg, 1.63 S and 0.5 P for the 0.1 mM NO_3^- treatment, and 5 NO_3^- N, 3.05 K, 0.6 Ca, 0.5 Mg, 0.5 S, 0.5 P for the 5 mM NO_3^- treatment. Both treatment solutions also contained (in µM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and ethylendiamine-N,N'-bis(2-hydroxyphenylacetic acid) (FeEDDHA)). Iron was supplemented twice weekly with the addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (8 mg L^{-1}) to avoid deficiency (Cramer *et al.*, 1994). Nutrient solutions were supplied at a pH between 5.8 – 5.9 and changed daily. Blank cigar-rolls (no seedlings) soaked with nutrient solution were positioned around the perimeter of those containing the seedlings to minimise any drying of the cigar-rolls containing seedlings.

Experimental design

The experiment was a split-plot design in two blocks with N treatment in main plots (trays), and both IRILs and parents on split-plots. Two replicates were staggered across the two blocks and each N treatment (low and sufficient NO_3^-). Each replicate consisted of one cigar-roll containing two plant samples of the same line acting as pseudo-replicates.

Plant phenotyping

Average seed mass was derived from the average mass of ten seeds before imbibition. All seedlings were harvested 15 d after imbibition (DAI), with the root being separated from the shoot and preserved in 30 % ethanol immediately. Seedling roots were then scanned as a digital image (Epson Expression 10000XL). Root morphological parameters (length, surface

area, volume, average diameter and root tip number) were determined from digital images using WinRHIZO Pro root image analysis software (V.2005b, Regent Instruments, Quebec, Canada). At this stage in growth, seedling root systems consisted of primary and seminal roots (together termed axial roots), with lateral roots (LR) emerging from them. Lateral roots were differentiated from axial roots on WinRHIZO using a distinguishing diameter of 0.370 mm (verified for lines and NO_3^- treatments). The average LR length was calculated by dividing the total length of the component root by the total number of root tips. Although the number of tips included the points where the root was cut, this was minimal (< 2 %). Lateral root density was calculated by dividing the total number of root tips by total axial root length. Plant roots and shoots were then dried at 80 °C for 3 d and weighed to determine dry-weights (DW). Root length efficiency (RLE) and surface area efficiency (RSAE) were calculated by dividing total root length or surface area by the total root DW, respectively.

Statistical analyses

Two-way analysis of variance (ANOVA) was carried out on the parental lines to determine whether any genotypic differences existed for any traits of interest. Similarly, two-way ANOVA was used to determine any NO_3^- treatment differences among the parents and IRILs. For each quantified trait, a linear mixed model was fitted that accounted for all sources of genetic and non-genetic variation. Specifically, non-genetic sources such as blocks and trays of the experimental design were fitted as random effects. The fixed component of each model contained a term that allowed separate estimation of the mean effect for the parental lines and an overall mean for the IRILs in each NO_3^- treatment. In each NO_3^- treatment, the genetic variation of the IRILs around this mean was estimated by including a random effect term that was assumed to have zero mean and a normally distributed genetic variance. From each of these fitted models, the best linear unbiased predictors (BLUPs) of the individual IRILs were extracted. All models were fitted and checked diagnostically using the flexible linear mixed

modelling software ASReml-R (Butler *et al.*, 2007) available as package in the R statistical software computing environment (R Development Core Team, 2012).

QTL mapping

Whole genome QTL analysis was conducted on each trait and NO_3^- treatment using the R package WGAIM (Taylor and Verbyla, 2011), which is a computational implementation of Verbyla *et al.* (2007) and Verbyla *et al.* (2012). Initially, a working linear mixed model is proposed that includes a whole genome contiguous block of QTL intervals as set of random covariates with a single marker variance. Using a type-I error of 0.05, the significance of this marker variance was tested using a simple likelihood ratio test. If it was significant, an outlier statistic was subsequently used to select the most likely putative QTL interval on the genome, which is then moved to the fixed part of the model. This process is repeated until no further QTL are detected. The simultaneous use of the whole genome in the analysis avoids repeated scans and the usual threshold calculations that are required for multiple testing problems. The final set of selected QTL interval are then summarised with their flanking markers, flanking marker distances, logarithm of odds (LOD) score and the percentage of genetic variation it accounted. The allele effects were also calculated and a negative allele effects indicate that Mo17 is favoured to increase the trait at that genetic location, whereas positive allele effects indicate B73 is favoured. The genotypic data for the IBM population was publically available on the maize genetics and genomics database (http://www.maizegdb.org/qtl-data.php). A total of 1,339 genetic markers and their map distances were obtained from the IBM2 map (http://www.maizegdb.org/map.php). The positions of the genetic markers in the genome were obtained through the integration of the IBM linkage map (http://www.maizegdb.org/qtl-data.php) and the maize genome sequence (Maize Genome AGP version 3; www.maizesequence.org). In order to determine the existence of any coincidence between the

putative QTL detected within this study and those already published in maize, the IBM2 2008 neighbours map was used (www.maizegdb.org/map.php).

RESULTS

Phenotypic variation

The phenotypic values for quantified traits among the parental lines (*Zea mays* var. B73 and Mo17) and IRILs at 15 d after imbibition (DAI) are presented in Table 1. No differences between parental lines and NO_3^- treatments were observed for most of the quantified traits. Large ranges for all phenotypic traits were observed within the IRILs, exceeding those of the parental lines, displaying transgressive segregation. However, the mean phenotypic values for the IRILs were generally within the range of the parental lines. Differences between the parental lines were observed for root length efficiency (root length per unit root DW; RLE) and root surface area efficiency (root surface area per unit root DW; RSAE), with B73 achieving greater values compared to Mo17. Between the parental lines, NO_3^- treatment differences were observed for RLE in Mo17, with RLE increasing in 0.1 mM NO_3^-, compared to the 5 mM treatment. Within the IRILs, NO_3^- treatment differences for mean phenotypic values were observed for RLE, with values being greater in 0.1 mM NO_3^-, with respect to the 5 mM treatment. Frequency distributions for quantified traits show that the phenotypic data for all traits within the IRIL population were normally distributed, however no major separation between NO_3^- treatments were observed (Fig. S1).

