Long- and short-term nitrate uptake regulation in maize

By

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Thesis submitted in fulfilment of the requirements for the degree of

Doctorate of Philosophy in the Faculty of Sciences at

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I dedicate this thesis

in loving memory of my dearest Nan

Audine Kay Holtham

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Luke Reid Holtham

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"Many of life's failures are people who did not realize how close they were to success when

they gave up"

- Thomas A. Edison -

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Abstract

Cereal crops supply a major proportion of the world's food and their production capacity is tightly linked to nitrogen (N) fertiliser use. With on average less than half of the applied N being captured by crops, there is scope and need to improve N uptake in cereals. With nitrate (NO_3^-) being the main form of N available to cereal crops there has been a significant global research effort to understand plant NO_3^- uptake. Despite this, our understanding of how the NO_3^- uptake system is regulated remains limited.

To advance our understanding of the NO_3^- uptake system and its regulation, three knowledge gaps were identified and explored in this thesis. Firstly, there is an identified need to better understand the NO_3^- uptake system and the signalling molecules which modulate it. Secondly, with the literature containing alternative approaches to studying NO_3^- uptake, there is a need to appreciate how these studies relate to better leverage the existing literature. And finally, with strong transcriptional control governing the NO_3^- uptake system, new leads were sought for modulating transcription of NO_3^- transporter genes.

To explore these knowledge gaps, dwarf maize (*Zea mays* L. var. Gaspe Flint) was grown hydroponically with either sufficient or limiting NO_3^- availability. During the vegetative growth period a subset of plants grown were moved from sufficient to limiting NO_3^- conditions and a range of physiological parameters were measured. The results showed: the high affinity NO_3^- uptake system (HATS) appears to contribute a major proportion of total NO_3^- uptake capacity and responds to N demand at external concentrations where it was previously thought to be saturated; NO_3^- itself appears to play a key role in modulating the NO_3^- uptake system, and; temporal variation of *NRT* transcripts are more variable than previously understood. The observed responses to reduction in NO_3^- revealed a series of responses leading to a new model for the control of the NO_3^- uptake system. Using the same growth system, plants were grown under steady state NO_3^- conditions and a starvation and re-

263   supply (primary nitrate response – PNR) response was explored in parallel. The information

264   generated provided data to relate the PNR literature to longer term steady state studies. The

265   *ZmNRT2.5* gene was highlighted as an interesting candidate for revealing cis-trans regulatory

266   elements associated with low N responses. To explore this, a combined phylogenomics and

267   co-expressed gene promoter analysis was undertaken. A number of evolutionarily and

268   functionally conserved regions were identified in the *ZmNRT2.5* promoter with six regions

269   showing no resemblance to known transcription factor binding sites. These sequences provide

270   a new resource for the discovery of cis-trans regulatory mechanisms associated with the low

271   N expression of *ZmNRT2.5*.

272   The findings in this thesis have identified key time points for future transcriptome analysis,

273   and revealed putative cis-elements as new leads for discovering novel cis-trans regulatory

274   elements associated with the regulation of NO_3^- uptake. Ultimately, further research may lead

275   to the identification of key regulatory genes as candidates for the improvement of N uptake

276   efficiency and overall N use efficiency in cereal crops.

List of Abbreviations

278	AA	amino acid
279	ANOVA	analysis of variance
280	bnt	billion tonnes
281	C	carbon
282	d	days
283	DAE	days after emergence
284	DW	dry weight
285	g	gram
286	HATS	high-affinity transport system
287	LATS	low-affinity transport system
288	N	nitrogen
289	NH_4^+	ammonium
290	NiR	nitrite reductase
291	NO_3^-	nitrate
292	NPF	nitrate transporter 1/peptide transporter family
293	NR	nitrate reductase
294	NRT	nitrate transporter
295	NUE	nitrogen use efficiency
296	NUpE	nitrogen uptake efficiency
297	NUtE	nitrogen utilisation efficiency
298	R:S	root to shoot biomass ratio
299	SEM	standard error of the mean
300	TAA	total amino acids
301	TFs	transcription factors

Chapter 1: Literature review

1.1 The importance of cereals

Cereals provide approximately 90% of the world's food energy intake with rice (*Oryza sativa* L.), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) accounting for around two thirds of this (Bruinsma, 2003). With world population set to grow by 35% from 2013 to 2050 (United Nations, 2013; World Bank, 2013), increases in per capita caloric consumption (Kearney, 2010), changes in diets leading to more consumption of grain consuming meat and dairy products (Du *et al.*, 2004), and increased use of cereal based biofuels (Nonhebel, 2012), demand for cereal crops is anticipated to continually rise in the foreseeable future (Alexandratos & Bruinsma, 2012).

1.2 Meeting global demand

World cereal production is anticipated to reach 2.52 billion tonnes (bnt) in 2014 and recent estimates expect demand to reach 3.28 bnt by 2050 meaning a further 30% increase may be required to reach 2050 demand (Fig. 1) (Alexandratos & Bruinsma, 2012; FAO, 2014a). Production increases to date have been achieved predominantly through increases in yield (78% contribution), with only minor contributions from increased cropland (15% contribution) and increased cropping intensity (7% contribution) (FAO, 2006; Foley *et al.*, 2011). Increases in average cereal crop yield appear to have grown linearly over time (Fig. 1), however, assessing growing regions separately has revealed that in recent years yield increases in some of the world's most important cropping regions have stagnated (Cassman, 1999; Peltonen-Sainio *et al.*, 2009; Brisson *et al.*, 2010; Finger, 2010). With recent reviews estimating that cereal yields in 24 – 39% of growing areas either never increase, have plateaued or are in decline (Ray *et al.*, 2012; Ray *et al.*, 2013), combined with land and water resource constraints (Hertel, 2011), soil degradation and salinisation (Cassman, 1999; Tilman *et al.*, 2011), land use competition and climate change (Lambin & Meyfroidt, 2011; Kurukulasuriya & Rosenthal, 2013), it is clear that there are significant challenges ahead to meet future cereal crop demands.
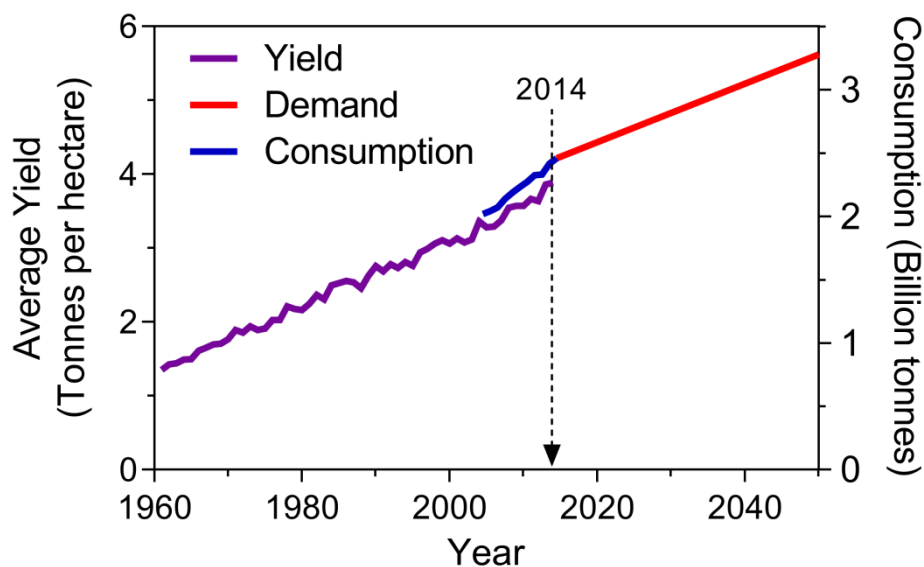
Fig. 1 Historical cereal yield data since 1961 compared to historical cereal consumption data since 2004 and anticipated 2050 cereal demand. Data sourced from (FAO, 2014a; FAO, 2014b).

1.3 Nitrogen in agriculture

1.3.1 A brief history

Nitrogen (N), the fifth most abundant element in our solar system, is a core component of amino acids which are essential for the synthesis of nucleic acids and proteins—the two most important building blocks of life (Canfield *et al.*, 2010). Of all the mineral nutrients that plants acquire from the soil to facilitate growth, N is required in the greatest amount (Marschner & Marschner, 2012). Some plant species, such as legumes, have the ability to acquire atmospheric N through a symbiotic relationship with N_2- fixing bacteria (Mylona *et al.*, 1995). Cereals however rely predominantly on N forms accessible via the root uptake system (Raun & Johnson, 1999). German chemist Justus von Liebig demonstrated in 1847 that N in its mineral form could be applied to the soil to facilitate plant growth, setting the stage for the development of a new industry and a substantial increase in crop production a century later (Liebig & Playfair, 1847). In 1909 the Nobel prize laureate Fritz Haber patented what was later termed the "Haber-Bosch" process enabling the economical production of N fertilizers at industrial scale by 1913 (Erisman *et al.*, 2008). This subsequently sparked the

rapid expansion of agriculture and fuelled human population growth during the 20th century

(Smil, 1999a; Smil, 2004; Erisman *et al.*, 2008). Since the 1960s, due to the continual demand

for increased cereal crop yield and the subsequent requirement for soil N, the use of N

fertilizers has grown steadily over the years in line with crop production (Fig. 2). This has

driven N fertiliser consumption to a volume of approximately 120 million tonnes globally in

2013 (Fig. 2) (FAO, 2014b).
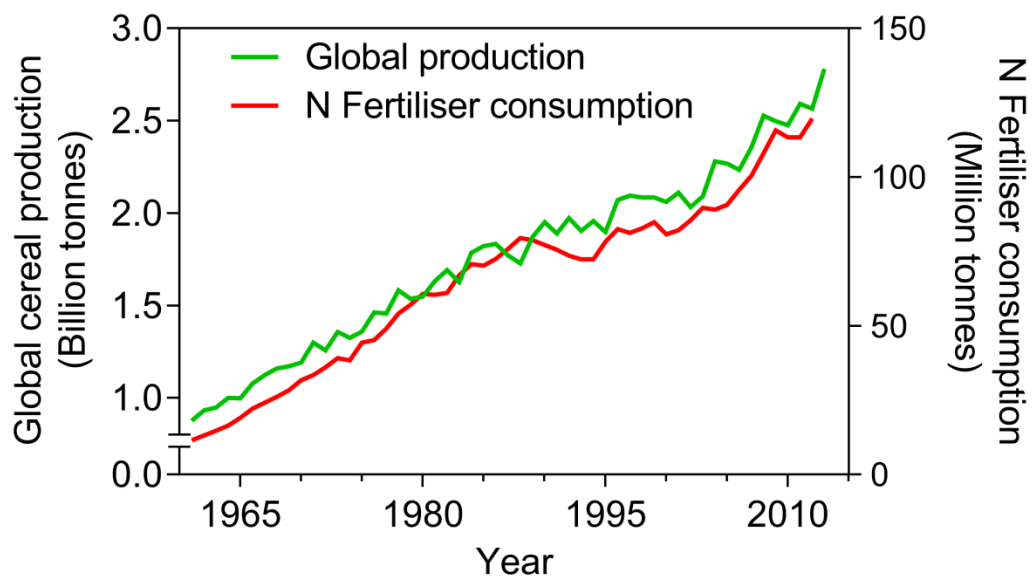
Fig. 2 Historical global cereal production data since 1961 compared to historical N fertiliser

consumption. Data sourced from (FAO, 2014b). N.B. The left Y axis has been truncated to

demonstrate the correlation between the two.

1.3.2 Economics

Total fertiliser production via the Haber-Bosch process is estimated to consume

approximately 2% of the global energy supply (Sutton *et al.*, 2013). The production of one

kilogram (kg) of N fertiliser consumes approximately 70,000 kJ (Helsel, 1992) which is

enough energy to power an average Australian household for an entire day (AER, 2014). With

such an energy hungry process, energy prices strongly influence global fertilizer prices

(Ramírez & Worrell, 2006). The main starting component for the production of N fertilizers is

natural gas, as a result natural gas price and availability also contribute strongly to N fertilizer

prices (GAO, 2003). Even if technological breakthroughs were able to decrease the cost of N

63   fertiliser production, N, like other crops nutrients, is a world market commodity and is

64   subsequently at the mercy of global economic forces such as market volatility and global

65   demand. With the dependence on energy and the influence of global market forces due to

66   demand, N fertiliser prices are anticipated to remain high into the future.

67   **1.3.3 Environmental impact**

68   The earth's N cycle has evolved over the past 2.7 billion years into a complex and delicate

69   process with natural feedback mechanisms and controls (Canfield *et al.*, 2010). Crop

70   production is the single largest cause of human alteration to the global N cycle with the

71   production and application of N fertilisers affecting the environment in multiple ways (Smil,

72   1999b). Excess N fertiliser not taken up by plants can run off or leach into waterways,

73   contaminating ground water and causing eutrophication of water bodies leading to the

74   formation of copious dead zones in rivers, lakes and oceans across the globe (Tilman *et al.*,

75   2002; Diaz & Rosenberg, 2008; Galloway *et al.*, 2008). In addition, soil microbial activity

76   and volatilisation of excess N produces significant volumes of greenhouse gases (N_2O, NO)

77   with a net neutral effect on total greenhouse gas levels due to carbon sequestration by the

78   plants (Snyder *et al.*, 2009; Burney *et al.*, 2010; de Vries *et al.*, 2011). This negates any

79   reverse effect on global warming that could be achieved through more efficient agricultural

80   production. With such significant impacts on the environment, the continued high use of N

81   fertilizers is not environmentally sustainable demanding improvements on the efficiency of N

82   fertilizer use.

83   **1.4 Nitrogen use efficiency**

84   **1.4.1 Defining NUE**

85   With the need to increase cereal production in an environmentally and economically

86   sustainable fashion, the potential to improve plant nitrogen use efficiency (NUE) has attracted

87   significant global attention. Many different descriptions of NUE exist, however, at their core

88   they all provide a measure of N input relative to plant output (Moll *et al.*, 1982; Good *et al.*,

89   2004; Masclaux-Daubresse *et al.*, 2010; Good & Beatty, 2011; Hawkesford, 2011; Hirel &

90   Lea, 2011; Kant *et al.*, 2011; Xu *et al.*, 2012). For the purpose of this thesis we have defined

91   NUE broadly as the yield (biomass or grain) per unit of available N (fertilizer + residual soil

92   N).

1.4.2 Agronomy

94   The use of improved N fertilizer management techniques can certainly improve NUE in cereal

95   crops (Keeney, 1982; Cassman *et al.*, 2002). Better synchrony between available soil N and

96   crop demand, controlled release fertilizers, nitrification inhibitors, advanced spatial

97   application, and improved estimation of N requirements hold promise for improving NUE

98   (Trenkel & Association, 1997; Shoji *et al.*, 2001; Shanahan *et al.*, 2008). With the rise of

99   information technology the concept of using sensors, robotics and automation for precision

100   agriculture is closer to becoming a reality (Gebbers & Adamchuk, 2010). These emerging

101   technologies have the potential to drive more efficient N fertilizer use, however, with the high

102   cost and early stage of these technologies it will likely be some time before they can make a

103   significant impact (Bongiovanni & Lowenberg-Deboer, 2004; Chen *et al.*, 2014).

1.4.3 Improving plant NUE

105   An attractive route for improving NUE in cereals is through improving the plants genetic

106   potential for NUE. There are two main aspects to improving cereal NUE. Firstly, N utilization

107   efficiency (NUtE) describes the plant's capacity to utilise accumulated N to facilitate efficient

108   conversion of N to grain yield (Good *et al.*, 2004). Plant NUtE is influenced by the plants

109   ability to efficiently assimilate N (N assimilation efficiency (NAE)) and to remobilize stored

110   N from vegetative tissues during seed maturation (N remobilization efficiency (NRE)) (Moll

111   *et al.*, 1982; Avice & Etienne, 2014). The second aspect to improving NUE is increasing N

112   uptake efficiency (NUpE) which is the plant's capacity to capture N from the soil (Garnett *et*

113   *al.*, 2009; Masclaux-Daubresse *et al.*, 2010). Because cereal crops only capture 40 – 50% of

114   the applied N fertiliser, there appears to be significant scope for the genetic improvement of

115   NUpE to deliver improvements in NUE for production, economic and environmental gains

116   (Peoples *et al.*, 1995; Sylvester-Bradley & Kindred, 2009). As a result this dissertation

117   focuses on the N uptake system with the aim of generating information useful for the

118   development of cereal crops with improved NUpE and overall increased NUE.