Pearson's correlation coefficients were calculated between all measured traits (Table 2). Correlation coefficients between all traits were similar for the two NO_3^- treatments. Among these correlations, traits correlating with average seed mass (AvgSM) were of interest to clarify if seed mass influenced seedling growth. Likewise, morphological root traits correlating with shoot DW were of interest to determine which traits may promote shoot growth. Across both NO_3^- treatments, AvgSM did not correlate with shoot DW. In 0.1 mM

NO_3^-, AvgSM did however positively correlate with all root traits except root:shoot (R:S); LR density; average LR volume (AvgLRV); RLE and RSAE. This was similarly observed in 5 mM NO_3^-, except that AvgSM also did not correlate with the number of root tips (tips) and total axial root length (TAxL). Across both NO_3^- treatments, shoot DW positively correlated with root DW; total root length (TRL), surface area (TRSA) and volume (TRV); average root diameter (AvgD); tips; TAxL, total axial root surface area (TAxSA) and volume (TAxV); total LR length (TLRL), surface area (TLRSA) and volume (TLRV); average LR length (AvgLRL) and surface area (AvgLRSA). However, a negative correlation was observed between shoot DW and root:shoot (R:S) across both NO_3^- treatments.

QTL detection

A total of 16 QTL were detected for morphological root traits across seven chromosomes (1, 2, 5, 6, 7, 9 and 10) (Table 3). Fourteen of these QTL were detected in 0.1 mM NO_3^-, whereas two were detected in the 5 mM treatment. In 0.1 mM NO_3^-, eight QTL were detected for TLRL across five chromosomes (1, 2, 5, 7 and 9). These QTL could each explain 3.6 – 20.3 % (total 69.3 %) of the TLRL variation within the IRIL population and B73 carried five of the favourable alleles for the QTLs associated with TLRL, whereas Mo17 carried three. A total of six QTL were detected for TLRSA in 0.1 mM NO_3^- across five chromosomes (1, 5, 6, 7 and 9). These QTL could each explain 3.6 – 14.2 % (total 55.7 %) of the TLRSA variation within the IRIL population, and both B73 and Mo17 each carried three of the favourable alleles for the QTL associated with TLRSA. In 5 mM NO_3^-, one QTL was detected for AvgLRV on chromosome 2, explaining 8.7 % of the AvgLRV variation within the IRIL population. Likewise, one QTL was detected for RLE in 5 mM NO_3^- on chromosome 10, explaining 12.1 % of the RLE variation within the IRIL population. Both the favourable alleles for the QTL associated with AvgLRV and RLE were carried by Mo17.

QTL comparison

Comparison of the QTL detected within this study showed that five QTL for TLRL in 0.1 mM NO_3^- co-located with those for TLRSA (Table 3). The QTL detected within this study were also compared with published QTL for traits associated with root morphology (Tuberosa *et al.*, 2002; Hund *et al.*, 2004; Zhu *et al.*, 2005; Zhu *et al.*, 2006; Liu *et al.*, 2008; Trachsel *et al.*, 2011) and N use efficiency (NUE) in maize (Agrama *et al.*, 1999; Bertin and Gallais, 2001; Ribaut *et al.*, 2007). Of the 11 different putative QTL detected within this study, four contained regions that overlapped with published QTL for morphological root traits only (Table 4). Among these, our putative QTL on chromosome 5, which was associated with TLRL in 0.1 mM NO_3^- (flanked by bnl5.24a/mmp118) overlapped with published QTL associated with primary root weight (Tuberosa *et al.*, 2002) and primary root diameter (Hund *et al.*, 2004). Another putative QTL on chromosome 5 for both TLRL and TLSA in 0.1 mM NO_3^- (flanked by bnl4.36/umc40) overlapped with a published QTL for average axial root length (Liu *et al.*, 2008). Similarly, our putative QTL on chromosome 7 for both TLRL and TLSA in 0.1 mM NO_3^- (flanked by ufg47/umc2095) also overlapped with a published QTL associated with average axial root length (Liu *et al.*, 2008).

DISCUSSION

Understanding the genetic basis for the regulation of morphological root traits contributing to above-ground yield and/or N uptake could help improve the low NUE of cereals. Across both NO_3^- treatments, shoot DW correlated with root DW and a number of axial and lateral root traits, suggesting that these root traits promote shoot growth within the IRIL population. This is consistent with recent studies reporting that increases to root DW (Sabermanesh, 2014a), TAxL, TAxSA, TLRL and TLRSA in low NO_3^-, with respect to sufficient NO_3^-, facilitated the maintenance of maximal shoot growth capacity in maize (*Zea mays* var. Mo17) (Sabermanesh, 2014b).

Among the root traits correlating with shoot DW, nine putative QTL were detected in low NO_3^- for TLRL and TLRSA. Five out of six QTL associated with TLRSA coincided with those for TLRL, likely due to the high correlation between these two phenotypic traits (> 0.90). Three of the nine QTL were however specific for TLRL, and one for TLRSA, suggesting that some genetic regulation specific for each trait may exist. The QTL associated with both TLRL and TLRSA in low NO_3^- were chiefly located on chromosomes 1 and 5. Among these, one major QTL on chromosome 1 (flanked by mmp56/cdo938a) could potentially explain 10.7 and 14.6 % of the TLRL and TLRSA variation within this population, respectively. Likewise, a major QTL detected on chromosome 5 (flanked by bnl4.36/umc40) associated with TLRL could potentially explain 20.3 and 6.8 % of the TLRL and TLRSA variation within this population, respectively. Two putative QTL associated with AvgLRV and RLE were detected in 5 mM NO_3^-, and although neither correlated with shoot DW, RLE (greater length per gram root or 'cheap' roots) has previously been associated with the theoretical ideotype for optimised water and N uptake in maize (Lynch, 2013). This is because cheap roots help achieve maximum absorptive area for uptake whilst minimising the C cost associated with constructing and maintaining the root system (Eissenstat, 1992). The QTL detected in 5 mM NO_3^- were associated with morphological traits contributing to root size/volume (RLE and AvgLRV), whereas in 0.1 mM NO_3^- they were associated with LR length and surface area. This may be because in 5 mM NO_3^-, stronger and ultimately detectable QTL were generated for traits associated with 'cheaper' roots, resulting from greater phenotypic variation within the IRIL population, whereas in 0.1 mM NO_3^-, the phenotypic variation for TLRL and TLRSA was greater as they substantially increase the effective absorptive area of the root to increase net N uptake when N is limited.

The QTL in 0.1 mM NO_3^- associated with TLRL and TLRSA on chromosome 5 (flanked by bnl4.36/umc40) coincided with published QTL for primary root weight (Tuberosa

et al., 2002) and LR diameter (Hund *et al.*, 2004). Likewise two QTL on chromosomes 5 and 7 for TLRL and TLRSA (flanked by bnl5.24a/mmp118 and umc1708/csu8, respectively) coincided with two published QTL for average axial root length (Liu *et al.*, 2008). Indeed, contrasting morphological root traits were associated between QTL within this study and those published. However, the coinciding QTL were detected in different mapping populations, which at least strengthens the reliability of their putative association with morphological root traits in general, given that QTL only detected within one population can be imprecise (Liu *et al.*, 2008). Regardless, this highlights the requirement for further testing using different maize mapping populations to validate the putative QTL detected within this study before incorporating them into marker-assisted breeding programs.