119   ## 1.5 The plant nitrogen management system

120   In order to focus on the N uptake system it is important to understand how plants manage N.

121   As N is an essential mineral element (Arnon & Stout, 1939), plants have a complex N

122   management system to facilitate N capture, assimilation, storage, and redistribution to

123   facilitate plant growth and reproduction. This section briefly explores key components of the

124   system to provide background for the subsequent focus of this thesis.

125   ### 1.5.1 N in soils

126   Plants acquire most of their essential nutrients from the soil via the root system (Marschner &

127   Rengel, 2012). The major sources of N in agricultural soils are nitrate (NO_3^-) and ammonium

128   (NH_4^+) (Wolt, 1994). Proportionally NH_4^+ is on average 10% of the soil NO_3^- concentration,

129   making NO_3^- the predominant form of N available to cereal crops (Miller *et al.*, 2007). Due to

130   its negative charge and solubility NO_3^- is highly mobile, and in cropping soils can vary by

131   four orders of magnitude from micromolar to millimolar (Ho *et al.*, 2009). As sessile

132   organisms, plants therefore need to be able to rapidly adapt to these variable soil NO_3^-

133   concentrations to optimize N capture.

134   ### 1.5.2 Nitrate uptake

135   To cope with such variable soil NO_3^- concentrations plants have two NO_3^- uptake systems: a

136   high affinity transport system (HATS) which is active when NO_3^- in the soil is low (< 250

137   μM); and a low affinity transport system (LATS) which predominates at high soil NO_3^-

138   concentration (> 250 μM) (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995; Garnett *et al.*, 2003).

139   This has been the accepted paradigm for many years. However, recent studies have shown the

140   HATS respond to plant N demand and contribute the majority of total uptake capacity at high

141 NO_3^- concentrations (> 2.5 mM) raising questions regarding the roles and activity of each

142 uptake system (Malagoli *et al.*, 2004; Garnett *et al.*, 2013). In Arabidopsis these LATS and

143 HATS uptake systems have been linked to the NO_3^- transporter (NRT) genes (*NRT1*/*NPF &*

144 *NRT2*) and their products NRT1.1/NRT1.2 (NPF6.3/NPF4.6) and

145 NRT2.1/NRT2.2/NRT2.4/NRT2.5 respectively (Huang, *et al.*, 1996; Okamoto *et al.*, 2003;

146 Li, *et al.*, 2007; Tsay *et al.*, 2007; Kiba *et al.*, 2012; Léran *et al.*, 2014; Lezhneva *et al.*, 2014).

147 However due to the dichotomy in the *NRT* gene families of dicots and grass species, and the

148 subsequent lack of direct orthologous gene pairs, the function of these genes cannot simply be

149 extrapolated into cereals based on sequence homology (Plett *et al.*, 2010).

150 The most extensively studied *NRT* gene is *NRT1.1* (*CHL1*/*NPF6.3*) which in Arabidopsis is

151 predominantly expressed in the epidermis of young root tips (Huang *et al.*, 1996). This gene is

152 NO_3^- inducible and has been shown to act as a dual affinity transporter with both HATS and

153 LATS activity (Liu *et al.*, 1999; Liu & Tsay, 2003; Parker & Newstead, 2014; Sun *et al.*,

154 2014), and as a transceptor with the ability to sense external NO_3^- and activate NO_3^--signalling

155 pathways (Remans, *et al.*, 2006; Ho *et al.*, 2009). Recently the AtNRT1.1 crystal structure has

156 been published revealing that AtNRT1.1 dimerises in the plasma membrane and operates as a

157 phosphorylation-controlled dimerisation switch (Parker & Newstead, 2014; Sun *et al.*, 2014).

158 Although NRT1.1 has been shown to possess both HATS and LATS activity, the extent to

159 which it contributes to overall HATS uptake capacity is still under debate (Glass & Kotur,

160 2013). Some cereal species have been shown to possess additional *AtNRT1.1* orthologues

161 although their functional roles are yet to be defined (Plett *et al.*, 2010; Buchner &

162 Hawkesford, 2014). Four co-orthologues have been identified in maize of which three showed

163 different expression patterns and responses to NO_3^- concentration over the lifecycle of dwarf

164 maize (*Zea mays* L. var. Gaspe Flint) (Garnett *et al.*, 2013). Similarly in wheat, four co-

165 orthologous genes were recently identified and shown to have different tissue specificity and

166 transcriptional responses to N supply (Buchner & Hawkesford, 2014), further confirming that

167 the functional roles need to be separately defined for cereals. In contrast to *NRT1.1*, *NRT1.2*

168   in Arabidopsis is primarily located in root hairs and the epidermis of both young root tips and

169   mature root regions and constitutively expressed (Huang *et al.*, 1999). In cereals a single

170   orthologous *NRT1.2* gene has been identified for each of the sequenced cereal species

171   meaning function may be more evolutionarily conserved (Plett *et al.*, 2010). In dwarf maize

172   Garnett *et al.* (2013) showed little difference in transcript levels of *ZmNRT1.2* between plants

173   grown at high and low NO_3^- concentration until late reproductive growth where expression

174   profiles differed between treatments. More recently however, a wheat orthologue has been

175   shown to be dramatically induced under N starvation (Guo *et al.*, 2014), again highlighting

176   the need for complete functional characterisation to confirm this genes contribution to NO_3^-

177   uptake in cereals.

178   In Arabidopsis *NRT2.1* and *NRT2.2* are 90.4% similar and located in tandem on chromosome

179   1 suggesting they are a product of a gene duplication event (Orsel *et al.*, 2002b). Despite their

180   similarity AtNRT2.1 has been demonstrated as the main component of the HATS under many

181   conditions with AtNRT2.2 providing only a minor contribution (Filleur *et al.*, 2001; Li *et al.*,

182   2007). However, when *AtNRT2.1* is knocked-out *AtNRT2.2* transcript levels have been shown

183   to increase and provide a greater contribution to HATS, partially compensating for the

184   AtNRT2.1 loss (Li *et al.*, 2007). Although the cereal orthologues are yet to be functionally

185   characterised, their transcriptional changes have shown strong correlation to NO_3^- uptake and

186   HATS activity indicating a similar role to their Arabidopsis counterparts (Quaggiotti *et al.*,

187   2003; Garnett *et al.*, 2013). In Arabidopsis, *NRT2.4* is expressed in both the epidermis of

188   lateral roots and in shoot tissue with affinity for NO_3^- at very low levels, suggesting this

189   protein plays a role in both the root and shoot during N starvation (Kiba *et al.*, 2012). Finally,

190   *NRT2.5* in Arabidopsis has been located in the epidermis and cortex of roots at the root hair

191   zone, and, is induced under N starvation (Okamoto *et al.*, 2003; Krapp *et al.*, 2011; Lezhneva

192   *et al.*, 2014) and suppressed by NO_3^- (Okamoto *et al.*, 2003; Orsel *et al.*, 2004). In rice the

193   orthologous gene *OsNRT2.5* (also known as *OsNRT2.3A*) is expressed predominantly in

194   xylem parenchyma cells of the root stele and has been demonstrated to play a role in the

195 transport of NO_3^- from root to shoot, again under low NO_3^- conditions (Tang *et al.*, 2012). In

196 both maize and wheat the *NRT2.5* orthologues also demonstrate induction under low NO_3^-

197 conditions (Garnett *et al.*, 2013; Guo *et al.*, 2014) indicating that the simple one to one

198 orthologous gene relationships for this gene may indicate conservation of function between

199 dicots and cereals (Plett *et al.*, 2010).

1.5.3 Assimilation and storage

201 Once NO_3^- has been acquired by the root it is either assimilated directly in the root, stored in

202 root vacuoles or transported to the shoot for assimilation or storage (Andrews, 1986;

203 Crawford, 1995). To date it has been shown that storage of NO_3^- within vacuoles is facilitated

204 via the chloride channels CLCa and CLCb (De Angeli *et al.*, 2006; von der Fecht-Bartenbach

205 *et al.*, 2010). Whether NO_3^- is assimilated in the root or the shoot varies based on species

206 (Smirnoff & Stewart, 1985), external NO_3^- concentration (Andrews, 1986; Andrews *et al.*,

207 2004) and internal N status (Stitt, 1999). The first step in the assimilation process is the

208 reduction of NO_3^- to nitrite (NO_2^-) in the cytosol by the enzyme nitrate reductase (NR)

209 (Maathuis, 2009). Subsequently NO_2^- is reduced to NH_4^+ via the enzyme nitrite reductase

210 (NiR) in the plastids (root) or chloroplast (shoot) (Meyer & Stitt, 2001). Due to its toxicity,

211 NH_4^+ is rapidly fixed into non-toxic organic N compounds (Hodges, 2002) via the enzymes

212 glutamine synthetase (GS) and glutamate synthase (GOGAT) as components of the

213 GS/GOGAT cycle (Oaks, 1994; Lea & Ireland, 1999). The major products from the

214 GS/GOGAT cycle are glutamine and glutamate which subsequently provide the backbone for

215 the biosynthesis of amino acids, nucleotides, chlorophylls, polyamines and alkaloids (Lea &

216 Ireland, 1999).

1.5.4 Transport within the plant

218 Nutritional needs change over a plant's developmental lifecycle (Leopold, 1964; Marschner &

219 Marschner, 2012). This, in conjunction with changing environmental conditions and

220 fluctuating nutrient availability, means that critical nutrients such as N need to be rapidly

221 transported between different tissues. As a result multiple N forms are readily transported

222 from root-to-shoot or shoot-to-root via the xylem and phloem, respectively, including: NO_3^-,

223 small amounts of NH_4^+ (Schjoerring *et al.*, 2002), amino acids and amides (Pate, 1973).

224 Translocation of NO_3^- from root-to-shoot occurs via the xylem (Marschner & Marschner,

225 2012). Loading of NO_3^- into the xylem has been shown to occur via non-specific anion

226 channels (Kohler *et al.*, 2002; Gilliham & Tester, 2005) and more recently certain NRTs (Lin

227 *et al.*, 2008; Li *et al.*, 2010; Tang *et al.*, 2012; Léran *et al.*, 2013). In Arabidopsis, AtNRT1.5

228 (NPF7.3) was shown to be located in root pericycle cells close to the xylem and subsequent

229 knockdown or knockout mutations led to reduced root-to-shoot NO_3^- transport (Lin *et al.*,

230 2008). More recently in rice OsNRT2.3A (orthologous to AtNRT2.5) was shown to be

231 located in the xylem parenchyma cells of the stele and subsequent knockdown impaired

232 xylem loading of NO_3^- (Tang *et al.*, 2012). The plasma membrane located NRT1.8 (NPF7.2)

233 is expressed mainly in the xylem parenchyma cells of roots and has been demonstrated to play

234 a role in NO_3^- removal from the xylem back into the root cells (Li *et al.*, 2010). In addition

235 Léran *et al.* (2013) has demonstrated that the NRT1.1 (NPF6.3) protein with its location in the

236 endodermis and stele, NO_3^- sensor properties, and bidirectional transport ability, could also

237 participate in sensing xylem NO_3^- and loading/unloading in the root stele.

238 Several NRTs have been shown to be involved in phloem NO_3^- transport. In Arabidopsis the

239 low affinity transporter AtNRT1.9 (NPF2.9) is expressed in companion cells of the root

240 phloem and mutants demonstrated reduced phloem NO_3^- concentration and less transport of

241 NO_3^- from shoot-to-root (Wang & Tsay, 2011). More recently NRT1.11/1.12

242 (NPF1.1/NPF1.2) have also been shown to be involved in xylem-to-phloem NO_3^- transfer

243 and to potentially play a role in facilitating redistribution of NO_3^- into developing leaves for

244 optimal growth (Hsu & Tsay, 2013).

245 **1.5.5 Remobilisation**

246 The majority of grain N is taken up by the plant prior to anthesis (Hirel *et al.*, 2007;

247 Marschner & Marschner, 2012). At that stage most of the plant's N exists within proteins,

with Rubisco accounting for 12 - 35% of leaf N in C_3 plants (Imai *et al.*, 2008). Consequently the majority of grain N (51 – 92%) is remobilised from protein (mainly Rubisco) within vegetative tissues during grain filling, with the remainder coming from stored inorganic forms of N such as NO_3^- and a minor contribution from active N uptake (Vansanford & Mackown, 1987; Palta & Fillery, 1995; Barbottin *et al.*, 2005; Kichey *et al.*, 2007). There is significant genetic variation for this trait in cereal crops such as wheat (Cox *et al.*, 1986; Papakosta & Gagianas, 1991; Barbottin *et al.*, 2005; Tahir & Nakata, 2005) and the genetic control of remobilization is linked to the regulation of leaf senescence (Sinclair & de Wit, 1975; Masclaux *et al.*, 2001; Uauy *et al.*, 2006; Gaju *et al.*, 2014).

For utilizing stored NO_3^- pools, a number of NRT transporters have been implicated in the remobilization of stored NO_3^- from source to sink tissues. In Arabidopsis NRT1.11/1.12 (NPF1.1/NPF1.2) and NRT1.7 (NPF2.13) have all been shown to participate in remobilizing NO_3^- from source leaves into developing tissues via the phloem (Fan *et al.*, 2009; Hsu & Tsay, 2013). Focusing on remobilization for grain development and filling, in Arabidopsis AtNRT1.6 (NPF2.12) is expressed in the vascular tissue of reproductive tissues (siliques and the funiculus) with mutants having less accumulated NO_3^- in mature seeds and high seed abortion rates (Almagro *et al.*, 2008). Also in Arabidopsis it has been demonstrated that AtNRT2.7 is located in reproductive organs and is most highly expressed in dry seeds with modification to the genes expression affecting seed NO_3^- concentration (Chopin *et al.*, 2007).

1.6 The controllers of nitrate uptake

In order to assess the key knowledge gaps to facilitate production of cereal crops with increased NUpE it is important to explore what is known about the control mechanisms governing the NO_3^- uptake system. There is evidence to suggest that NO_3^- uptake is controlled at the transcriptional, translational and post-translational levels. Consequently, this section will provide an overview of the current literature in these areas to put into context the approach of this thesis.

1.6.1 Transcriptional

275 Transcriptional control of NO_3^- uptake is well documented. When Arabidopsis and barley

276 plants are subjected to NO_3^- starvation and resupply, the observed changes in transcript levels

277 of nitrate-inducible *NRT2* genes follow the changes in HATS NO_3^- uptake capacity (Minotti *et*

278 *al.*, 1969; Jackson *et al.*, 1973; Goyal & Huffaker, 1986; Aslam *et al.*, 1993; Henriksen &

279 Spanswick, 1993; Trueman *et al.*, 1996; Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et*

280 *al.*, 2003). Mutant analyses of these genes have confirmed that they are indeed the major

281 drivers of the changes in NO_3^- uptake capacity supporting the link between *NRT2* transcription

282 and uptake capacity (Cerezo *et al.*, 2001; Filleur *et al.*, 2001; Orsel *et al.*, 2004; Li *et al.*,

283 2007). Longer term lifecycle analysis has also shown distinct correlation between the NO_3^-

284 uptake capacity changes and transcript levels of the *NRT2s* across the lifecycle of maize

285 (Garnett *et al.*, 2013). In Arabidopsis, maize and wheat transcript levels of some *NRT2s* have

286 been shown to increase in response to reduction in N availability, aligning with an observed

287 increase in NO_3^- uptake capacity (Okamoto *et al.*, 2003; Krapp *et al.*, 2011; Buchner &

288 Hawkesford, 2014).

289 Transcription factors (TFs) act as master switches for regulatory networks (Guilfoyle, 1997;

290 Spitz & Furlong, 2012; Porto *et al.*, 2014). A number of TFs have been shown to influence

291 the expression of *NRT* genes in *Arabidopsis* including: MADS box (*NRT2.1*) (; Gan *et al.*,

292 2005), NLP (*NRT2.1 & NRT2.2*) (Loren Castaings, 2009; Konishi & Yanagisawa, 2013a;

293 Konishi & Yanagisawa, 2013b; Liseron-Monfils *et al.*, 2013; Marchive *et al.*, 2013), LBD

294 (*NRT1.1*, *NRT2.1 & NRT2.2*) (Rubin *et al.*, 2009) and bZIP (*NRT1.1*) (Jonassen *et al.*, 2009).