Some limitations associated with the experimental system and setup were evident within this study. Firstly, only two true replicates were used to derive mean values for quantified traits, allowing for marked variance. This is evidenced by the low number of traits displaying NO_3^- treatment differences, despite the percentage difference for mean values (LN – HN %) exceeding 20 % in some instances. Had more replicates been used, NO_3^- treatment differences for phenotypic values may have been detected as the variance may have been reduced. Secondly, seedlings were harvested at 15 DAI as axial roots reached the bottom of the cigar-roll, which may have limited further root growth. Further, this allowed approximately 10 % of the root system to be submerged in nutrient solution, which may have delayed the occurrence of any NO_3^- treatment effects, as the axial roots may have been able to supply the seedling with all its N requirements during this early growth period when N demand and uptake may have been low (Sabermanesh, 2014a). We expected NO_3^- treatment differences to occur before 15 DAI as Sabermanesh (2014b) reported NO_3^- treatment differences in maize root morphology occurring 14 - 17 DAI when comparing 0.5 mM to 2.5 mM NO_3^-, and our NO_3^- treatment concentrations were beyond these values (0.1 mM vs. 5

mM NO_3^-, respectively). However, the flux density at canopy level within this study was lower than the mentioned study (*c.* 450 µmol m^{-2} s^{-1} vs. 650 µmol m^{-2} s^{-1}), likely lowering seedlings growth rate (Blackman and Wilson, 1951; Mitchell, 1953), and ultimately N demand. If this is the case, we hypothesise that more time was required for the seedlings N demand to exceed N supply with the light intensity provided.

QTL for axial root traits were not detected within this study. This may be because the axial roots reached the bottom of the cigar-roll, providing inaccurate variation within this population, compared to what may have been observed if further root growth was not limited. Indeed, QTL for axial root traits have previously been detected in maize using the cigar-roll culture system (Zhu *et al.*, 2006), however a larger RIL population was used consisting of 162 RILs, reducing the probability of type-II errors (QTL with small effects not being detected), in comparison to smaller populations (Zeng, 1994).

The cigar-roll culture system was employed for this study to expose roots to a gradient of nutrient concentrations along the roll, better reflecting the nutrient gradient down the soil profile, in comparison to hydroponics. Also, it provided a high-throughput alternative to grow and harvest roots with minimal impact on the seedlings native morphology, unlike what is required when phenotyping soil grown roots. However, given the described caveats associated with limited root growth by the cigar-culture system, we suggest this system may not be ideal for maize roots. If this study was performed again to confirm any putative QTL for root traits, an alternate experimental approach should be employed, enabling seedling roots to grow for 21 DAI without any restriction from the culture system. This would permit ample time for N treatment effects to occur on growth and morphology, as observed in hydroponics (Liu *et al.*, 2008; Sabermanesh, 2014a) and soil (Maizlish *et al.*, 1980). In addition, we suggest either using more replicates to minimise the standard error of mean phenotypic values, in order to

provide more accurate input data for QTL analysis, or utilising a larger maize mapping population to minimise type-II error and detect more putative QTL. We acknowledge that this will increase the labour involved in growing and phenotyping roots, however this may be unavoidable.

In conclusion, 11 putative QTL for morphological root traits were detected across two NO_3^- treatments. QTL in low NO_3^- were associated with LR traits that positively correlate with shoot growth, whereas those in sufficient NO_3^- were associated root size/volume. Three of the putative QTL associated with LR traits coincide with published QTL for morphological root traits, strengthening their putative role in regulating morphological roots traits. With further validation, these putative QTL could be used as potential markers towards breeding new maize cultivars with optimised root morphology to maximise above-ground yield and possibly N uptake through marker-assisted selection approaches.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Frequency distributions for quantified phenotypic traits.

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FIGURES

Table 1 Means for quantified phenotypic values

Phenoypic trait	NO_3^- treatment	Parental lines			IRILs	
		B73	Mo17		Mean	Range
AvgSM (g)		0.279	0.330		0.272	0.181 - 0.373
Shoot DW (g.plant^{-1})	0.1 mM	0.061	0.085	ns	0.063	0.026 - 0.106
	5 mM	0.062	0.100	ns	0.062	0.024 - 0.109
	0.1 mM - 5 mM (%)	-2.0 ns	-18.5 ns		1.3 ns	
Root DW (g.plant^{-1})	0.1 mM	0.066	0.101	ns	0.080	0.030 - 0.115
	5 mM	0.074	0.110	ns	0.075	0.02 - 0.110
	0.1 mM - 5 mM (%)	-11.1 ns	-9.2 ns		5.8 ns	
R:S (DW)	0.1 mM	1.08	1.28	ns	1.31	0.77 - 2.08
	5 mM	1.20	1.11	ns	1.28	0.77 - 2.01
	0.1 mM - 5 mM (%)	-10.6 ns	13.7 ns		2.3 ns	
TRL (cm)	0.1 mM	287.4	311.1	ns	326.3	111.7 - 626.2
	5 mM	312.3	305.0	ns	302.1	108.0 - 631.6
	0.1 mM - 5 mM (%)	- 8.6 ns	2.0 ns		7.4 ns	
TRSA (cm^2)	0.1 mM	52.24	66.68	ns	59.61	24.76 - 112.71
	5 mM	59.38	66.63	ns	58.07	20.75 - 129.83
	0.1 mM - 5 mM (%)	-13.7 ns	0.1 ns		2.6 ns	
TRV (cm^3)	0.1 mM	0.757	1.139	ns	0.874	0.438 - 1.632
	5 mM	0.899	1.159	ns	0.890	0.509 - 1.450
	0.1 mM - 5 mM (%)	-18.75 ns	-1.7 ns		-1.9 ns	
AvgD (mm)	0.1 mM	0.572	0.682	ns	0.590	0.499 - 0.720
	5 mM	0.606	0.695	ns	0.614	0.528 - 0.736
	0.1 mM - 5 mM (%)	-6.0 ns	-1.9 ns		-4.2 ns	
Number of root tips	0.1 mM	382.25	356.33	ns	429.61	126.33 - 748.67
	5 mM	362.75	358.00	ns	385.84	181.00 - 610.33
	0.1 mM - 5 mM (%)	5.1 ns	-0.5 ns		10.2 ns	
TAxL (cm)	0.1 mM	206.23	206.55	ns	224.35	96.74 - 379.99
	5 mM	242.50	233.63	ns	211.32	124.17 - 296.57
	0.1 mM - 5 mM (%)	-17.6 ns	-13.1 ns		5.8 ns	
TAxSA (cm^2)	0.1 mM	43.94	56.10	ns	49.34	22.97 - 87.81
	5 mM	50.43	58.60	ns	48.27	18.4 - 69.85
	0.1 mM - 5 mM (%)	-14.8 ns	-4.4 ns		2.2 ns	
TAxV (mm^3)	0.1 mM	796.40	1322.73	ns	954.60	371.80 - 2131.61
	5 mM	889.51	1284.20	ns	936.90	234.42 - 2299.13
	0.1 mM - 5 mM (%)	-10.4 ns	-3.0 ns		1.0 ns	
LR density (tips.cm axial root^{-1})	0.1 mM	1.9	1.7	ns	1.9	1.3 - 3.6
	5 mM	1.5	1.5	ns	1.8	1.5 - 3.1
	0.1 mM - 5 mM (%)	19.3 ns	11.2 ns		6.7 ns	

Table 1 (*Continued from previous page*)

Phenoypic trait	NO_3^- treatment	Parents			IRILs	
		B73	Mo17		Mean	Range
TLRL (cm)	0.1 mM	81.06	104.45	ns	101.83	14.97 - 254.17
	5 mM	69.68	71.31	ns	92.06	24.35 - 176.94
	0.1 mM - 5 mM (%)	14.0 ns	31.7 ns		9.6 ns	
TLRSA (cm^2)	0.1 mM	4.91	6.70	ns	6.16	0.87 - 15.77
	5 mM	4.19	4.38	ns	5.79	0.84 - 11.64
	0.1 mM - 5 mM (%)	14.7 ns	34.6 ns		6.0 ns	
TLRV (mm^3)	0.1 mM	33.10	35.75	ns	35.30	5.00 - 103.6
	5 mM	24.05	25.20	ns	40.26	2.80 - 75.10
	0.1 mM - 5 mM (%)	37.6 ns	41.9 ns		-12.5 ns	
AvgLRL (cm)	0.1 mM	0.213	0.291	ns	0.226	0.068 - 0.385
	5 mM	0.199	0.195	ns	0.232	0.094 - 0.361
	0.1 mM - 5 mM (%)	6.7 ns	33.0 ns		-2.4 ns	
AvgLRSA (cm^2)	0.1 mM	0.013	0.019	ns	0.014	0.003 - 0.029
	5 mM	0.012	0.012	ns	0.015	0.004 - 0.026
	0.1 mM - 5 mM (%)	8.0 ns	35.9 ns		-7.5 ns	
AvgLRV (mm^3)	0.1 mM	0.078	0.090	ns	0.112	0.010 - 0.449
	5 mM	0.070	0.069	ns	0.104	0.016 - 0.310
	0.1 mM - 5 mM (%)	11.8 ns	31.2 ns		7.7 ns	
RLE (cm.g root DW^{-1})	0.1 mM	4478	3131	*	4581	2462 - 6388
	5 mM	4440	2766	*	4252	2318 - 5732
	0.1 mM - 5 mM (%)	0.9 ns	11.7	*	7.2 *	
RSAE (cm^2.g root DW^{-1})	0.1 mM	800	669	*	838	604 - 1338
	5 mM	848	604	*	810	558 - 1182
	0.1 mM - 5 mM (%)	-5.9 ns	9.7 ns		3.3 ns	

Values are means from plants harvested 15 DAI (n = 2). 0.1 mM - 5 mM (%) represents the percentage difference of mean values between 0.1 mM and the 5 mM NO_3^- treatment. * Mean values that are significantly different between either the parental lines or the two growth conditions ($P < 0.05$). Ns represents comparisons of means that are not significantly different.

AvgSM, average seed mass; DW, dry-weight; R:S, root:shoot; TLR, total root length; TRSA, total root surface area; TRV, total root volume; AvgD, average diameter; tips, number of root tips; TAxL, total axial root length; TAxSA, total axial root surface area; TAxV, total axial root volume;TLRL, total lateral root length; TLRSA, total lateral root surface area; TLRV, total lateral root volume; AvgLRL, average lateral root length; AvgLRSA, average lateral root surface area; AvgLRV, average lateral root volume; RLE, root length efficiency; RSAE, root surface area efficiency.

Table 2 Pearson's correlation coefficients for average seed mass and morphological root traits in maize grown with low or sufficient NO_3^- supply