295 Commonly, TFs elicit their control by interacting with cis-acting elements and/or with other

296 TFs to control gene expression (Guilfoyle, 1997; Spitz & Furlong, 2012; Porto *et al.*, 2014).

297 To date, identifying NO_3^- specific cis-trans regulatory elements has focused heavily on finding

298 NO_3^--responsive cis-elements (NREs) involved in triggering the NO_3^- inducible expression

299 associated with the primary nitrate response (PNR) (reviewed in subsequent section). The

300 promoter regions of the nitrate reductase genes (*NIA1 & NIA2*) have been extensively studied

301   in Arabidopsis and spinach revealing a number of key cis-elements with the ability to drive

302   NO_3^- induced expression in minimal promoter studies (Hwang *et al.*, 1997; Rastogi *et al.*,

303   1997; Konishi & Yanagisawa, 2010; Konishi & Yanagisawa, 2011). For the *NRTs,* the

304   Arabidopsis *AtNRT2.1* promoter has been analysed using a minimal promoter approach which

305   identified a 150 bp sequence required for the gene's NO_3^- expression and N metabolite

306   repression responses (Girin *et al.*, 2007). Deletion analysis of the rice *OsNAR2.1* (Os*NRT3.1* –

307   see Plett *et al.* (2010)) promoter identified a 311 bp region necessary for the NO_3^- responsive

308   transcriptional activation of the gene (Feng *et al.*, 2011). Subsequent motif analysis of that

309   sequence revealed three putative nitrate-responsive cis-elements which had all previously

310   been associated with the NO_3^- responsiveness of the *NIA* genes in Arabidopsis and Spinach:

311   5'-GATA-3' (Rastogi *et al.*, 1997; Bi *et al.*, 2005), 5'-A(c/G)TCA-3' (Hwang *et al.*, 1997),

312   and 5'-GACt<u>CTT</u>N10<u>AAG</u>-3' (Konishi & Yanagisawa, 2010; Konishi & Yanagisawa, 2011).

313   Another transcriptional mechanism that has been demonstrated to play a role in regulating

314   expression of the *NRTs* is histone methylation and subsequent chromatin modification. In

315   Arabidopsis, using a mutant impaired in the systemic feedback repression that is well

316   characterised for *NRT2.1* at high N supply, the group identified the mutant gene to be a

317   component of the RNA polymerase II complex. Subsequently Widiez *et al*. (2011)

318   demonstrated that the mechanism through which the gene acts in roots to repress *NRT2.1*

319   transcription in response to high N supply was associated with an increase in histone H3

320   lysine 27 trimethylation at the *NRT2.1* locus.

321   **1.6.2 Post Transcriptional**

322   Evidence exists to suggest that post transcriptional regulation of NRTs may play a

323   predominant role in the control of the NO_3^- uptake system (Laugier *et al*., 2012). Micro RNAs

324   (miRNAs) have recently emerged as another mode of master regulation governing gene

325   expression in plants (Jones-Rhoades *et al.*, 2006; Voinnet, 2009). Many studies have now

326   revealed that miRNAs can regulate plant adaptive responses to nutrient deprivation (Jones-

327   Rhoades & Bartel, 2004; Fujii *et al.*, 2005; Sunkar *et al.*, 2007; Hsieh *et al.*, 2009; Pant *et al.*,

328   2009; Sunkar, 2010). Significant differences in miRNA accumulation have been observed in

329   response to NO_3^- availability, especially under low NO_3^- conditions (Xu *et al.*, 2011; Zhao *et*

330   *al.*, 2012; Zhao *et al.*, 2013). The repression of six miRNAs (miR528a/b, miR528a*/b*,

331   miR169i/j/k, miR169i*/j*/k*) in maize roots in response to prolonged low NO_3^- provision has

332   been suggested to play a key role in integrating NO_3^- signals into root developmental changes

333   (Trevisan *et al.*, 2012). The small RNA mi167 has been shown to mediate lateral root

334   initiation and growth in response to NO_3^- in Arabidopsis (Gifford *et al.*, 2008). Pant et al

335   (2009) found several NO_3^- responsive miRNAs in Arabidopsis and different members of the

336   mi169 family have been shown to be involved in the long distance signaling that regulates

337   NO_3^- starvation responses (Zhao *et al.*, 2011). The NO_3^- induced miR393 was identified in a

338   transcriptomics study and shown to target an auxin receptor *AFB3*, revealing an N-responsive

339   module that controls root system architecture in response to external and internal N

340   availability in Arabidopsis (Vidal *et al.*, 2010). Compared to modifying transcriptional and

341   post-transcriptional activation, it is anticipated that miRNA transcription and processing may

342   be less energy intensive (Fischer *et al.*, 2013). Subsequently it has recently been proposed that

343   modification of miRNAs may be an attractive option for improving NUE in plants (Fischer *et*

344   *al.*, 2013). However, at this stage no miRNAs have been shown to specifically target and

345   regulate the *NRT*s. With that said, given the increasing research interest in this area it appears

346   likely that it may only be a matter of time until *NRT* specific miRNAs are identified which

347   would open new opportunities for improving NUpE for improved NUE in cereals.

348   **1.6.3 Post translational**

349   Post-translational regulation has also been demonstrated as an important mechanism

350   controlling NO_3^- uptake and assimilation (Tischner, 2000; Kaiser & Huber, 2001; Krouk *et*

351   *al.*, 2010). The post-translational control of NR activity is well characterised. The NR enzyme

352   is inactivated by a two-step process involving the phosphorylation of Ser residue 543,

353   followed by the inhibitory binding of a 14-3-3 protein kinase (see review by (Lillo *et al.*,

354   2004)). Focusing on the NRTs, AtNRT1.1 (CHL1/NPF6.3) has been demonstrated as a dual

affinity transporter under post-translational control. When AtNRT1.1 is phosphorylated at T101 by CIPK23, AtNRT1.1 functions as a high affinity NO_3^- transporter and when T101 is dephosphorylated it functions as a low-affinity nitrate transporter (Liu & Tsay, 2003; Ho *et al.*, 2009; Parker & Newstead, 2014; Sun *et al.*, 2014). A calcineurin B-like (CBL)-interaction protein kinase CIPK8 has also been shown to mediate nitrate sensing and to positively regulate the nitrate-induced expression of PNR associated genes including *NRT1.1* (CHL1/NPF6.3), *NRT2.1* and *NRT2.2* (Hu *et al.*, 2009). A number of conserved protein kinase C recognition motifs have been identified in the N- and C-terminal domains of NRT2.1 (Forde, 2000) suggesting that phosphorylation events may be involved in regulating NRT2.1 activity as has been demonstrated for NRT1.1, however, this has yet to be demonstrated experimentally. Most notably, the AtNAR2.1 (AtNRT3.1) protein has been shown to constitute part of a two-component nitrate HATS system which is essential for high affinity NO_3^- transport (Orsel *et al.*, 2007). The AtNAR2.1 protein is not a transporter itself but is a partner protein which has been shown to interact with AtNRT2.1 on a protein level at the plasma membrane (Orsel *et al.*, 2006). Subsequently it has been shown that AtNRT2.1 may only function when in a complex with AtNAR2.1 in the plasma membrane, and may exist as a tetramer consisting of two subunits each of AtNRT2.1 and AtNAR2.1 (Yong *et al.*, 2010). In Arabidopsis, all NRT2s with the exception of AtNRT2.7 appear to require interaction with AtNAR2.1 to facilitate NO_3^- transport (Kotur *et al.*, 2012). This two component NO_3^- uptake system has also been shown to hold true in barley (*Hordeum vulgare*) and rice (*Oryza sativa*) for orthologous NRT2 and NAR2.1 proteins (Ishikawa *et al.*, 2009; Feng *et al.*, 2011). Partial proteolysis has also be hypothesised as a post-translational mechanism regulating the NRT2s. This was raised by Wirth *et al*. (2007) where they demonstrated that the NRT2.1 C terminus is cleaved, resulting in the presence of both intact and truncated forms of NRT2.1. Together this information highlights the influence of post-translational control mechanisms on the NO_3^- uptake system.

1.6.4 Signalling

There has been a significant amount of work attempting to unravel what molecules act as signals for communicating NO_3^- supply and demand to trigger changes in the plants NO_3^- uptake system. Nitrate itself has been shown to act as a signal molecule that regulates its own uptake (Crawford & Glass, 1998; Forde, 2000; Orsel *et al.*, 2002a) which is a property not shared by other ions and their associated transport systems. Reduced nitrogen sources have also been shown to regulate NO_3^- uptake with NH_4^+ inducing strong inhibitory effects on NO_3^- uptake (Kronzucker *et al.*, 1999). Supplying amino acids as the sole nitrogen source exerts strong inhibition on NO_3^- uptake (Muller & Touraine, 1992). Individual amino acid levels, particularly glutamine, have been strongly linked to gene expression and feedback repression of genes involved in NO_3^- uptake and assimilation (Zhuo *et al.*, 1999; Vidmar, *et al.*, 2000b). To date no one metabolite has been identified as the key signalling molecule regulating the NO_3^- uptake system and this remains a key area of interest amongst the scientific community.

1.7 Understanding the system

A considerable proportion of the literature attempting to unravel the NO_3^- transport system and its regulation describes experiments growing plants for a period without NO_3^- (starvation) and then analysing the response of the plants immediately following exposure to NO_3^- (induction); named the "primary nitrate response" (PNR) (Medici & Krouk, 2014). The PNR was first described by Gowri *et al.* (1992) and further defined a year later by the same group (Redinbaugh & Campbell, 1993). This response has since been widely used for studying and understanding plant response to NO_3^- availability at the molecular and physiological levels. In the PNR, HATS NO_3^- uptake capacity exhibits strong induction peaking after 6 h, followed by repression after a period of sufficient NO_3^- provision in Arabidopsis, and barley (Minotti *et al.*, 1969; Jackson *et al.*, 1973; Goyal & Huffaker, 1986; Aslam *et al.*, 1993; Henriksen & Spanswick, 1993; Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et al.*, 2003). This pattern is consistent with the transcript level response of *NRT2.1* and *NRT2.2* in barley and Arabidopsis (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et al.*, 2003) and subsequent mutant analyses has confirmed that these genes were indeed the major drivers of the PNR

409 (Cerezo *et al.*, 2001; Filleur *et al.*, 2001; Orsel *et al.*, 2004; Li *et al.*, 2007). The induction

410 response has been shown to involve up to 1000 genes and has consequently been fruitful for

411 discovery of genes associated with NO_3^- uptake and its regulation (Wang *et al.*, 2000; Wang

412 *et al.*, 2003; Scheible *et al.*, 2004; Gutierrez *et al.*, 2007; Medici & Krouk, 2014). To date it

413 has not been shown whether the results from this N response can be directly related to

414 agriculturally relevant growth environments.

415 In contrast to the PNR, other attempts to understand the NO_3^- uptake system and its regulation

416 have assessed the role that N demand plays on the NO_3^- uptake system and how this varies

417 with growth and developmental stage. Early studies assessed growth rates and NO_3^- uptake

418 kinetics by growing plants at different relative rates of nitrate-N addition which gave early

419 insight into N demand effects on the NO_3^- uptake system (Oscarson & Larsson, 1986;

420 Oscarson *et al.*, 1989b; Oscarson *et al.*, 1989a; Mattsson *et al.*, 1991). Malagoli *et al.* (2004)

421 measured uptake capacity of the HATS and LATS in oilseed rape throughout development

422 and combined this analysis with field N data to develop models suggesting that the HATS

423 may play a dominant role in total N uptake over the plant lifecycle. More recently Garnett *et*

424 *al.* (2013) grew maize under both low and sufficient steady state NO_3^- conditions and

425 demonstrated substantial demand driven variation in NO_3^- uptake across the lifecycle which

426 correlated with the transcript levels of the *ZmNRT2.1, ZmNRT2.2 and ZmNRT2.5*. Currently it

427 is unclear how the widely published PNR results relate to the data from these longer term

428 studies which integrate N supply with plant N demand effects.

429 ## 1.8 The gaps for improving nitrate uptake efficiency

430 From the literature review three main knowledge gaps have been identified which are

431 fundamental for progressing towards the development of cereal crops with high NUpE.

432 ### 1.8.1 Gap 1 – The uptake systems and signalling molecules

433 As highlighted previously the accepted paradigm describing the LATS and HATS

434 contribution to total NO_3^- uptake has recently been challenged by showing that the HATS is

435   also responsive to N demand at high NO_3^- concentrations and appears to be responsible for a

436   major proportion of the plants NO_3^- uptake capacity (Garnett *et al.*, 2013). Resolving the

437   ambiguity around the contribution of each system to NO_3^- uptake is important for focusing

438   NUpE improvement efforts on specific NRT transporters. In addition, the same study by

439   Garnett *et al.* (2013) revealed that the NO_3^- uptake system changes dynamically in response to

440   N demand and that both NO_3^- and amino acids may be involved in regulating these responses.

441   However, due to the time resolution the researchers were unable to directly correlate tissue

442   concentrations of NO_3^- or any assimilates with the observed changes in the NO_3^- uptake

443   system. In light of this information, a more detailed fine timescale lifecycle analysis of the

444   NO_3^- uptake system, NO_3^-, and its assimilates appears to be the next step towards: confirming

445   the role of the HATS and LATS uptake systems in NO_3^- uptake; and revealing the signals

446   modulating the NO_3^- uptake system in response to NO_3^- supply and demand.

447   **1.8.2 Gap 2 - Leveraging the PNR literature**

448   With the majority of the literature regarding NO_3^- uptake focused around PNR NO_3^- starvation

449   and re-supply experiments (Medici & Krouk, 2014), it is important to understand how the

450   results stimulated by this perturbation may relate to other literature assessing the NO_3^- uptake

451   system. A few lifecycle studies have begun to integrate NO_3^- availability and NO_3^- demand

452   responses providing a different dimension in understanding how the NO_3^- uptake system

453   responds to NO_3^- supply and demand (Malagoli *et al.*, 2004; Garnett *et al.*, 2013). To

454   efficiently make use of the substantial PNR data to improve NUpE in cereals, understanding

455   the relationships between these experimental models could provide key insight into the

456   complex regulation networks governing the NO_3^- uptake system.

457   **1.8.3 Gap 3 - New leads for transcriptional control**

458   With such a core role in all aspects of plant function there is evidence that TFs have played a

459   major role in crop improvement over the years of crop domestication and breeding (Doebley

460   *et al.*, 2006; Kovach *et al.*, 2007; Pourkheirandish & Komatsuda, 2007). Consequently TFs

461   have been suggested as attractive candidates for engineering complex traits such as NUpE and

NUE (Yanagisawa *et al.*, 2004; Century *et al.*, 2008). As highlighted previously, with evidence of such strong transcriptional control over the *NRTs* there is the potential to exploit key cis-trans regulatory elements to increase functional NRT levels for improved NUpE. Consequently discovery of novel *NRT* cis-trans regulatory elements appears to be an attractive step to enable the production of cereals with increased NUpE and overall improved NUE.

1.9 Aims and objectives

Based on the identified gaps, the research objectives of this thesis are:

i)   to clarify the contribution of the HATS and LATS to total NO_3^- uptake in cereals

ii)   to unravel the roles of NO_3^- and its assimilates in signalling plant N status and regulating the NO_3^- uptake system

iii)   to understand how data from the PNR literature relates to longer term lifecycle analysis studies

iv)   to identify novel *NRT* cis-trans regulatory elements

Chapter 2 examines the daily effect of NO_3^- supply and demand on HATS and LATS uptake capacity, *NRT* transcripts, NO_3^- and its assimilates throughout vegetative growth in maize grown under steady state low and high NO_3^- availability.

Chapter 3 investigates a PNR experiment alongside a separate long term steady-state analysis to compare and contrast lifecycle analysis studies to the widely published PNR literature.

Chapter 4 uses a phylogenomics and co-expressed gene promoter analysis approach to identify functionally and evolutionarily conserved cis-elements in the low N induced *ZmNRT2.5* promoter region.

Chapter 5 summarises the outcomes of this thesis highlighting the key findings and proposing future directions.

Due to the challenges with growing full size maize plants in hydroponics and to relate the results of this thesis to our previous work (Garnett *et al*., 2013), for the growth studies in Chapters 2 and 3 the dwarf maize variety 'Gaspe Flint' was used (Hourcade *et al*., 1986).

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Chapter 2: Adaptive responses to low nitrogen supply in maize

1 **Adaptive responses to low nitrogen supply in maize**

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SUMMARY

- Understanding how the nitrate (NO_3^-) uptake system is controlled in response to exogenous nitrogen (N) supply and endogenous N demand may aid in the development of crops with improved nitrogen use efficiency (NUE).