	AvgSM	Shoot DW	Root DW	R:S	TRL	TRSA	TRV	AvgD	Tips	TAxL	TAxSA	TAxV	LR density	TLRL	TLRSA	TLRV	AvgLRL	AvgLRSA	AvgLRV	RLE	RSAE
AvgSM		**0.25**	**0.32 ***	**0.09**	**0.34 ***	**0.37 ***	**0.37***	**0.35***	**0.25**	**0.25**	**0.33***	**0.42***	**0.10**	**0.37***	**0.39***	**0.40***	**0.34***	**0.34***	**0.32**	**0.13**	**0.18**
Shoot DW	0.25		**0.72 ***	**-0.44 ***	**0.56 ***	**0.64 ***	**0.66 ***	**0.54 ***	**0.53 ***	**0.47 ***	**0.61 ***	**0.66 ***	**0.28 ***	**0.57 ***	**0.55 ***	**0.54 ***	**0.44 ***	**0.42 ***	**0.40**	**-0.16**	**-0.21**
Root DW	0.30*	0.71 *		**0.30 ***	**0.77 ***	**0.88 ***	**0.91 ***	**0.80 ***	**0.60 ***	**0.79 ***	**0.90 ***	**0.90 ***	**0.09**	**0.59 ***	**0.56 ***	**0.55 ***	**0.41 ***	**0.39 ***	**0.37**	**-0.15**	**-0.16**
R:S	0.04	-0.45 *	0.31 *		**0.25**	**0.26**	**0.28 ***	**0.29 ***	**0.04**	**0.38 ***	**0.32 ***	**0.26**	**-0.28**	**-0.03**	**-0.05**	**-0.03**	**-0.07**	**-0.08**	**-0.07**	**0.01**	**0.07**
TRL	0.32*	0.53 *	0.78 *	0.26 *		**0.95 ***	**0.81 ***	**0.97 ***	**0.79 ***	**0.90 ***	**0.89 ***	**0.83 ***	**0.27 ***	**0.86 ***	**0.83 ***	**0.82 ***	**0.66 ***	**0.64 ***	**0.62**	**0.40 ***	**0.31 ***
TRSA	0.35*	0.60 *	0.88 *	0.30 *	0.95 *		**0.95 ***	**0.93 ***	**0.71 ***	**0.92 ***	**0.98 ***	**0.95 ***	**0.15**	**0.76 ***	**0.75 ***	**0.75 ***	**0.59 ***	**0.59 ***	**0.57**	**0.21**	**0.21**
TRV	0.35*	0.63 *	0.89 *	0.28 *	0.81 *	0.95 *		**0.79 ***	**0.56***	**0.85***	**0.97***	**0.99***	**0.02**	**0.58***	**0.58***	**0.58***	**0.45***	**0.45***	**0.44***	**-0.03**	**0.06**
AvgD	0.33*	0.52*	0.79*	0.27*	0.99*	0.95*	0.80*		**0.79***	**0.91***	**0.88***	**0.80***	**0.24**	**0.82***	**0.79***	**0.79***	**0.61***	**0.60***	**0.57***	**0.39***	**0.31***
Tips	0.24	0.52*	0.65*	0.10	0.77*	0.72*	0.57*	0.77*		**0.62***	**0.63***	**0.57***	**0.71***	**0.83***	**0.74***	**0.68***	**0.41***	**0.37***	**0.32***	**0.39***	**0.24**
TAxL	0.23	0.40*	0.78*	0.45*	0.88*	0.91*	0.84*	0.89*	0.60*		**0.94***	**0.83***	**-0.03**	**0.59***	**0.56***	**0.57***	**0.41***	**0.41***	**0.40***	**0.26**	**0.26**
TAxSA	0.32	0.57*	0.89*	0.36*	0.88*	0.97*	0.97*	0.89*	0.64*	0.94*		**0.96***	**0.02**	**0.63***	**0.61***	**0.61***	**0.46***	**0.46***	**0.44***	**0.11**	**0.15**
TAxV	0.42*	0.67*	0.89*	0.23	0.79*	0.93*	0.98*	0.79*	0.59*	0.79*	0.94*		**0.05**	**0.63***	**0.62***	**0.63***	**0.51***	**0.51***	**0.50***	**0.00**	**0.07**
LR density	0.10	0.28*	0.12	-0.25	0.26	0.15	0.02	0.25	0.71*	-0.05	0.02	0.07		**0.55***	**0.46***	**0.39***	**0.20**	**0.14**	**0.09**	**0.28***	**0.10**
TLRL	0.34*	0.56*	0.63*	0.03	0.86*	0.77*	0.59*	0.84*	0.81*	0.56*	0.62*	0.62*	0.55*		**0.98***	**0.95***	**0.82***	**0.79***	**0.75***	**0.46***	**0.30***
TLRSA	0.37*	0.54*	0.59*	0.00	0.82*	0.75*	0.58*	0.80*	0.72*	0.53*	0.59*	0.62*	0.46*	0.96*		**0.99***	**0.88***	**0.86***	**0.84***	**0.49***	**0.35***
TLRV	0.36*	0.52*	0.56*	0.00	0.79*	0.73*	0.57*	0.78*	0.64*	0.51*	0.57*	0.60*	0.39	0.93*	0.99*		**0.89***	**0.89***	**0.88***	**0.48***	**0.36***
AvgLRL	0.34*	0.48*	0.47*	-0.06	0.67*	0.59*	0.45*	0.64*	0.42*	0.38*	0.44*	0.51*	0.22	0.82*	0.88*	0.90*		**0.99***	**0.97***	**0.40***	**0.29***
AvgLRSA	0.33*	0.47*	0.45*	-0.06	0.64*	0.58*	0.45*	0.62*	0.36*	0.38*	0.44*	0.51*	0.15	0.78*	0.86*	0.89*	0.99*		**0.99***	**0.40***	**0.32**
AvgLRV	0.32	0.44*	0.43*	-0.05	0.62*	0.57*	0.45*	0.60*	0.31*	0.37*	0.42*	0.49*	0.10	0.74*	0.83*	0.87*	0.97*	0.99*		**0.37***	**0.31**
RLE	0.16	-0.13	-0.06	0.10	0.44*	0.26	0.03	0.44*	0.41*	0.28*	0.16	0.03	0.30*	0.46*	0.50*	0.49*	0.40*	0.38*	0.37*		**0.91***
RSAE	0.21	-0.18	-0.06	0.16	0.36*	0.27	0.13	0.36*	0.27	0.27	0.20	0.11	0.12	0.32*	0.38*	0.39*	0.29*	0.30*	0.31	0.92*	

Values in the lower portion of the table (not bold) represent the 0.1 mM NO_3^- treatment, whereas those in the upper portion (bold) represent the 5 mM NO_3^- treatment. * Coefficients that are significantly different from zero ($P < 0.01$).

AvgSM, average seed mass; DW, dry-weight; R:S, root:shoot; TLR, total root length; TRSA, total root surface area; TRV, total root volume; AvgD, average diameter; tips, number of root tips; TAxL, total axial root length; TAxSA, total axial root surface area; TAxV, total axial root volume;TLRL, total lateral root length; TLRSA, total lateral root surface area; TLRV, total lateral root volume; AvgLRL, average lateral root length; AvgLRSA, average lateral root surface area; AvgLRV, average lateral root volume; RLE, root length efficiency; RSAE, root surface area efficiency.

Table 3 Putative QTL detected in maize grown with low or sufficient NO_3^- supply using whole genome QTL analysis

NO_3^- treatment	Trait	Chromosome no.	Flanking markers[a]	Interval (IcM)[b]	AGP coordinate interval[c]	Co-locating QTL[d]	LOD	r^2 (%)[e]	Allele effect[f]
0.1 mM	**TLRL**	1	mmp56 - cdo938a	495.0 - 498.72	54,737,591 - 54,924,847	●	4.32	7.9	19.95
		1	umc1118 - umc1744	1595.84 - 1613.61	290,523,810 - 292,796,493	○	4.03	6.1	-19.03
		2	bnlg1893 - umc36a	879.09 - 889.89	232,577,740 - 232,717,048		2.68	3.8	14.26
		5	ufg49 - bnl6.10	417.79 - 419.9	43,661,909 - 44,053,209		4.45	9.6	21.75
		5	bnl4.36 - umc40	481.02 - 493.85	80,842,741 - 87,415,251	□	9.37	20.3	-34.42
		5	bnl5.24a - mmp118	886.19 - 901.21	205,552,861 - 216,915,529		4.68	7.3	20.48
		7	umc1708 - csu8	710.52 - 718.72	163,184,932 - 163,209,668	Δ	6.79	10.7	-23.98
		9	ufg47-umc2095	558.99 - 573.7	134,304,956 - 136,017,208	†	2.23	3.6	14.06
							Total	**69.3**	
	TLRSA	1	mmp56 - cdo938a	495.0 - 498.72	54,737,591 - 54,924,847	●	4.76	14.2	26.70
		1	umc1118 - umc1744	1595.84 - 1613.61	290,523,810 - 292,796,493	○	2.46	3.6	-13.95
		5	bnl4.36 - umc40	481.02 - 493.85	80,842,741 - 87,415,251	□	2.65	6.8	-19.32
		6	umc62 - npi561	774.51 - 792.61	164,802,736 - 165,517,084		2.23	5.5	17.93
		7	umc1708 - csu8	710.52 - 718.72	163,184,932 - 163,209,668	Δ	6.18	14.6	-27.91
		9	ufg47 - umc2095	558.99 - 573.7	134,304,956 - 136,017,208	†	4.43	11	25.19
							Total	**55.7**	
5 mM	**AvgLRV**	2	npi208c - umc1824	133.78 - 137.48	6,460,688 - 8,018,406		2.29	8.7	-14.13
							Total	**8.7**	
	RLE	10	umc2021 - php20568a	761.77 - 778.68	147,933,074 - 147,970,601		2.45	12.1	-7.04
							Total	**12.1**	