- Nitrate uptake capacity, transcript levels of putative high and low affinity NO_3^- transporters, NO_3^- concentration and amino acids were profiled daily during vegetative growth of dwarf maize (*Zea mays*) plants grown at 0.5 mM and 5 mM NO_3^-. To compare between long term NO_3^- demand responses and short term NO_3^- supply responses a subset of plants was transferred from 5 mM to 0.5 mM part way through the growth period.

- Under steady state NO_3^- growth conditions transcript levels of several putative *NRTs* showed dramatic temporal variation with up to three-fold changes between consecutive daily measurements. All putative *NRTs* exhibited higher transcript levels in 0.5 mM with the exception of *ZmNRT1.1B*.

- The high affinity transport system was responsible for a major proportion of total uptake capacity at both 5 mM and 0.5 mM NO_3^- levels under steady state NO_3^- conditions and was responsive to reduction in NO_3^- supply highlighting the plasticity within the HATS.

- Reduction in NO_3^- availability revealed a series of responses correlated with changes in tissue NO_3^- concentration starting with transcriptional increases related to root-to-shoot NO_3^- translocation (*ZmNRT1.5A* and *ZmNRT2.5*) and then up regulation of the HATS transporters (*ZmNRT2.1* and *ZmNRT2.2*) before changes in growth were observed.

- The results from this study reveal new insight into the adaptive responses in reaction to N supply and demand, and strengthens the potential role of NO_3^- in regulating its own uptake system.

INTRODUCTION

Nitrogen (N) is a key growth and yield enhancing nutrient for plants. Consequently more than 100 million T of N fertilisers are applied annually to crops (Heffer & Prud'homme, 2013). Given that 40 – 60% of crop yields are attributable to fertiliser inputs (Stewart & Roberts, 2012) and global population is set to rise by almost 30% by 2050 (United Nations, 2009) it is evident that the pressures on global food production will continue to drive increasing N fertiliser use. Unfortunately, cereal crops capture only 40 - 50% of the applied N (Peoples *et al.*, 1995; Sylvester-Bradley & Kindred, 2009) leaving a considerable proportion free in the aqueous (via runoff and leaching) and atmospheric (via volatilisation and microbial activity) environments with significant environmental impact (Good & Beatty, 2011). Given the eminent reliance on N fertilizer and the inefficiency of cereals in using this fertiliser, improving this appears an achievable goal to deliver considerable global impact.

Nitrate (NO_3^-) is the principal form of N available to crops in most high-input agricultural soils, and concentrations in the soil can vary by four orders of magnitude from micromolar to millimolar (Wolt, 1994; Miller et al., 2007). To cope with these variable concentrations, plants have two NO_3^- uptake systems: a high affinity transport system (HATS) when NO_3^- present in the soil is low (< 250μM); and a low affinity transport system (LATS) which dominates at high soil NO_3^- concentration (>250 μM) (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995a; Garnett *et al.*, 2003). In Arabidopsis NO_3^- uptake via LATS and HATS activity has been linked to the NO_3^- transporter (*NRT/NPF*) genes and their related proteins NRT1.1/NRT1.2 and NRT2.1/NRT2.2/NRT2.4/NRT2.5 respectively (Tsay *et al.*, 2007; Kiba *et al.*, 2012; Léran *et al.*, 2014; Lezhneva *et al.*, 2014). Given that the NO_3^- concentrations in most agricultural soils are above the saturation point of the HATS (*c.* 250 μM) (Wolt, 1994; Miller *et al.*, 2007) and *NRT2* genes are repressed at high NO_3^- (Lejay *et al.*, 1999) it has been proposed that the LATS system is responsible for the majority of NO_3^- uptake in an agricultural setting (Glass, 2003). Conversely, by linking NO_3^- uptake to field total N measurements, Malagoli *et al.* (2004) demonstrated that the HATS could supply most of the

78 plants N requirements at high N availability. More recently Garnett *et al.* (2013) demonstrated

79 that, at relatively high steady state NO_3^- concentrations (2.5 mM), HATS activity could

80 account for most of the total NO_3 uptake activity across the lifecycle of maize. Given these

81 recent insights, the contribution of each uptake system and the importance of their associated

82 transporters to total net NO_3^- uptake remain in question.

83 Nitrogen uptake and its regulation is a complex system tightly controlled in response to

84 exogenous N supply and endogenous N demand (Gutiérrez, 2012). A considerable proportion

85 of the literature attempting to unravel the system and its regulation has focused on

86 experiments involving NO_3^- starvation and re-supply (Medici & Krouk, 2014). Plants subject

87 to a period of NO_3^- starvation followed by re-exposure show strong HATS induction followed

88 by repression after a period of sufficient NO_3^- (Minotti *et al.*, 1969; Jackson *et al.*, 1973;

89 Goyal & Huffaker, 1986; Aslam *et al.*, 1993; Henriksen & Spanswick, 1993; Zhuo *et al.*,

90 1999). This strong induction and repression is highlighted by the transcript levels of

91 *AtNRT2.1* and *AtNRT2.2*, which follow the changes in NO_3^- uptake capacity (Zhuo *et al.*,

92 1999; Okamoto *et al.*, 2003). This pattern is widely known as the primary NO_3^- response

93 (PNR) after it was first described by Gowri *et al.* (1992). The induction response has been

94 shown to involve expression of up to 1000 genes and has consequently been fruitful for the

95 discovery of genes involved in NO_3^- sensing, uptake and assimilation (Wang *et al.*, 2000;

96 Scheible *et al.*, 2004; Gutierrez *et al.*, 2007; Medici & Krouk, 2014). Understanding the PNR

97 has helped in our understanding of the NO_3^- uptake system, however, it is unclear how this

98 artificial perturbation relates to N responses experienced by plants in the field. As a result,

99 much remains unknown about how NO_3^- uptake is regulated over the lifecycle of field grown

100 plants.

101 Despite a rich scientific literature on this topic much remains unknown about the molecular

102 mechanisms that regulate NO_3^- uptake in response to supply and demand (McAllister *et al.*,

103 2012). Nitrate itself has been shown to act as a signalling molecule regulating gene expression

104 (Crawford & Glass, 1998; Forde, 2000; Llamas *et al.*, 2002; Rexach *et al.*, 2002).

105 Downstream assimilates such as amino acids (AA) have been strongly linked to gene

106 expression and feedback repression of genes involved in NO_3^- uptake and assimilation (Zhuo

107 *et al.*, 1999; Vidmar *et al.*, 2000). Additionally, there is evidence suggesting that the NO_3^-

108 uptake system is controlled at the transcriptional, translational and post translational level (see

109 review by Krapp *et al.* (2014)). Recent analysis over the lifecycle of maize revealed that the

110 NO_3^- uptake system changes dynamically in response to N demand and that both NO_3^- and AA

111 could be involved in regulating these responses (Garnett *et al.*, 2013). However, the

112 researchers were unable to directly correlate tissue concentrations of NO_3^- or any assimilates

113 with the observed changes in the NO_3^- uptake system potentially due to the limited time

114 resolution (Garnett *et al.*, 2013). In light of this information, a more detailed fine timescale

115 lifecycle analysis of the NO_3^- uptake system, NO_3^-, and its assimilates may reveal how the

116 uptake is controlled in response to NO_3^- supply and demand.

117 In this study we examined the effect of N demand on HATS and LATS uptake capacity daily

118 throughout vegetative growth in maize in response to steady state low or high NO_3^-

119 availability. In addition, by reducing NO_3^- availability during the growth period we were able

120 to study the dynamic responses to change in exogenous NO_3^- supply without starvation.

121 Combined, this study provides important insights into how the uptake system is controlled in

122 response to NO_3^- supply and demand.

123 **MATERIALS AND METHODS**

124 **Plant Growth**

125 Seeds of the dwarf maize (*Zea mays* var. Gaspe Flint) were pre-treated by washing thoroughly

126 with sterile MilliQ water, followed by a 5 min treatment with a combination of Captan®

127 (Farmalinx) and Spinflo® (NuFarm) fungicides at rates of 1.25 g L^{-1} and 2 ml L^{-1}

128 respectively. Following fungicide treatment the seeds were then thoroughly washed and then

129 imbibed by soaking in sterile MilliQ water for 24 h with continuous aeration. The seeds were

then germinated on filter paper moistened with 0.5 mM $CaCl_2$ (3 d at 26°C in the dark). A total of 80 seedlings were then transferred to each of six 120 L ebb and flow hydroponic systems with the fill/drain cycles completed in 15 min. Plants were grown on mesh collars within tubes (300 mm x 50 mm) which kept roots of adjacent plants separate but allowed free access to solution. The hydroponic system was situated in a controlled environment room with a day : night cycle of 14 h : 10 h, 25°C : 20°C, at a flux density of 500 µmol m^{-2} s^{-1} at canopy level which was maintained throughout the experiment. The nutrient solution was a modified Johnson's solution (Johnson *et al.*, 1957) containing either (in mM) 0.5 NO_3^--N, 3.05 K, 1.25 Ca, 0.5 Mg, 1.63 S, and 0.5 P for the 0.5 mM NO_3^- treatment or (in mM): 5 NO_3^--N, 3.05 K, 1.25 Ca, 0.5 Mg, 0.5 S, and 0.5 P for the 5 mM NO_3^- treatment. Both treatment solutions contained (in µM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and 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 5.9 and 6.1. Solution NO_3^- was monitored using a NO_3^- electrode (TPS, Springwood, Australia) and nutrient solutions were changed every 7 d. 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.

Preliminary Experiment

For the preliminary experiments the plants were grown in the same conditions as above with a modified Johnson's solution (Johnson *et al.*, 1957) containing either (in mM) 0.2 NO_3^--N, 5.55 K, 2.5 Ca, 0.5 Mg, 2.955 S, and 0.5 P for the 0.2 mM NO_3^- treatment, 0.5 NO_3^--N, 5.55 K, 2.5 Ca, 0.5 Mg, 2.88 S, and 0.5 P for the 0.5 mM NO_3^- treatment, or 10 NO_3^--N, 5.55 K, 2.5 Ca, 0.5 Mg, 0.5 S, and 0.5 P for the 10 mM NO_3^- treatment. Plants were harvested between 11:00 and 13:00 h (light period began at 06:00 h) at 18, 27, 32 and 62 d after emergence. Roots and shoots (and cob for final harvest) were separated, weighed, dried at 65°C for 7 d, and then weighed again.

Flux measurement

On sampling days, between 11:00 and 13:00 h (light period began at 06:00 h), plants were transferred to a controlled environment room with conditions matching growth conditions (light, temperature, relative humidity and growth solutions). The roots were then given a 5-min rinse with the same nutrient solution but with either 100 or 2500 μM NO_3^-, followed by 10 min exposure to the same solution but with ^{15}N labelled NO_3^- (^{15}N 10%). At the end of the flux period roots were rinsed for 2 min in matching but unlabelled solution. Two identical solutions were used for this rinse to allow an initial 5 s rinse to remove labelled solution adhering to the root surface. The flux timing was based on that used by Kronzucker *et. al* (1995b). Roots were blotted, and then roots and separated shoots were weighed and dried at 65°C for 7 d after which the roots were ground to a fine powder. Total N and ^{15}N in the plant samples were determined with an isotope ratio mass spectrometer (Sercon, Cheshire, UK). Unidirectional NO_3^- HATS and LATS flux (i.e. high-affinity and low affinity NO_3^- uptake capacity) into the root was calculated based on ^{15}N content of the root and shoot at both 100 and 2500 μM external NO_3^- flux conditions (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995b; Garnett *et al.*, 2003; Garnett *et al.*, 2013). LATS uptake capacity was then determined by subtracting the 100 μM (HATS) from the 2500 μM (Total). The unidirectional NO_3^- influx measured in this study is described as the uptake capacity of the plant.

Quantitative real time PCR

On sampling days root material was harvested between 11:00 and 13:00 h. The whole root was excised and snap-frozen in liquid nitrogen and stored at -80°C. Homogenous fine-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. RNA integrity was checked on a 1.2% (w/v)

184   agarose gel. cDNA synthesis was performed on 1 µg of total RNA with oligo(dT)$_{19}$ using

185   SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the

186   manufacturer's instructions. Quantitative real time PCR (Q-PCR) was carried out as outlined

187   in Burton *et al.* (2008). In this method, the amount of each amplicon in each cDNA is

188   quantified with respect to a standard curve of the expected amplicon (typically, PCR

189   efficiencies ranged between 0.85 and 1.05). Four control genes (*ZmGaPDh*, *ZmActin*,

190   *ZmTubulin* and *ZmElF1*) were utilised for the calculation of the normalisation factor. Q-PCR

191   normalisation was carried out as detailed in Vandesompele *et al.* (2002) and Burton *et al.*

192   (2004). Q-PCR primers were as per Garnett *et al.* (2013). Q-PCR products were verified by

193   sequencing, agarose gel electrophoresis and melt-curve analysis to confirm a single PCR

194   product was being amplified.

195   **Xylem sap sampling**

196   Preliminary work was carried out to establish an efficient method for sampling xylem sap

197   from young plants in hydroponics. Cut height from the root:shoot interface was optimized to

198   1.5 cm to achieve maximum xylem sap yield with < 1 cm and > 3 cm yielding little to no

199   xylem sap. There was no observed difference in xylem sap yield between 15 and 30 min

200   collection times so the shorter time of 15 min was chosen to avoid results being skewed by

201   stress responses to shoot severance. Plants were cut using a sterilized scalpel and xylem sap

202   was accrued for 15 min on the surface of the cut shoot. Xylem sap was then harvested using a

203   pipette, stored in a 0.5 ml tube, snap frozen snap-frozen in liquid nitrogen and stored at -80°C.

204   **Nitrate determination**

205   Tissue NO$_3^-$ content was determined via a previously published method (Cataldo *et al.*, 1975).

206   Cryogenically fine-ground tissue (20 - 25 mg) was aliquoted into 1.5 ml tubes, 1 ml of

207   deionised H$_2$O added, and then boiled for 20 min. Sampled were then cooled on ice and the

208   supernatant collected after centrifugation for 15 min at 12,000 x g. Supernatant samples were

209   then stored at -80°C until required. Xylem sap samples were aliquoted and diluted prior to

210   analysis. In 1.5 ml tubes 10 µl of sample (tissue extracted supernatant or xylem sap) was then

211   mixed with 40 µl of 5% (w/v) salicyclic acid in concentrated H_2SO_4, mixed, and then

212   incubated at room temperature for 20 min. To this 0.95 ml of 2 N NaOH was then added,

213   mixed well, and incubated at room temperature for 20 min. For each sample 200 µl was

214   transferred to a 96 well flat bottom plate (Greiner Bio-One, Vic, Australia) and absorbance

215   was measured at 410 nm (POLARstar Optima, BMG Labtech, Germany). To determine NO_3^-

216   concentration 200 µl of KNO_3 standard samples subject to the same reaction (0 – 10 mM)

217   were run on each plate and processed the same as the samples above. Root and shoot tissue

218   samples nitrate content was expressed as µmol of NO_3^- per g of tissue FW. For xylem sap

219   samples, nitrate content was expressed as µmol of NO_3^- per ml of xylem sap.

220   **Amino acid determination**

221   Tissue AA concentration was determined using liquid chromatography electrospray

222   ionization-mass spectrometry as described by Broughton *et al.* (2011) once the samples had

223   been derivatised following the method of Cohen and Michaud (1993). Outliers were removed

224   using the Grubbs' test, also known as extreme studentized deviate method (ESD) with a

225   significance cut-off level of 0.05 (two-sided) (Grubbs, 1969).

226   **Statistical analyses**

227   The experiment was designed with three independent internal replicate growth systems for

228   each treatment and plants were randomly harvested. There was no statistical difference for all

229   measured parameters between the replicate systems. All statistical analyses within this study

230   were carried out using two-way analysis of variance (ANOVA) unless otherwise described.

231   **Correlation Analysis**

232   Each individual timecourse dataset was mean centred as per the equation $Xc = Xi \div \bar{X}$ where

233   Xi is the mean value for a given time point and $\bar{X}$ is the mean of all timepoint mean values for

234   a given dataset. The mean centred data was then converted to a $\log_{10}$ scale. The data was then

235   imported into Genesis Gene Expression Similarity Investigation Suite (Sturn *et al.*, 2002).

236   Hieratical clustering was then calculated under the following parameters: adjust = $\log_{10}$ to

237    $\log_2$, distance = Pearsons correlation.