[a] Markers that flank the LOD confidence interval. [b] The position that defines the interval around the position of peak likelihood for the QTL. [c] The genome location of each genetic marker was obtained through the integration of the IBM linkage map (http:/www.maizegdb.org/qtl-data.php) and the maize B73 genome sequence (Maize Genome AGP version 3; www.maizesequence.org). [d] Symbols in column represent differing pairs of QTL flanked by matching markers. [e] r^2 represents the proportion of phenotypic variation explained by the QTL. [f] A positive value indicates that B73 carries the allele contributing towards an increase in the phenotypic trait, whereas a negative value means that the allele is carried by Mo17.

Table 4 Comparison of QTL detected within this study and those published in the literature

Phenotypic trait	Chromosome no.	Flanking/associated markers	AGP coordinate interval[a]	Population	Treatment	Reference
average axial root length	5	umc2373 - umc1060	80,842,184 - 136,342,406	Z3 × 87-1	**low N**	Liu *et al.* (2008)
primary root weight	5	php10017	205,440,973 - 217,012,402	Lo964 × Lo1016	**drought stress**	Tuberosa *et al.* (2002)
lateral root diameter	5	php10017	205,440,973 - 217,012,402	Lo964 × Lo1016	**cold stress**	Hund *et al.* (2004)
average axial root length	7	umc1782 - phi328175	162,153,302 - 163,184,932	Z3 × 87-1	**low N**	Liu *et al.* (2008)

[a] The genome location of each genetic marker was obtained through the integration of the IBM linkage map (http:/www.maizegdb.org/qtl-data.php) and the maize B73 genome sequence (Maize Genome AGP version 3; www.maizesequence.org).

SUPPLEMENTARY MATERIAL

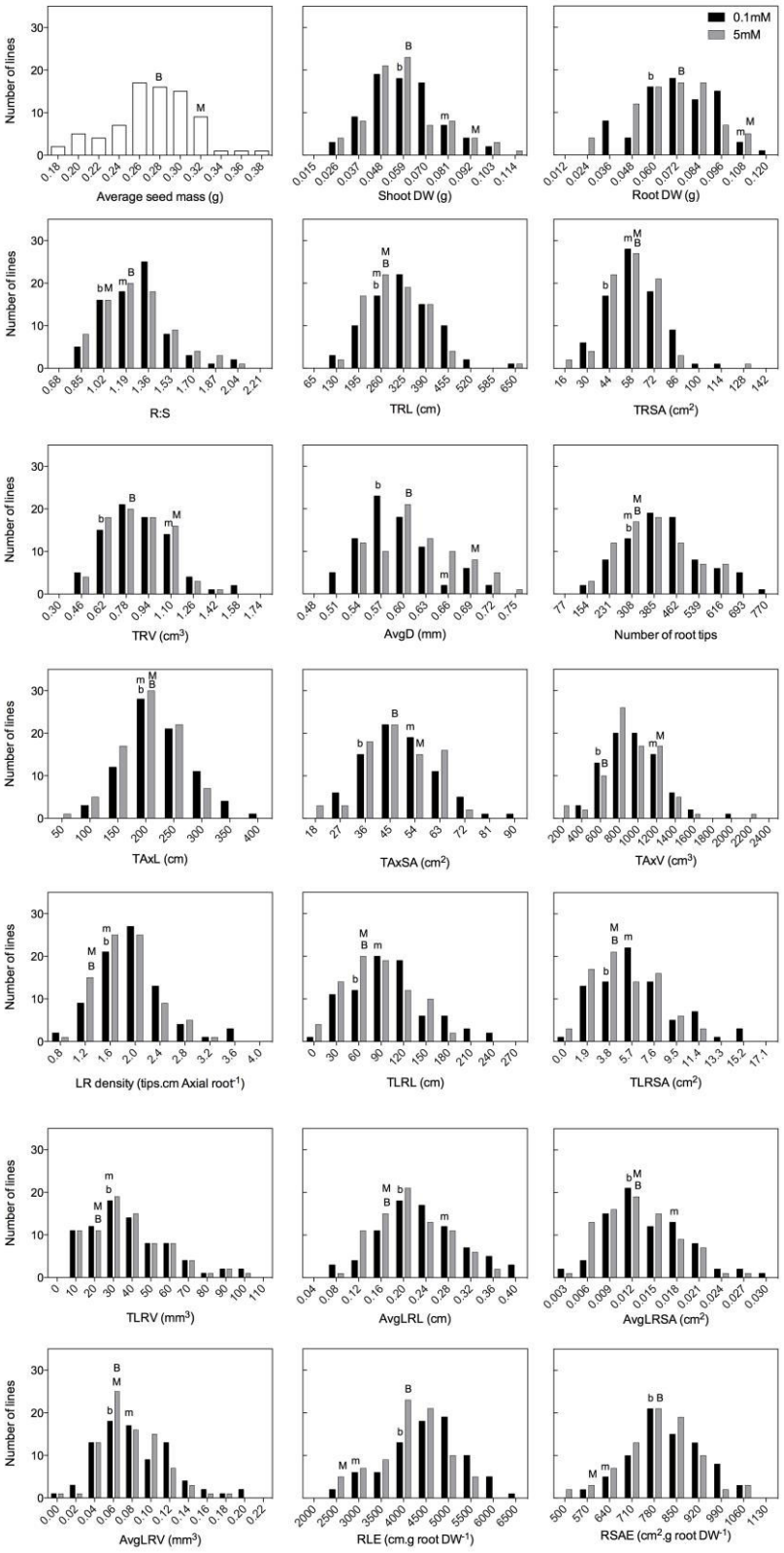

Fig. S1 Histograms of frequency distributions for phenotypic traits quantified from the IRILs. Average seed mass (AvgSM) was measured prior to imbibition (white column; n = 10). Values for shoot DW, root DW, root:shoot (R:S) and all morphological root traits are the mean of plants harvested at 15 DAI (n = 2). Plants were grown in either 0.1 mM (black column) or 5 mM (grey column) NO_3^-. Lower-case b and m above columns represent the means of B73 and Mo17 in 0.1 mM NO_3^- respectively, whereas the upper-case letters represent their means in 5 mM NO_3^-.