238   **RESULTS**

239   **Growth responses**

240   A preliminary lifecycle experiment examining the effect of various steady state NO_3^-

241   concentrations on growth and yield was undertaken (Fig. S1). The magnitude of the observed

242   differences in root growth between 0.5 mM and 10 mM indicated that 0.5 mM and 5 mM

243   NO_3^- should provide a sufficient treatment difference to show NO_3^- induced growth

244   differences.

245   Under steady-state hydroponic conditions, higher root and lower shoot biomass (plants at

246   each of the growth stages can be seen in Supporting Information Figure S2) was observed

247   when plants were grown in nutrient solution containing 0.5 mM NO_3^- concentration compared

248   to 5 mM (Figs. 1a,b). From the first measured time point 10 d after emergence (DAE) the 0.5

249   mM treated plants had a higher root to shoot ratio (R:S) compared to 5 mM treated plants

250   (Fig. 1c). Overall, R:S decreased from 10 to 15 DAE in both NO_3^- treatments (Fig. 1c).

251   Across the experiment 0.5 mM treated plants maintained a higher R:S compared to 5 mM

252   treated plants, with a noteworthy increase from 14 to 16 DAE (46% higher at 14 DAE to

253   77% higher at 16 DAE) attributed to a large increase in root growth (Figs. 1b,c). This increase

254   in root growth was followed by a reduction in shoot growth starting from 18 DAE which

255   resulted in smaller shoots in 0.5 mM treated plants compared to 5 mM at the final measured

256   time point (Fig. 1a).

257   To compare between long term NO_3^- demand responses and short term NO_3^- supply

258   responses, at 15 DAE a subset of plants was transferred from 5 mM to 0.5 mM (D15 Red.).

259   Shoot growth was reduced by 23% after 5 d post decrease in external NO_3^- concentration

260   resulting in plants with 16% smaller shoots compared to 5 mM at 22 DAE (Fig. 1a). After 6 d

261 post reduction in NO_3^- availability an increase in root growth was observed resulting in plants

262 with 18% larger roots compared to 5 mM plants (Fig. 1b).

263 **Nitrate flux capacity**

264 Root and shoot dry weight (DW) and tissue ^{15}N labelled NO_3^- was used to determine uptake

265 capacity of the plants (see methods). Total NO_3^- uptake capacity was dominated by the HATS

266 contribution with on average >80% of total uptake being attributed to the measured HATS

267 activity (Figs. 2a & S3). For 0.5 mM treated plants, on average the measured HATS NO_3^-

268 uptake capacity was 5x higher than the calculated LATS capacity across the experiment

269 (excluding 20 DAE where no LATS activity was calculated) (Fig. 2b). Similarly, 5 mM

270 treated plants exhibited, on average, double the HATS NO_3^- uptake capacity compared to the

271 calculated LATS capacity (Figs. 2a,b). Comparing NO_3^- uptake capacity between treatments,

272 with the exception of 22 DAE we observed higher HATS NO_3^- uptake capacity for 0.5 mM

273 treated plants which was most pronounced at the earlier time points (Fig. 2a). For 0.5 mM

274 treated plants we observed an overall decreasing trend in HATS NO_3^- uptake capacity post 14

275 DAE. The calculated LATS NO_3^- uptake capacity for 0.5 mM treated plants was comparable

276 to 5 mM treated plants until post 14 DAE whereupon it decreased until there was little to no

277 LATS activity calculated from 18 DAE in 0.5 mM plants (Fig. 2b). Plants subject to a

278 reduction in NO_3^- demonstrated a transient increase in HATS NO_3^- uptake capacity from 3 d

279 post reduction in external NO_3^- concentration which was followed by a decrease in HATS

280 activity from 5 d post reduction in external NO_3^- concentration in line with 0.5 mM treated

281 plants (Fig. 2a). In contrast LATS decreased in response to reduction in NO_3^- supply within 3

282 d and then continued to decrease more sharply, in line with the decline in HATS capacity

283 after 5 d post NO_3^- reduction in external NO_3^- concentration (Fig. 2a,b).

284 To understand the relationship between NO_3^- uptake capacity and total N uptake, root and

285 shoot growth together with tissue N were used to calculate net N uptake (Table S1). Both the

286 root and shoot DW followed an exponential function with coefficients of determination (R^2)

of 0.992 and 0.994 respectively. Net N uptake of the plants at 0.5 mM and 5 mM was calculated as $(N_{tot}(t) = N_S \cdot DW_S(t) + N_R \cdot DW_R(t))$ to give net N uptake per g DW_R and overlayed onto the experimentally determined NO_3^- uptake capacity figures (Fig. 2). Plants grown at 5 mM showed a relatively constant net N uptake rate of around 80 µmoles per g^{-1} DW h^{-1} whereas 0.5 mM plants exhibited a 2.8 fold lower N uptake rate of approximately 30 µmoles per g^{-1} DW h^{-1} with a declining trend over time.

Nitrogen and Carbon

Root and shoot total N were measured to examine plant N status over the experiment. From the first time point (10 DAE) the N concentration was lower in 0.5 mM treated plants (*c.* 16% and 14% lower than 5 mM for root and shoot tissue respectively) and this trend continued in both root and shoot across the experiment (Figs. 3a,b). For plants grown at 5 mM, root N concentration remained relatively stable ranging from 3.6 to 4 mmol g^{-1} DW whereas a gradual decline from 4.4 to 3.1 mmol g^{-1} DW was observed in shoot tissue. For 0.5 mM treated plants we observed a pattern of variation in both root and shoot tissue involving a reduction between 14 and 16 DAE (*c.* 28% and 27% reduction in root and shoot tissue respectively) followed by a transient stabilisation/recovery at 18 DAE and a subsequent decline (Figs. 3a,b). Reducing NO_3^- concentration caused N concentration to decline in the shoots after 5 d, however, the root response for these plants was more rapid with a decrease after 3 d post reduction in external NO_3^- concentration (Figs. 3a,b).

Given the tight balance between carbon (C) and N, total C concentration was also examined. Carbon concentration in both root and shoot was constant and comparable between 0.5 mM and 5 mM treated plants across the experiment (Figs. 3c,d). Reducing NO_3^- concentration had little effect on root C concentration, but caused a rapid 18% increase in the measured C concentration in the shoot tissue (Figs. 3c,d). Examining the C/N the stark increase in shoot C concentration for D15 Red. plants appeared to be complementary to an increase in shoot N, resulting little to no initial change in C/N (Figs. 3a,c,e). With little variance in C for 0.5 mM

313   and 5 mM plants, the C/N changes were essentially an inverse relationship of the N changes

314   with 0.5 mM being higher from 10 DAE compared to 5 mM plants and the differences

315   between the two treatments becoming larger overtime (Figs. 3e,f).

316   **Nitrate**

317   The observed changes in NO_3^- concentration across the experiment for 0.5 mM and 5 mM

318   plants showed similar trends in root, shoot and xylem. The NO_3^- concentrations measured in

319   root, shoot and xylem sap were significantly lower in 0.5 mM treated plants compared to 5

320   mM across the experiment (Fig. 4). From the first measured time point at 10 DAE, the NO_3^-

321   concentration was already lower in both root and shoot tissues (*c.* 24 and 41% lower in root

322   and shoot respectively) (Figs 4a,b). Plants treated with 5 mM showed a slow decrease in NO_3^-

323   concentration throughout vegetative growth from first (10 DAE) to last (24 DAE)

324   measurement in root, shoot and xylem sap (Fig. 4). In contrast we observed that plants grown

325   in the 0.5 mM treatment exhibited a more rapid and prominent reduction in NO_3^-

326   concentration reduction in root, shoot and xylem sap across the experiment (Fig. 4).

327   Furthermore for 0.5 mM plants it was observed that the majority of the decrease in NO_3^-

328   concentration was between 10 and 16 DAE (*c.* 63, 75, 58% decrease for root, shoot and xylem

329   sap respectively), which was followed by a transient increase (*c.* 26, 23, 42% increase for

330   root, shoot and xylem sap respectively), and then a final decrease with close to zero measured

331   NO_3^- content in root and shoot, and very low measurements in xylem sap post 21 DAE (Fig.

332   4). After reducing NO_3^- concentration, D15 Red. plants were able to briefly maintain root and

333   shoot NO_3^- concentration similar to that of the 5 mM plants before exhibiting a rapid decrease

334   in NO_3^- concentration after 3 d (root) and 4 d (shoot) post reduction in external NO_3^-

335   concentration (Fig. 4a,b). In contrast, xylem sap NO_3^- concentration in D15 Red. plants

336   decreased immediately in response to reduced NO_3^- availability and displayed the same

337   transient increase as 0.5 mM treated plants (Fig. 4c).

Amino Acids

The total free amino acid concentrations (TAA) showed different trends and treatment differences in root, shoot and xylem sap. Across the experiment TAA in 0.5 mM plants were lower in root tissue and higher in shoot tissue compared to 5 mM plants (Figs. 5a,b). In root tissue 0.5 mM plants maintained a low and constant TAA level (average 5 µmoles g^{-1} FW) and conversely in shoot tissue 5 mM plants maintained a low and stable TAA level with the exception of 14 DAE (average 3.2 µmoles g^{-1} FW excluding 14 DAE) (Figs. 5a,b). In the root from 10 to 16 DAE, TAA was on average 33% higher for 5 mM plants compared to 0.5 mM plants until 17 DAE where an increase was observed with TAA in 5 mM plants reaching three-fold the concentration of 0.5 mM plants at 17 DAE (Fig. 5b). Three transient peaks in shoot tissue TAA were measured in 0.5 mM plants over the experiment which hit sharper peaks over time (*c.* TAA 3, 4 & 4 fold higher in 0.5 mM plants compared to 5 mM at peaks 1, 2 & 3 respectively) (Fig. 5a). Xylem sap TAA was similar for both 5 mM and 0.5 mM plants until 20 DAE where 5 mM maintained high levels whilst 0.5 mM plants exhibited a rapid reduction in TAA (Fig. 5c).

When plants were subjected to a decrease in NO_3^- supply, rapid changes in TAA were observed with a reduction in the root (*c.* 31% reduction) and significant increase in the shoot (*c.* 2 fold increase) within 1 d post reduction in external NO_3^- concentration. After this adjustment, levels in both root and shoot remained relatively consistent, with D15 Red. root TAA levels matching 0.5 mM plants at around 5µmoles g^{-1} FW and shoot TAA maintaining an approximately 2 fold higher TAA level than 0.5 mM plants at approx. 6 µmoles g^{-1} FW. Changes in xylem sap TAA concentration for D15 Red. plants occurred after 3 d post reduction in external NO_3^- concentration, later than in shoot and root tissue (Fig. 5c). Hierarchical clustering was performed on the individual amino acid concentration profiles to understand which AA followed the same trends. Approximately one third of the AA (cluster RA) contributed to the trends of the calculated total root TAA, due to their high concentration (Figs. S4,S5). In the root there was another trend (cluster RB) with homoserine, GABA,

365   alanine, proline, serine, glycine, beta-alanine and valine exhibiting a prominent peak at 19

366   DAE in 5 mM grown plants. The remaining root trend (cluster RC) was for putrescine,

367   arginine, lysine, isoleucine, leucine, tryptophan which all exhibited similar tends between

368   treatments (Figs. S4,S5). In shoot tissue 15 of the 29 measured AA (cluster SB) followed the

369   trend of the calculated shoot total TAA (Figs. S4,6). The remaining measured AA in the shoot

370   (cluster SA) all followed a general trend, whereby 0.5 mM and D15 Red. plants exhibited

371   increasing AA concentrations post 19 DAE (Figs. S4,S6). Half of the AA measured in the

372   xylem sap followed the TAA profile (cluster XA), apart from this there was a second

373   observed trend (cluster XB) with a substantial peak in glycine, aspartic acid, glutamic acid,

374   tyrosine and phenylalanine at 20 DAE in 5 mM plants (Figs. S4,S7).

375   *NRT* **transcript levels**

376   Based on our previous observations for putative *NRT* transcript responses in maize (Garnett *et*

377   *al.*, 2013), specific *NRTs* of interest were selected for examination in this study. *ZmNRT3.1A*

378   was most highly represented in the total RNA pool compared to the *NRT2* and *NRT1* genes

379   examined (Fig. 6a). This was closely followed by the putative HATS genes *ZmNRT2.1* and

380   *ZmNRT2.2* which exhibited on average two-fold lower transcript levels than *ZmNRT3.1* (Figs.

381   6b,c). All the putative *NRTs,* with the exception of *ZmNRT1.1B,* showed higher transcript

382   levels in 0.5 mM treated plants compared to 5 mM across the experiment. Of note was the

383   distinct similarity between the transcript profiles of both 5 mM and 0.5 mM plants for

384   *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A*, *ZmNRT1.1A* and *ZmNRT1.5A* from 15 DAE onwards.

385   This group of genes showed an increase in transcript levels post 15 DAE, with three peaks at

386   17, 19 and 21 DAE (Fig. 6). *ZmNRT2.5* expression was only detectable in plants grown under

387   0.5 mM with transcript levels demonstrating a general increasing trend over time (Fig. 6d).

388   Different speeds of transcriptional response were observed in reaction to reducing NO_3^-

389   availability. Both *ZmNRT3.1A* and *ZmNRT1.5A* responded within 1 d post reduction in

390   external NO_3^- concentration in D15 Red. plants, increasing transcript levels compared to 5

mM treated plants (*c.* 2.6x and 3.4x increase compared to 5 mM for *ZmNRT3.1A* and *ZmNRT1.5A* respectively). The *ZmNRT2.1* and *ZmNRT2.2* genes were next to respond with significant increases in expression from 2 d post reduction in external NO_3^- concentration (*c.* 2.2x and 2.8x increase compared to 5 mM for *ZmNRT2.1* and *ZmNRT3.1* respectively). Both *ZmNRT1.1A* and *ZmNRT2.5* exhibited higher expression in D15 Red. plants compared to 5 mM within 4 d post reduction in external NO_3^- concentration and *ZmNRT1.1B* was the slowest to exhibit a difference becoming lower in D15 Red. plants compared to 5 mM after 5 d post reduction in external NO_3^- concentration (Figs. 4d,e,f)

Correlations

In order to investigate the relationships between growth, NO_3^- uptake capacity, *NRT* expression, NO_3^- concentrations, total N, total C and AA concentrations, a correlation analysis was performed. All data was mean centred and subjected to a hierarchical clustering analysis to identify measured parameters which exhibited similar patterns of variation, treatment differences and response to change in NO_3^- availability (Fig. 7). As some parameters were measured daily whilst others were measured every second day, gaps were left blank and represented with grey boxes. One distinct cluster "C1" was identified, highlighting a common pattern of change and treatment response between all NO_3^-, total N measurements, and *ZmNRT1.1B* (Fig. 7). Another cluster "C2" was identified correlating the root and shoot growth curves with the observed changes in proline and putrescine concentration over time (Fig. 7). For the AA profiles, a slight correlation between root glutamine, aspartic acid and the "C1" cluster was indicating a putative relationship between these AA, NO_3^-, total N measurements and *ZmNRT1.1B* (Fig. 7). Finally, the changes in R:S correlated loosely with many of the *NRTs* due to their shared increase between 15 and 17 DAE in 0.5 mM treated plants (Fig. 7).

DISCUSSION

N status and growth responses to steady state N limitation

In our previous work we examined the effect of two non-growth limiting NO_3^- concentrations on the NO_3^- uptake system (Garnett *et al.*, 2013). In this study, growth limiting and non-growth limiting NO_3^- concentrations were selected to compare the responses of the NO_3^- uptake system when N stress was greater. For plants grown under 5 mM NO_3^- conditions, root and shoot growth reflective of non N-limited conditions was observed (Figs. 1). This non N-limited growth was evident through the N status of these plants reflecting sufficient tissue total N and NO_3^- concentrations as suggested by Reuter and Robinson (1997) (Figs 3, 4). In contrast 0.5 mM treated plants exhibited decreased shoot growth, increased root growth and an overall higher R:S compared to 5 mM plants (Figs. 1). For these plants total N and NO_3^- concentration were substantially lower reflecting a shoot growth limiting N status with N levels reaching around the reported critical N concentration of approximately 2 mmol g^{-1} dry weight (Reuter & Robinson, 1997) (Figs 3, 4). In the root 5 mM treated plants maintained a high and stable total N and NO_3^- concentration whereas the 0.5 mM plants showed a sharp decline (Fig. 3, 4). In the shoot, plants grown under 5 mM conditions exhibited a slow decline in total N and NO_3^- concentration whereas for 0.5 mM plants the shoot total N changes closely reflected the root trends in the same plants (Fig. 3, 4). The observed decrease in shoot N concentration at non-limiting N supply has been shown before and is thought to be due to an increase in the proportion of plant structural and storage tissues (Lemaire & Gastal, 1997; Plénet & Lemaire, 1999).

Interestingly 0.5 mM treated plants had a higher R:S from the first measured time point indicating that the plants had responded to external N conditions as early seedlings (Fig. 1). Seed reserves can support growth for up to 7 d (Watt & Cresswell, 1987) and external NO_3^- has little effect on growth during this period (Srivastava *et al.*, 1976) indicating that after seed N reserves are depleted the plants promptly respond to external N supply. Both root and shoot growth rates for 0.5 mM plants closely followed the 5 mM plants until 17 DAE, where a

442 substantial increase in root mass was observed. This increase corresponded with a dip in both

443 root and shoot total N and NO_3^- concentration suggesting plasticity of the root system in

444 response to internal N status. This is in line with previous studies that have demonstrated the

445 responsiveness of root growth to low tissue N levels (Brouwer, 1962; Raper *et al.*, 1977; Bhat

446 *et al.*, 1979; Freijsen & Otten, 1984; Tolley-Henry & Raper, 1986; Scheible *et al.*, 1997;

447 Ågren & Franklin, 2003; Ikram *et al.*, 2012). After the increase in root mass a reduction in

448 shoot growth rate by 22 DAE was observed for 0.5 mM plants which aligned with the low

449 point in root and shoot NO_3^- concentration (Fig. 1, 4). The concept of a critical N level and its

450 effect on shoot growth rates has been heavily explored in many plant species (Ulrich, 1952;

451 Lemaire & Salette, 1984; Greenwood *et al.*, 1991; Justes *et al.*, 1994; Lemaire & Gastal,

452 1997; Colnenne *et al.*, 1998; Plénet & Lemaire, 1999). The observed correlation between

453 shoot growth changes and tissue NO_3^- concentration here may indicate a threshold critical

454 NO_3^- level required to maintain shoot maximum growth rate which has been suggested

455 previously in wheat and tomato (Papastylianou *et al.*, 1982; Cárdenas-Navarro *et al.*, 1999).

456 **Changes in nitrate uptake capacity in response to N supply and demand**

457 Across the experiment it was observed that NO_3^- uptake capacity was dominated by the

458 activity of the HATS for both 5 mM and 0.5 mM plants (Fig. 2). This supports our previous

459 work (Garnett *et al.*, 2013) further demonstrating, that under sufficient NO_3^- growth

460 conditions ($\geq$ 2.5 mM) the HATS appear to contribute a major portion of total NO_3^- uptake

461 capacity (in this study on average ~ 65% of total uptake capacity for plants grown at 5 mM

462 NO_3^-) where previously the LATS were thought to predominate (Siddiqi *et al.*, 1990;

463 Kronzucker *et al.*, 1995b; Garnett *et al.*, 2003; Malagoli *et al.*, 2004). The uptake capacity of

464 the HATS was *c.* 50% of the net uptake rate which was similar to the HATS proportion of net

465 uptake capacity (*c.* 65%) further supporting the uptake capacity results and highlighting that

466 the HATS do not appear to be supressed at sufficient NO_3^- concentrations as has been reported

467 in many PNR studies (Minotti *et al.*, 1969; Jackson *et al.*, 1973; Goyal & Huffaker, 1986;

468 Aslam *et al.*, 1993; Henriksen & Spanswick, 1993; Zhuo *et al.*, 1999). It is well described that

469 total NO_3^- uptake capacity for plants grown with low NO_3^- supply (≤ 0.5 mM) is supported

470 mainly via HATS activity and indeed our results support this with the HATS contributing on

471 average $\geq 80\%$ of total NO_3^- uptake capacity for 0.5 mM grown plants (Siddiqi *et al.*, 1989;

472 Siddiqi *et al.*, 1990; Okamoto *et al.*, 2003; Garnett *et al.*, 2013). As expected the calculated

473 net NO_3^- uptake was low for 0.5 mM plants in comparison to 5 mM. These differential

474 contributions of HATS and LATS to 0.5 mM and 2.5 mM NO_3^- supply are in support of the

475 recent model proposed by Le Deunff and Malagoli (2014).

476 In line with our previous work (Garnett *et al.*, 2013), when NO_3^- availability was reduced

477 from 5 mM to 0.5 mM the HATS responded by increasing HATS uptake capacity from 3 d

478 post reduction in external NO_3^- concentration (Fig. 2). In contrast the LATS slowly decreased

479 post reduction in NO_3^- supply perhaps highlighting the shut down and transition from LATS

480 to HATS as would be expected under the current models and activity range described for the

481 LATS (Fig. 2) (Le Deunff & Malagoli, 2014). Previous studies have described the inducible

482 HATS (iHATS) and its role in mediating uptake in response to low NO_3^- (Siddiqi *et al.*, 1989;

483 Okamoto *et al.*, 2003; Cerezo *et al.*, 2007; Li *et al.*, 2007; Le Deunff & Malagoli, 2014). The

484 observed correlation between the total uptake capacity increase (Fig. S3) and HATS uptake

485 capacity increase (Fig. 2) in response to changing N appears to reflect the iHATS response

486 and highlights the role of the HATS in facilitating the plasticity (response to reduced N)

487 within the NO_3^- uptake system. In many plant species the link between HATS uptake capacity

488 and *NRT2* transcript levels, specifically *NRT2.1* and *NRT2.2*, has been clearly demonstrated

489 (Krapp *et al.*, 1998; Cerezo *et al.*, 2001; Filleur *et al.*, 2001; Orsel *et al.*, 2004; Li *et al.*, 2007;

490 Garnett *et al.*, 2013). With the observed increase in *ZmNRT2.1* and *ZmNRT2.2* transcripts

491 prior to the measured increase in NO_3^- uptake capacity in response to reduction in NO_3^- supply

492 it raises the question: would daily measurements of NO_3^- uptake capacity reveal a more

493 prompt increase in HATS capacity in response to reduction in NO_3^- availability (e.g. 2 d).

494

Dynamic transcriptional variation in response to steady state N limitation

Across the measured growth period all of the *NRTs* examined with the exception of *ZmNRT1.1B* showed higher transcript levels under 0.5 mM conditions. For orthologous *NRT* genes this has been shown before with higher transcript levels in response to N starvation and N limitation in Arabidopsis, *Nicotiana plumbaginifolia*, maize and wheat (Krapp *et al.*, 1998; Remans *et al.*, 2006; Garnett *et al.*, 2013; Buchner & Hawkesford, 2014). In our previous work we reported the substantial variation in *NRT* transcript levels when plants were grown under steady state NO_3^- conditions (Garnett *et al.*, 2013). In that study plants were sampled every $2 - 5$ d and two distinct peaks in the transcript levels of a number of putative *NRTs* were observed which were correlated with developmental stage and its associated N demand. In this study a focus on the vegetative growth period and a finer time resolution revealed substantially greater and more dynamic variation over time with examples of change in excess of 3 fold between consecutive daily measurements. A distinct three-peak transcription pattern between 16 - 22 DAE was observed for *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT3.1A*, *ZmNRT1.1A* and *ZmNRT1.5A* for plants grown under low N conditions (Fig. 6). This pattern may present evidence of some level of oscillation associated with the membrane transport of NO_3^- as has been widely reported for calcium (Evans *et al.*, 2001) and membrane-transport more generally (Shabala *et al.*, 2006). This new insight into the temporal variability of the *NRTs* highlights that much remains unknown about the transcriptional control of the *NRTs*. Understanding the nature of this variation could be key to understanding the regulation of NO_3^- uptake. This transcriptional variation should also be an important consideration for the design of future experiments as it could skew analysis of experimental data if experiments are not designed to take this into account.

Root to shoot xylem movement of nitrate and its assimilates

When plants were grown at 0.5 mM and 5 mM NO_3^-, changes in xylem sap NO_3^- levels complemented the changes in root and shoot NO_3^- concentration. The low NO_3^- concentration in 0.5 mM plants compared to 5 mM is supported by previous work in soybean where xylem

522   sap NO_3^- in N starved plants has been shown to be approximately 2% of N sufficient plants

523   during early pod filling (Layzell & LaRue, 1982). When NO_3^- supply was reduced, xylem sap

524   NO_3^- concentration dropped rapidly within 1 d, and by 3 d matched plants continuously grown

525   at 0.5 mM NO_3^- and followed the same temporal changes (Fig.4c). There are few studies

526   examining xylem sap NO_3^- concentration with which to compare these results, however, the

527   results indicate that NO_3^- concentration xylem sap varies greatly in response to changes in

528   NO_3^- supply and demand.

529   Xylem TAA levels remained similar between treatments until late in the vegetative growth

530   period when levels declined in 0.5 mM treated plants (Fig. 5c). This decline in TAA for 0.5

531   mM plants aligned with the observed decrease in xylem, root and shoot NO_3^- concentrations

532   and may represent an internal NO_3^- threshold where plants increase TAA utilisation (Figs. 4

533   and 5c). In response to reduction in NO_3^- supply TAA levels stayed constant for 5 d and did

534   not peak in line with 0.5 mM and 5 mM plants. At 5 d post reduction in NO_3^- availability,

535   xylem sap TAA concentration for D15Red. plants matched 0.5 mM grown plants. At this

536   same time point root and shoot NO_3^- for D15Red. plants had also dropped to align with 0.5

537   mM plants. Again this may highlight an internal NO_3^- threshold where plants increase TAA

538   utilisation, however, this remains to be investigated. With limited studies in the literature

539   exploring changes in xylem sap NO_3^- and AA these results set a good basis for future work

540   understanding root to shoot N transport in response to N supply and demand.

541   **Signalling molecules**

542   The regulation of NO_3^- uptake by plant N status has been widely reported (Cooper &

543   Clarkson, 1989; Imsande & Touraine, 1994; Forde, 2002; Miller *et al.*, 2008; Gojon *et al.*,

544   2009). These studies have highlighted both tissue concentration of NO_3^- itself or down-stream

545   assimilates such as AA being potential signals of N status and regulators of the NO_3^- uptake

546   system. Our previous lifecycle study (Garnett *et al.*, 2013) indicated that both of these were

547   plausible and with a higher time resolution it was anticipated that it may be possible to more

548   effectively correlate NO_3^- or assimilates directly with the observed changes in the NO_3^- uptake

549   system. From the data presented in this study, at a higher time resolution a loose correlation

550   was observed between shoot concentrations of arginine, aspartic acid, citruline, glutamate,

551   tyramine, phenethylamine and HATS uptake capacity (Fig. 7). The influence of AA on NO_3^-

552   uptake capacity and *NRT* transcript levels has been proposed as a negative feedback system

553   whereby certain assimilates (specifically glutamate, glutamine, aspartate and asparagine)

554   suppress *NRT* transcription and uptake capacity at high levels (Zhuo *et al.*, 1999; Vidmar *et*

555   *al.*, 2000; Gansel *et al.*, 2001; Nazoa *et al.*, 2003; Miller *et al.*, 2008). Under the conditions of

556   this study we did not see evidence to support this inverse correlation between *NRT* transcript

557   levels, NO_3^- uptake capacity and AA concentrations. In contrast the 0.5 mM declining trend,

558   the flat stable 5 mM profile, and the transient increase in response to reduction in NO_3^- supply

559   observed for root, shoot and xylem sap NO_3^- concentrations when assessed separately with the

560   corresponding treatment profiles for HATS NO_3^- uptake capacity showed a strong correlation

561   (Figs. 2a & 4). In addition the induction of *NRT* expression at 16 - 17 DAE coincides with the

562   low point in measured NO_3^- pools throughout the plant suggesting an internal NO_3^- threshold

563   and subsequent transcriptional trigger. These results strengthen the case for NO_3^- acting as a

564   signalling molecule to regulate the NO_3^- uptake system.

565   **Series of responses to reduction in N availability**

566   By examining numerous physiological parameters in response to steady state NO_3^- supply a

567   baseline was set to effectively analyse plant response to reducing NO_3^- supply. After 5 d post

568   reduction in external NO_3^- concentration a decrease in shoot growth was observed and an

569   increase in root growth was seen 2 d later, however, the observed effects on NO_3^- and its

570   uptake system preceded this. Xylem sap NO_3^- decreased within 24 h along with a decrease in

571   root TAA and an increase in shoot TAA in response to reduction in NO_3^- supply. This was in

572   line with the general responses to steady state NO_3^- supply with 0.5 mM plants consistently

573   having lower and higher TAA concentration in the root and shoot respectively (Fig. 5). High

574   concentration of shoot TAA have been reported previously in response to persistent N

starvation and described as a product of leaf senescence (via nucleic acid and protein breakdown) (Schulze *et al.*, 1994; Crafts-Brandner *et al.*, 1998; Masclaux *et al.*, 2000; Hörtensteiner & Feller, 2002). The reduction in root TAA concentration could reflect either a reduction in root assimilation or a rapid utilisation of root TAA in response to the reduction in NO_3^- supply. Interestingly a rapid increase in shoot C was observed which highlights the tight link between N and C which has been widely reported (Stitt, 1999; Stitt *et al.*, 2002; Commichau *et al.*, 2006).

In support of our previous work (Garnett *et al.*, 2013), here with a finer time resolution we observed increases in transcript levels of *ZmNRT.1A*, *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT2.5* and *ZmNRT3.1A* in response to reducing NO_3^- supply. Within one day of reducing NO_3^- supply a transcriptional increase was observed for *ZmNRT3.1A*, *ZmNRT2.5* and *ZmNRT1.5A*. The low N induced transcription of *NRT1.5* genes has not been reported previously with all published data being focused on NO_3^- starvation and induction (Lin *et al.*, 2008; Buchner & Hawkesford, 2014). In Arabidopsis AtNRT1.5 has been characterised as a low affinity NO_3^- transporter and shown to be located in root pericycle cells close to the xylem (Lin *et al.*, 2008). Subsequent knockdown or knockout mutations have led to reduced root-to-shoot NO_3^- transport implicating a role for this protein in xylem loading of NO_3^- (Lin *et al.*, 2008). The *NRT2.5* orthologues are induced under low NO_3^- conditions in both maize and wheat (Garnett *et al.*, 2013; Guo *et al.*, 2014) and N starvation in Arabidopsis (Okamoto *et al.*, 2003; Krapp *et al.*, 2011; Lezhneva *et al.*, 2014). In rice OsNRT2.3A (orthologous to AtNRT2.5) has been located in the xylem parenchyma cells of the stele and subsequent knockdown impaired xylem loading of NO_3^- (Tang *et al.*, 2012). With the rapid observed changes in xylem sap NO_3^- and the transcriptional increase in *NRTs* with putative roles in xylem loading of NO_3^- it appears that an early response to reduced NO_3^- may be to increase root-to-shoot NO_3^- transport to maintain shoot growth. The ZmNRT3.1 ortholog in Arabidopsis, AtNAR2.1, has been shown to constitute part of a two-component NO_3^- HATS system which is essential for high affinity NO_3^- transport (Okamoto *et al.*, 2006; Orsel *et al.*, 2006; Yong *et al.*, 2010). This

two component NO_3^- uptake system has also been identified in barley (*Hordeum vulgare*) and rice (*Oryza sativa*) for orthologous NRT2 and NAR2.1 proteins (Ishikawa *et al.*, 2009; Feng *et al.*, 2011). In Arabidopsis, all NRT2s with the exception of AtNRT2.7 appear to require interaction with AtNAR2.1 to facilitate NO_3^- transport (Kotur *et al.*, 2012). With *ZmNRT2.5* being the only *NRT2* gene showing a transcriptional increase at the same time as *ATNRT3.1*, this may indicate the requirement for both ZmNRT3.1 and ZmNRT2.5 together to facilitate xylem loading of NO_3^-.