Chapter 5: General discussion

It has been proposed that the poor NUpE of cereals could be improved by increasing NO_3^- uptake capacity and optimising root morphology to maximise root N uptake (Garnett *et al.*, 2009). However, in order to improve these components of root N uptake, the biology behind increasing root NO_3^- uptake capacity to meet demand, and adaptation of root morphology to N supply needs to be understood. Recent work within our laboratory indicates that some maize lines maintain net N uptake and shoot biomass when NO_3^- supply is limited, relative to sufficient NO_3^- supply, without altering root biomass or root:shoot (21 DAI) (Garnett *et al.* unpublished data). Conversely, other lines already decreased net N uptake and shoot growth, and increased root:shoot by 21 DAI. This highlights the existence of natural variation in the capacity of maize seedlings to maximise N uptake and shoot growth in low N environments, and that N supply can affect growth very early in development. Maximising growth during seedling establishment is important as differences early in development often affect final harvested yield (Claassen and Shaw, 1970; Koutroubas *et al.*, 1998; Grant *et al.*, 2001). The research described in this dissertation used early seedling development to:

i) understand how seedlings manage the transition from seed N use to external N capture;
ii) determine how seedlings adapt to NO_3^- supply by changing root morphology;
iii) identify genetic loci associated with morphological root traits contributing to shoot dry matter accumulation and/or N uptake, relative to NO_3^- supply.

5.1 Advances in knowledge from this study

A model was developed in Chapter 2 encompassing the physiological processes involved in managing the transition from seed N use to external N capture in maize. As seedlings developed, shoot N% as well as root and shoot free amino acid concentrations decreased.

Once root and shoot free amino acid concentrations reached critical levels (8 DAI), seedlings rapidly increased root NO_3^- uptake capacity (10 DAI) to maintain tissue N concentrations. Then, as root NO_3^- uptake reached maximum capacity, seedlings adapted to N limitation by increasing root:shoot, relative to the sufficient N treatment if tissue N concentrations were not stabilised (12 DAI).

No particular free amino acid was identified to regulate root NO_3^- uptake. However, asparagine and glutamine have previously been suggested to have a role, and the results here do not conflict with this (Muller and Touraine, 1992; Sivasankar *et al.*, 1997; Vidmar *et al.*, 2000). The increase in root NO_3^- uptake capacity correlated with transcript profiles of genes encoding putative high-affinity NO_3^- transporters *ZmNRT2.1*, *ZmNRT2.2* and NO_3^- uptake related protein *ZmNRT3.1A*. This highlights their putative roles in mediating NO_3^- uptake capacity in maize, as has been shown in *Arabidopsis* (reviewed by Wang *et al.* (2012)). This suggests that we could possibly apply our existing knowledge of the functional role of the *Arabidopsis* orthologues of these *NRT*s to maize, despite the dichotomy in the coding sequence of these genes between *Arabidopsis* and cereals (Plett *et al.*, 2010). Understanding how seedlings manage this transition provides insight into how and why plants up-regulate their NO_3^- uptake system to meet N demand.

The timing of the physiological processes outlined in the model were similar for both lines tested (*Zea mays* var. B73 and Mo17), suggesting that the model may also be applicable to other maize lines. However, an increase in B73 root:shoot corresponded to decreased net N uptake and shoot growth, whilst no change to root growth rate was observed. Conversely, Mo17 increased root mass when NO_3^- supply was limited, and maintained both net N uptake and shoot growth. Consequently, Chapter 3 investigated whether the adaptive growth responses of B73 and Mo17 to NO_3^- limitation are reflected by any changes to root morphology. Results indicated that the maintenance of Mo17 shoot growth in low NO_3^- could

have been attributed to the increased effective absorptive area of the root, relative to the sufficient NO_3^- treatment. This resulted from increases in lateral and axial root length prior to increasing root mass. Conversely, B73 roots were unresponsive to NO_3^- limitation, perhaps leading to decreased shoot growth, with respect to the sufficient NO_3^- treatment. Together this highlights that some maize lines are able to adapt to NO_3^- limitation by increasing the absorptive area of the root via a change in morphology rather than root mass. By understanding which morphological root responses help maintain shoot growth when NO_3^- supply is limited, we can understand which traits may contribute to a root system optimised for maximal N capture. These traits could then be utilised as phenotypic markers within breeding programs to generate new varieties with sustained/improved yields in low-input agricultural systems.

By observing the processes encompassing how maize seedlings meet N demand and maximise shoot growth relative to NO_3^- supply, a possible 'order of responses' can be derived. When a seedling's N demand increases, they appear to:

i) Increase root NO_3^- uptake capacity;
ii) Modify root morphology, independent of root mass;
iii) Increase root:shoot.

This order infers that seedlings initially try and meet their N demand by up-regulating their NO_3^- uptake system. This strategy involves increasing NO_3^- capture per unit root surface area, whilst maintaining minimal root infrastructure to maximise shoot growth. If the growing N demand is not met with maximal root NO_3^- uptake capacity, seedlings may then modify their root system to increase the total absorbing area. This could help increase net root NO_3^- uptake by increasing the effective absorbing area, however validation of this inference is required being derived from results across two separate experiments. Also, this may only be

an adaptive response employed by some maize lines, as it was only observed in Mo17. If all prior attempts to capture enough N fail to meet the seedling's growing N demand, seedlings resort to changing biomass allocation, reflected in increased root:shoot, possibly reducing shoot growth potential. This adaptive root:shoot increase differs within maize, whether it is decreased shoot growth rate, possibly to minimise the increase in N demand, or increased root growth to increase the absorptive area of the root and maintain net NO_3^- uptake and maximise shoot growth. This order may exist to ensure the C cost of developing and maintaining the root system is kept minimal, in order to maximise shoot development and ultimately photosynthetic capacity.