After the observed increase in putative xylem loading associated *NRTs*, an up regulation of the main HATS *NRT* transporters was seen. It is well characterised that NRT2.1 and NRT2.2 in Arabidopsis facilitate the majority of NO_3^- uptake at low N (Filleur *et al.*, 2001; Li *et al.*, 2007). Both *ZmNRT2.1* and *ZmNRT2.2* transcript levels increased from 2 d post reduction in NO_3^- availability. This was followed by a subsequent increase in HATS NO_3^- uptake capacity which putatively restored xylem sap NO_3^- concentration by 3 d post reduction in NO_3^- availability (Figs 2a & 4c). At this same time point a substantial reduction in root NO_3^- and N concentration was observed indicating that due to the low external NO_3^- availability that uptake was not able to meet shoot demand and root NO_3^- stores were accessed (Figs. 3b, 4b). With this increase in uptake not meeting N demand from the shoot, after 5 d post reduction in NO_3^- availability both root and shoot NO_3^- and total N decreased rapidly (Figs. 3 & 4). This decrease in overall plant N status corresponded with a shutdown of HATS uptake capacity together with a reduction in shoot growth rate compared to 5 mM plants. With N pools throughout the plant hitting a low at $6 - 7$ d post reduction in NO_3^- availability it appears plants compensated by increasing root mass to increase N capture area. When maximum NO_3^- uptake capacity is unable to meet N demand it is widely reported that plants invest in root growth to increase N capture (Brouwer, 1962; Raper *et al.*, 1977; Bhat *et al.*, 1979; Freijsen & Otten, 1984; Tolley-Henry & Raper, 1986; Scheible *et al.*, 1997; Ågren & Franklin, 2003; Ikram *et al.*, 2012). This daily series of responses to reduction in NO_3^- supply reveals a lot

628 about the steps which take place when plants sense and respond to change in NO_3^-

629 availability.

A dynamic nitrate uptake system

631 Overall the data presented here reveals that the temporal changes in *NRT* expression and

632 concentrations are significantly greater and more dynamic than previously understood,

633 highlighting the need to consider this variability in the design of future experiments. With

634 both 0.5 mM and 5 mM NO_3^- grown plants it was demonstrated that the HATS appears to be

635 responsible for the majority of total uptake capacity and mediate the plasticity within the NO_3^-

636 uptake system in response to N supply and demand. There was also evidence to support that

637 NO_3^- in root, shoot and xylem sap appears to be playing a major role in signalling plant N

638 status and modulating the NO_3^- uptake system. A series of responses were observed in

639 response to N demand and changes in N supply delivering new insight into the NO_3^- uptake

640 system and its control. Future work will be focused on analysing NRT protein levels, global

641 gene expression to further elucidate how NO_3^- transport is regulated in response to N supply

642 and demand. Through understanding the physiological and biochemical mechanisms

643 governing the NO_3^- uptake system in response to N supply and demand we may move closer

644 toward the development of plants with increased NUE and more specifically N uptake

645 efficiency.

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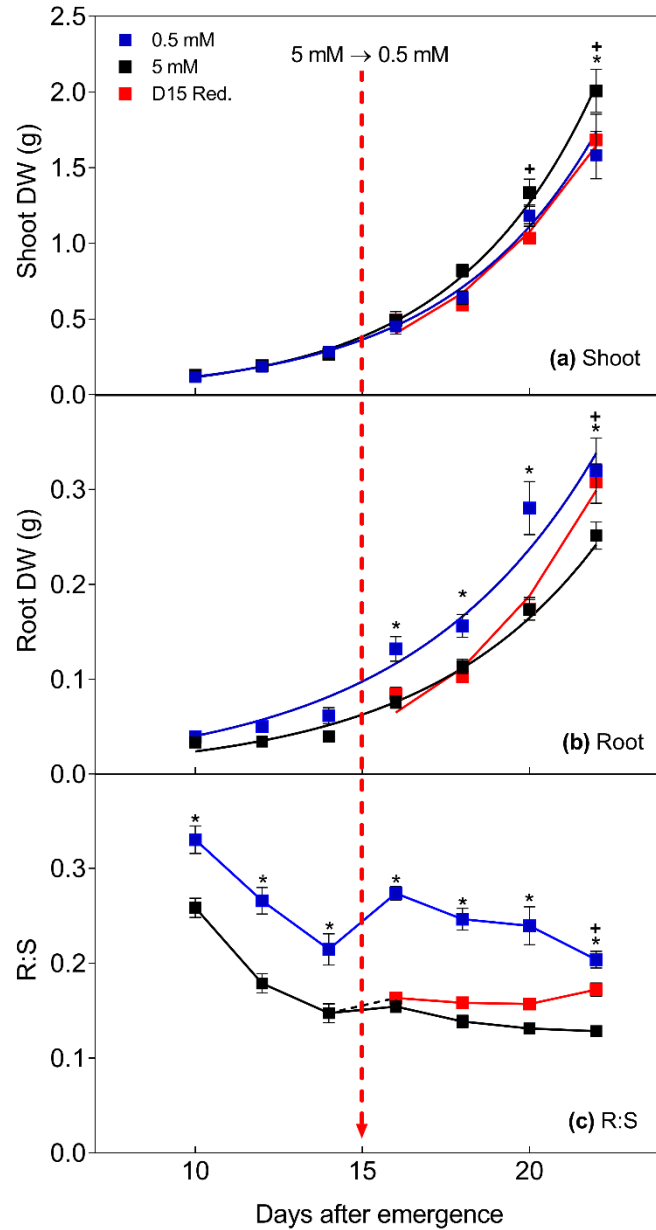
FIGURES

Figure 1. Root growth, shoot growth and R:S of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. (a) Shoot dry weight (DW), (b) root (DW) and (c) root to shoot ratio. Fitted curves for root and shoot growth are as described in the text. Significant differences were observed for root and shoot biomass between treatments. Values are ± SEM (n=14). *Points significantly different between 0.5 mM and 5 mM growth conditions ($P<0.05$). [+]Points significantly different between 5 mM and D15 Red. growth conditions ($P<0.05$).
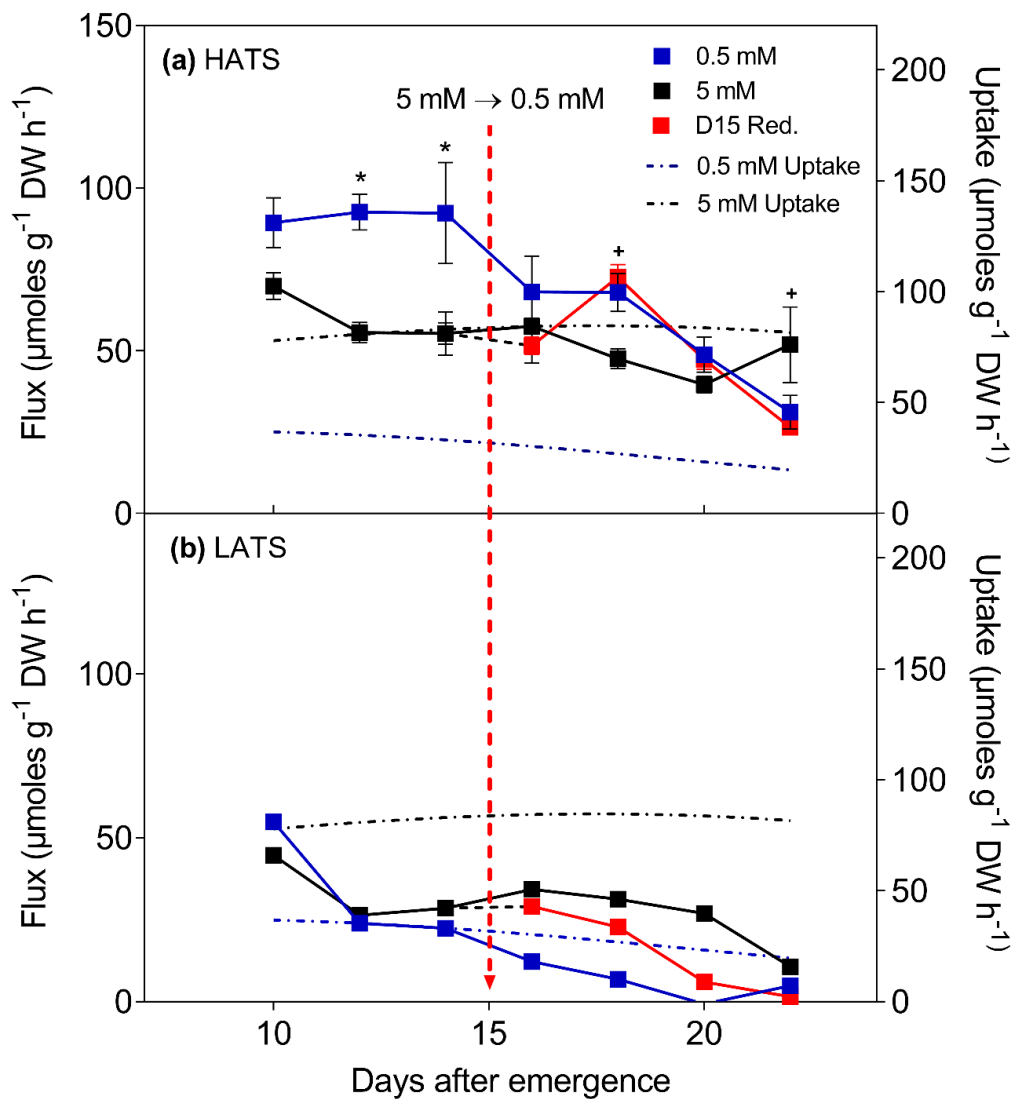
Figure 2. Unidirectional NO_3^- influx into the roots of dwarf (*Zea mays*) Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). Net uptake at 0.5 mM NO_3^- (blue dotted line) and 5 mM NO_3^- (black dotted line) was calculated from the fitted curves for shoot DW, root DW (Fig. 1) and shoot N (Fig. 3) as described in the text. The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. (a) HATS measured at 100 μM ^{15}N labelled NO_3^-. (b) LATS calculated by subtracting measured flux of ^{15}N labelled NO_3^- at 100μM from 2500μM. Significant differences were observed for root and shoot biomass between treatments. Values are ± SEM (n=4). *Points significantly different between 0.5 mM and 5 mM growth conditions ($P<0.05$). +Points significantly different between 5 mM and D15 Red growth conditions ($P<0.05$).
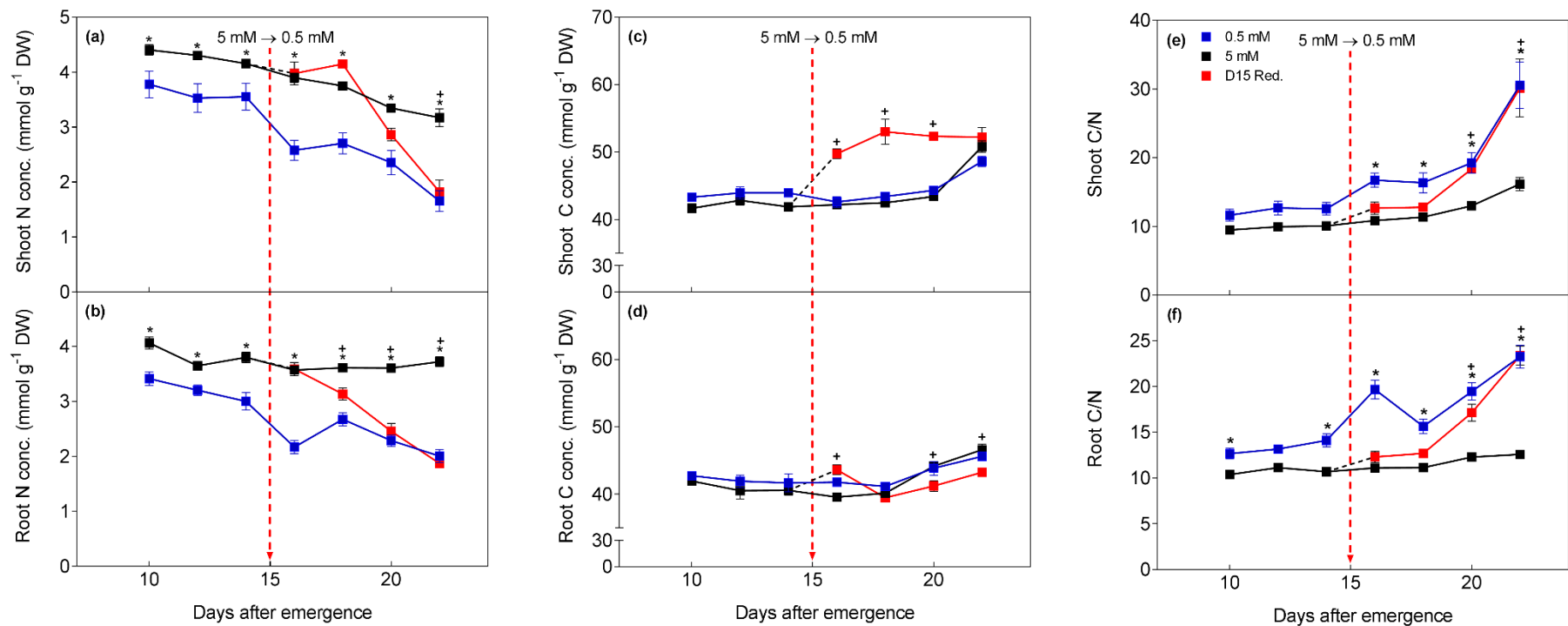
Figure 3. Nitrogen (N) and carbon (C) concentration (mmol g^{-1} DW), and carbon to nitrogen ratio (C/N) in the roots and shoots of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO$_3^-$ (blue squares), 5 mM NO$_3^-$ (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO$_3^-$ at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. (a) shoot tissue N concentration, (b) root tissue N concentration, (c) shoot tissue C concentration, (d) root tissue C concentration, (e) shoot tissue carbon to nitrogen ratio, and (f) root tissue carbon to nitrogen ratio. Values for root are ± SEM (n=8). Values for shoot are ± SEM (n=4). *Points significantly different between 0.5 mM and 5 mM growth conditions ($P<0.05$). +Points significantly different between 5 mM and D15 Red. growth conditions ($P<0.05$).
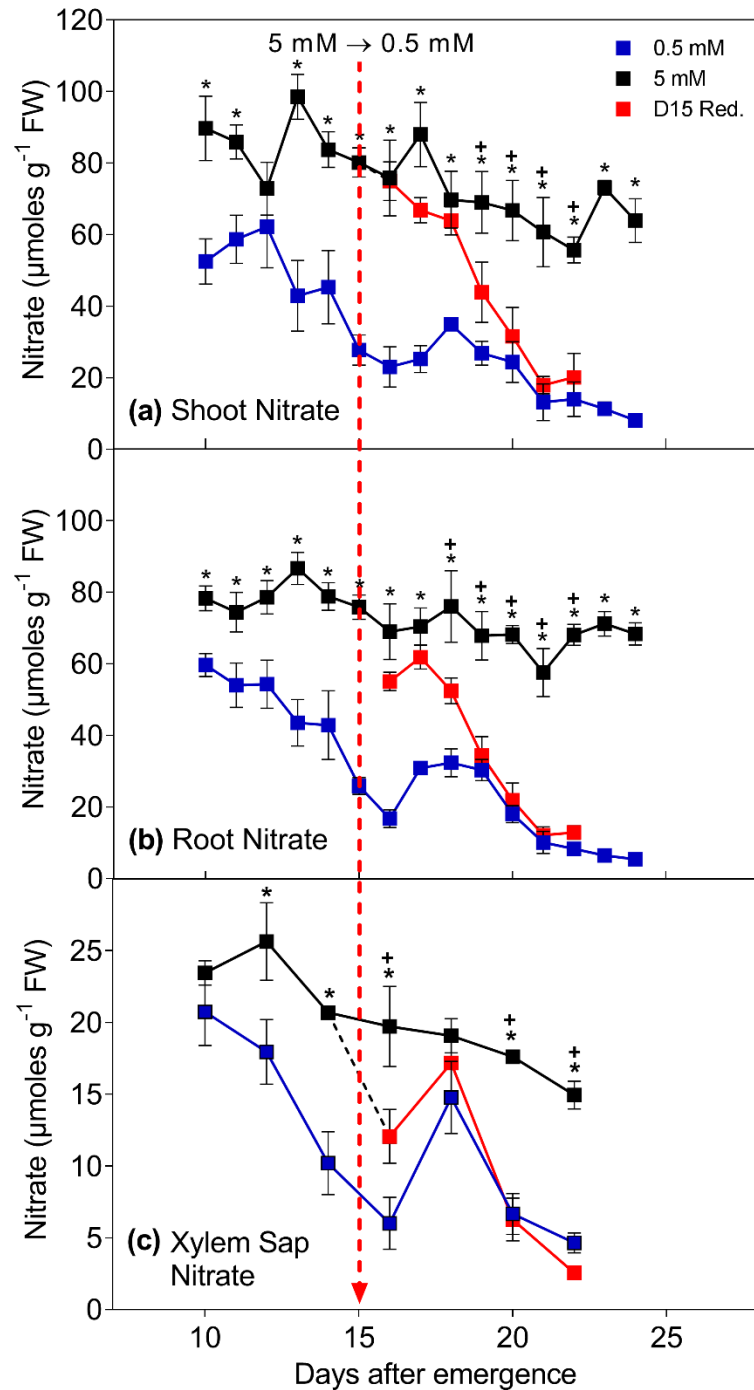
Figure 4. Nitrate concentration in (a) shoot, (b) root and (c) xylem sap of dwarf (*Zea mays*) Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. For root and shoot data values are ± SEM (n=4). For xylem sap data values are ± SEM (n=between 2 & 6). *Points significantly different between 0.5 mM and 5 mM growth conditions (*P*<0.05). +Points significantly different between 5 mM and D15 Red. growth conditions (*P*<0.05).