Chapter 4 identified genetic loci for morphological root traits contributing to greater shoot growth and/or previously associated with the theoretical ideotype of maize root morphology for optimised N uptake (Lynch, 2013), relative to NO_3^- supply. Across both NO_3^- treatments, a panel of morphological root traits were positively correlated with shoot growth, including the length and surface area of lateral roots. In low NO_3^-, nine putative QTL associated with the length and surface area of lateral roots were detected, with three of these coinciding with published QTL for morphological root traits. Although discrepancy existed for the morphological root traits these coinciding QTL were associated with, their putative association with the genetic regulation of some morphological root traits in general were strengthened, as the QTL were detected using different mapping populations. In sufficient NO_3^-, a putative QTL associated with root length per unit root DW was detected. This trait was previously described as 'cheap roots' and was associated with the theoretical ideotype for optimised water and N uptake in maize, as maximum surface area can be achieved whilst minimising the metabolic cost associated with constructing and maintaining a root system which favours shoot growth (Lynch, 2013). Together, a total of eight new putative QTL and three co-localising with published QTL were detected for morphological root traits

contributing to greater shoot growth and/or N uptake. With further testing, the repeatability and validity of these putative QTL can be clarified and used as genetic markers within breeding programs towards the generation of new maize varieties with enhanced shoot growth and N uptake.

5.2 Future directions

Critical developmental time-points associated with the up-regulation of the NO_3^- uptake system and adaptive responses to N limitation were identified across two separate experiments. Consequently, another growth experiment is required to capture all the processes detailed within the model from Chapter 2, and the adaptive morphological root responses to NO_3^- supply. Greater separation in NO_3^- concentrations between treatments should be incorporated (e.g. 0.2 mM vs. 5 mM, rather than 0.5 mM vs. 2.5 mM) to better exploit any NO_3^- treatment effects on the NO_3^- uptake system and growth, whilst also investigating how this model performs across other NO_3^- concentrations. The transcription networks underpinning these responses can then be dissected by analysing gene expression at critical developmental time-points:

i) 9 DAI – Just prior to the up-regulation of *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT3.1A* transcription and NO_3^- uptake capacity;
ii) 11 DAI – *ZmNRT2.1*, *ZmNRT2.2* and *ZmNRT3.1A* transcription and NO_3^- uptake capacity increasing;
iii) 12 DAI – Increase in root:shoot occurs when NO_3^- is limited, relative to sufficient NO_3^-;
iv) 15 DAI – NO_3^- uptake capacity at maximum capacity.

By performing transcriptomics on time-points before, during, and after the processes involved in up-regulating the NO_3^- uptake system and changing biomass allocation, candidate

regulatory and N-responsive genes regulating these processes can be identified. These genes can then be manipulated in the attempt to generate maize with greater NO_3^- uptake capacity and ultimately NUpE.

The proposed model for the up-regulation of root NO_3^- uptake across the transition from seed N use to external N capture fits early seedling establishment. However, whether this is also applicable to later growth stages when root N uptake increases to meet demand has not been clarified. Garnett *et al.* (2013) showed that the NO_3^- uptake system responds to demand across the lifecycle of maize, with peak uptake rates occurring during early vegetative growth and towards anthesis. This provides scope to validate the model at these growth stages. If the model is valid for later growth stages, then the use of the transition of seedlings from seed N use to external N capture may be the ideal system to dissect the regulation of NO_3^- uptake capacity and adaptation to N supply, as it provides researchers the opportunity to conduct shorter growth experiments, in comparison to using mature plants. Future directions should also be aimed at quantifying how the early effects of NO_3^- supply on net N uptake and growth influence later growth to clarify which seedling traits could maximise final harvestable yield. It must also be kept in mind that the observations within this dissertation are derived in hydroponics; a system where water and nutrients are constantly available to plant roots. Consequently, the responses to N supply should be clarified in soil/field conditions to validate their applicability to more agriculturally comparable environments.

Chapters 3 and 4 highlighted how morphological root traits may contribute towards shoot growth, relative to NO_3^- supply, however their relative contributions towards NO_3^- uptake and uptake capacity remains to be clarified. This could be approached by quantifying NO_3^- uptake capacity relative to root surface area rather than root mass. It could also be

approached by using methods that are able to measure uptake in specific root tissues, such as those utilising NO_3^- specific microelectrodes for ion flux measurements. This could be used in conjunction with Q-PCR of putative *ZmNRT* genes on individual root components (e.g. lateral roots, axial roots, root tips) when NO_3^- uptake capacity is at maximum rates (15 DAI). By quantifying NO_3^- uptake rates on individual root components, their relative contribution to net NO_3^- uptake could be determined, which would help determine how maize root morphology could be optimised to maximise NO_3^- uptake.

B73 and Mo17 were used throughout this dissertation as important genetic resources are available for these lines. Thus, if considerable differences in quantified traits existed between these two lines, their genetic regulation could be dissected using forward genetics. However, substantially contrasting profiles for NO_3^- uptake capacity; net N uptake; *ZmNRT1* and *ZmNRT2* transcripts; and root and shoot growth were not observed between these lines, suggesting this pair may not have been ideal to detect genetic differences for these traits within maize. If lines with greater contrast in any of the mentioned profiles were identified within the maize diversity set, this would provide the basis to dissect the genetic regulation for these contrasting traits using forward genetics. The lack of difference between the lines tested may have also been contributed by the NO_3^- concentrations used. Had a greater separation in NO_3^- concentrations been used (0.2 mM vs. 5 mM rather than 0.5 mM vs. 2.5 mM), differences in these profiles may have been detected.

Despite using substantially different concentrations for the low and sufficient NO_3^- treatments in Chapter 4, minimal treatment effects on root morphology were detected, hindering the opportunity to detect QTL for morphological root traits, relative to NO_3^- supply. This lack of NO_3^- treatment differences may have been contributed by the early harvest of seedling roots (due to the roots reaching the end of the cigar-roll), or the lower flux density at

canopy level in comparison to what was used in Chapters 2 and 3 (*c.* 450 µmol m^{-2} s^{-1} vs. 650 µmol m^{-2} s^{-1}). Future studies should employ a different growth system that permits unrestricted root growth for 21 DAI, as NO_3^- treatment differences in growth were established by this time in Chapters 2 and 3. A proposed growth system could consist of individual pots (narrow but deep) filled with very sandy soil (or silicate sand), within an ebb and flow hydroponic system. This will expose roots to an environment where they penetrate through the strata to establish themselves whilst still exposed to uniform nutrient supply. Also, using sandy soil could help minimise the adherence of soil particles to the root system, which would minimise the presence of artefacts in subsequent image analysis.

Understanding how the NO_3^- uptake system up-regulates, and how seedlings adapt to N supply to maintain maximal growth provides insight into how root N uptake is maximised relative to N supply. Future investigation into the global gene expression across these key developmental time-points during this stage of plant growth could help elucidate the molecular regulation of NO_3^- uptake capacity and root morphology. This knowledge may ultimately help develop cereal crops with enhanced NO_3^- uptake capacities and shoot growth.

Chapter 6: Literature cited (Literature review & general discussion)

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