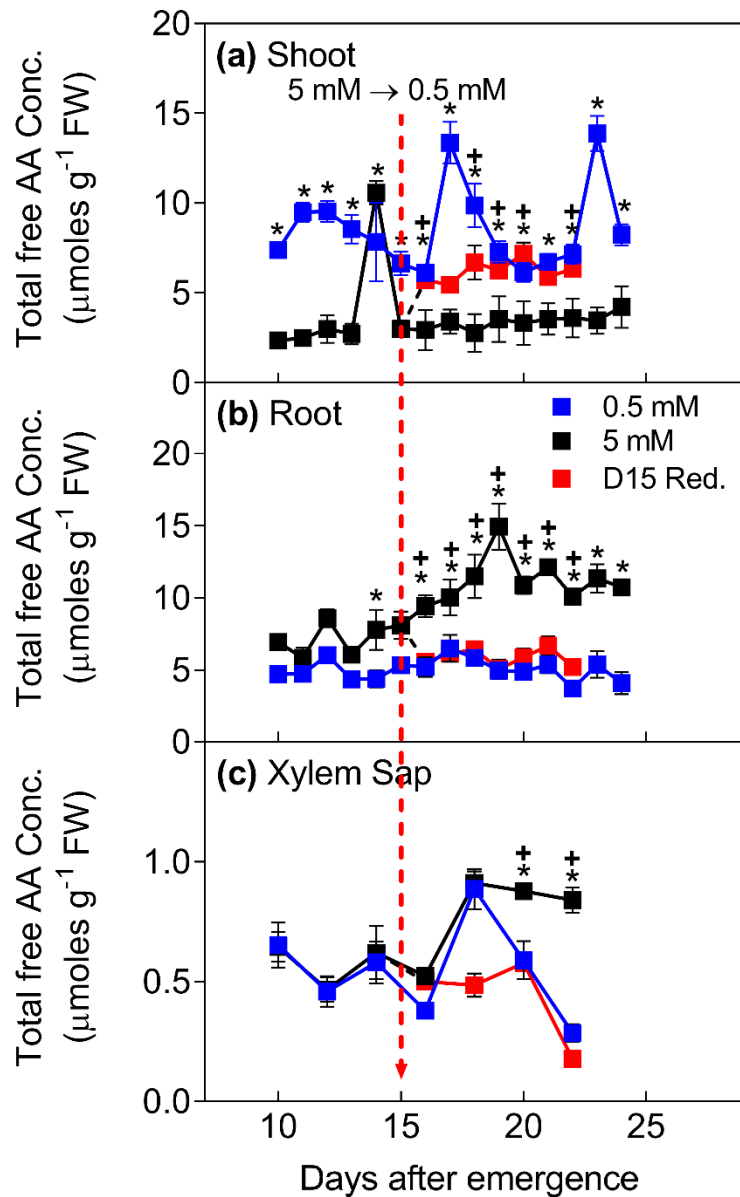**Figure 5.** Total free amino acid concentration (TAA) in (a) shoot, (b) xylem sap and (c) root of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants are indicated by the dotted red arrowed line. Root ad shoot values are ± SEM (n=4). For xylem sap data values are ± SEM (n=between 2 & 6) *Points significantly different between 0.5 mM and 5 mM growth conditions (*P*<0.05). +Points significantly different between 5 mM and D15 Red. growth conditions (*P*<0.05)

Figure 6. Root transcript levels of various putative high- and low-affinity (*NRT1*, *NRT2* and *NRT3*) NO_3^- transporters in dwarf (*Zea mays*) Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line.). Values are ± SEM (n=4). *Points significantly different between 0.5 mM and 5 mM growth conditions (*P*<0.05). +Points significantly different between 5 mM and D15 Red. growth conditions (*P*<0.05).

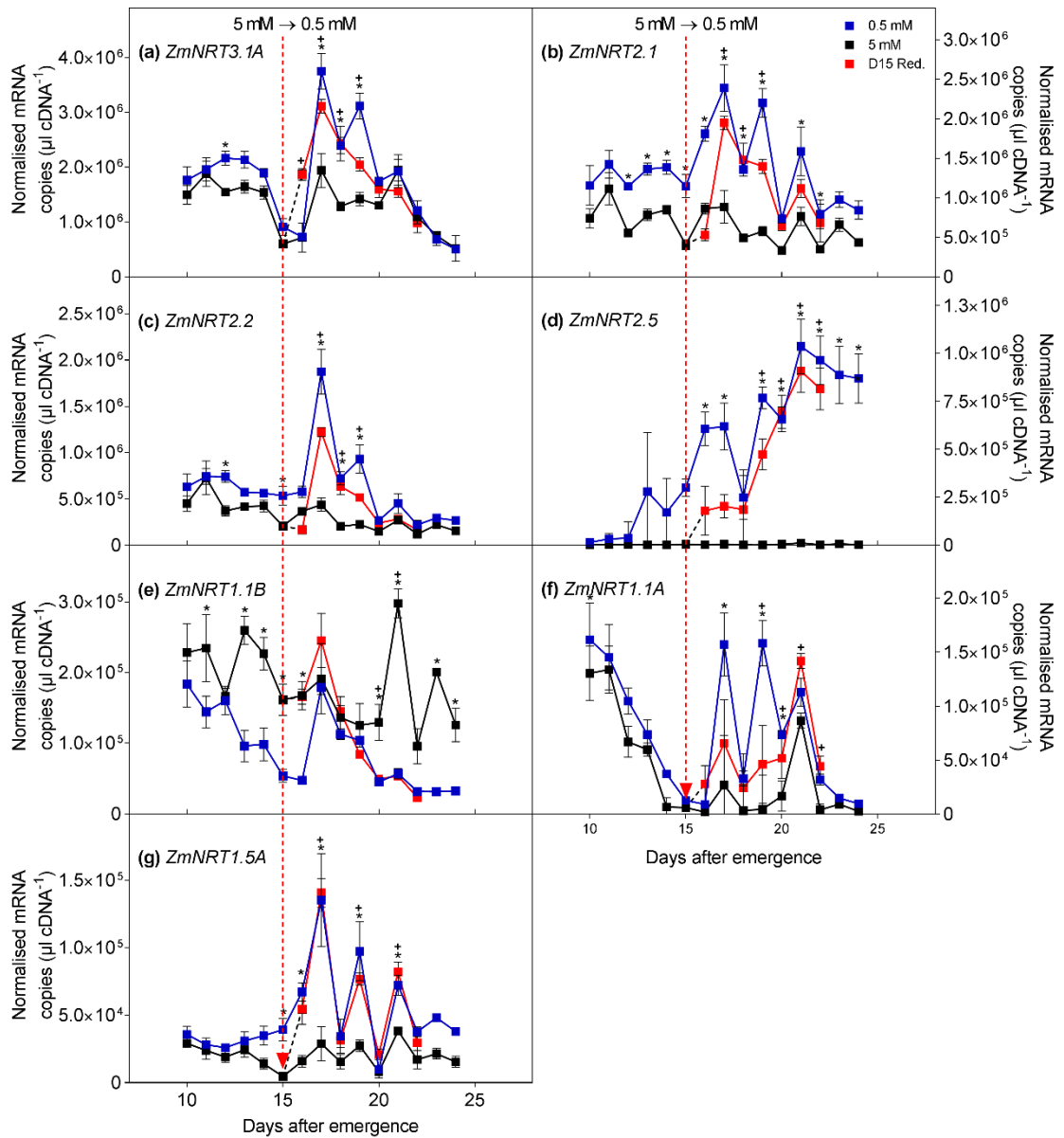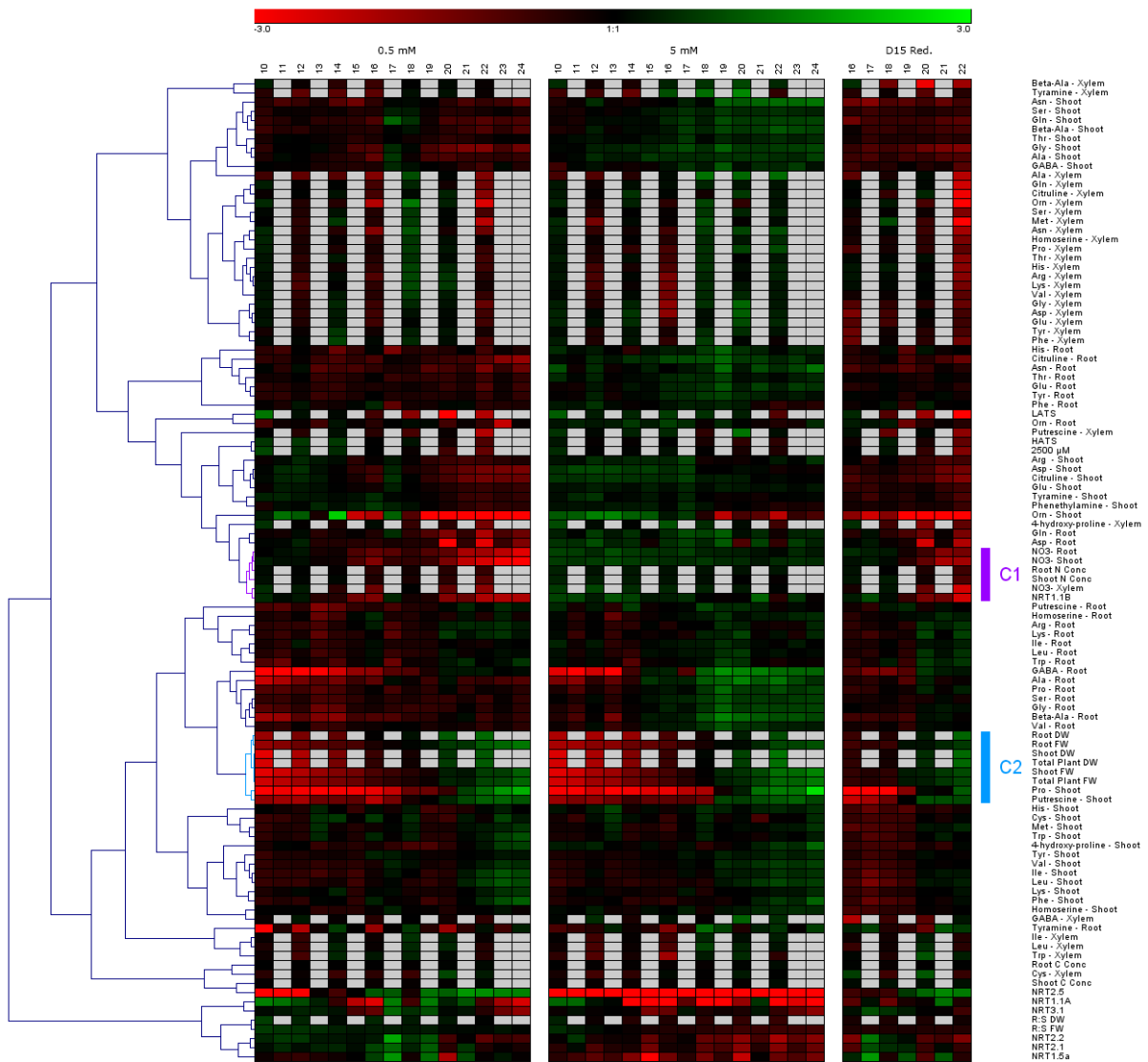**Figure 7.** Hierarchical clustering of all measured parameters in dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^-, 5 mM NO_3^-, or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red.). Data was mean centred and adjusted to $\log_2$ scale. Hierarchical clustering was performed using average linkage clustering agglomeration rule and distance based on Pearson correlation. Grey boxes indicate no value for parameters recorded every 2 d. Analysis performed using Genesis V1.7.6.

SUPPORTING INFORMATION FIGURES
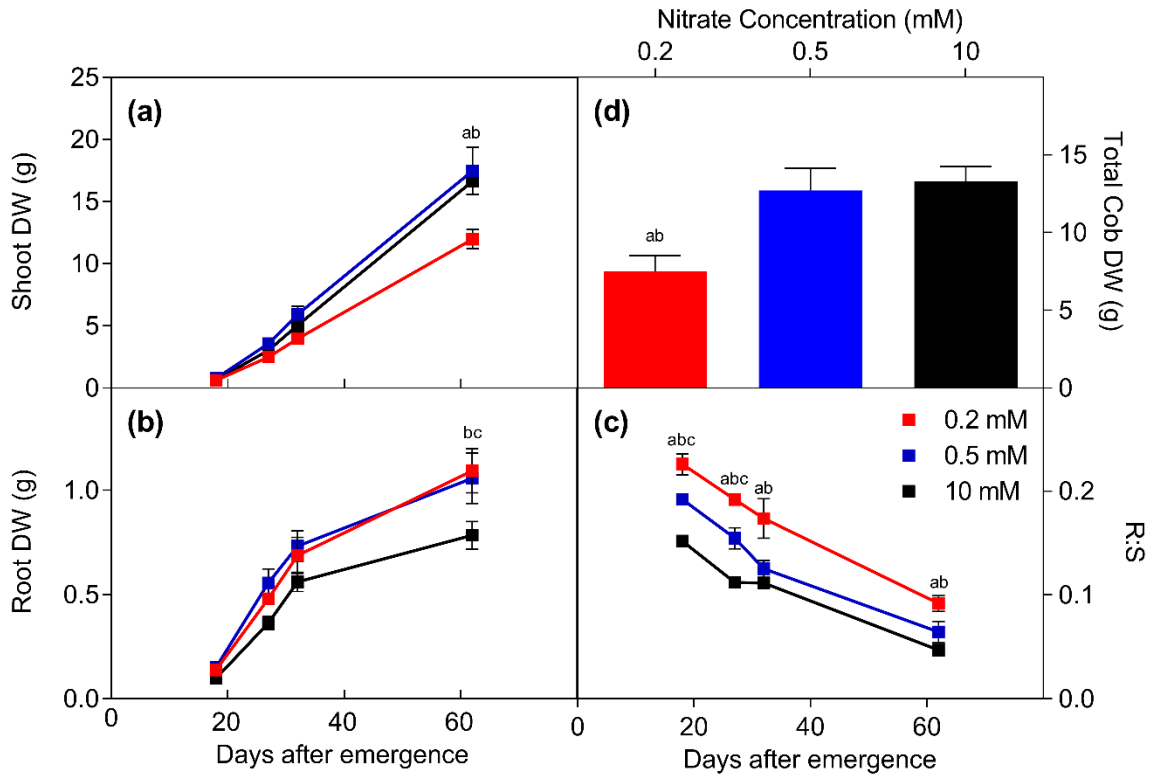
1013   **Supporting Information Figure S1.** Preliminary experiment analysing the response to

1014   different steady state nitrate concentrations across the lifecycle of dwarf (*Zea mays)* Gaspe

1015   Flint plants grown at either 0.2 mM NO_3^- (red squares), 0.5 mM NO_3^- (blue squares), or 10

1016   mM NO_3^- (black squares). (a) Shoot DW, (b) Root DW, (c) DW root:shoot ratio, (d) Total cob

1017   dry weight as a representation of yield. Root, shoot and R:S values are ± SEM (n=4 except for

1018   day 62 where n=6). Cob DW values are ± SEM (n=4). [a]Points significantly different between

1019   0.2 mM and 0.5 mM growth conditions ($P<0.05$). [b]Points significantly different between 0.2

1020   mM and 10 mM ($P<0.05$). [c]Points significantly different between 0.5 mM and 10 mM

1021   growth condition.
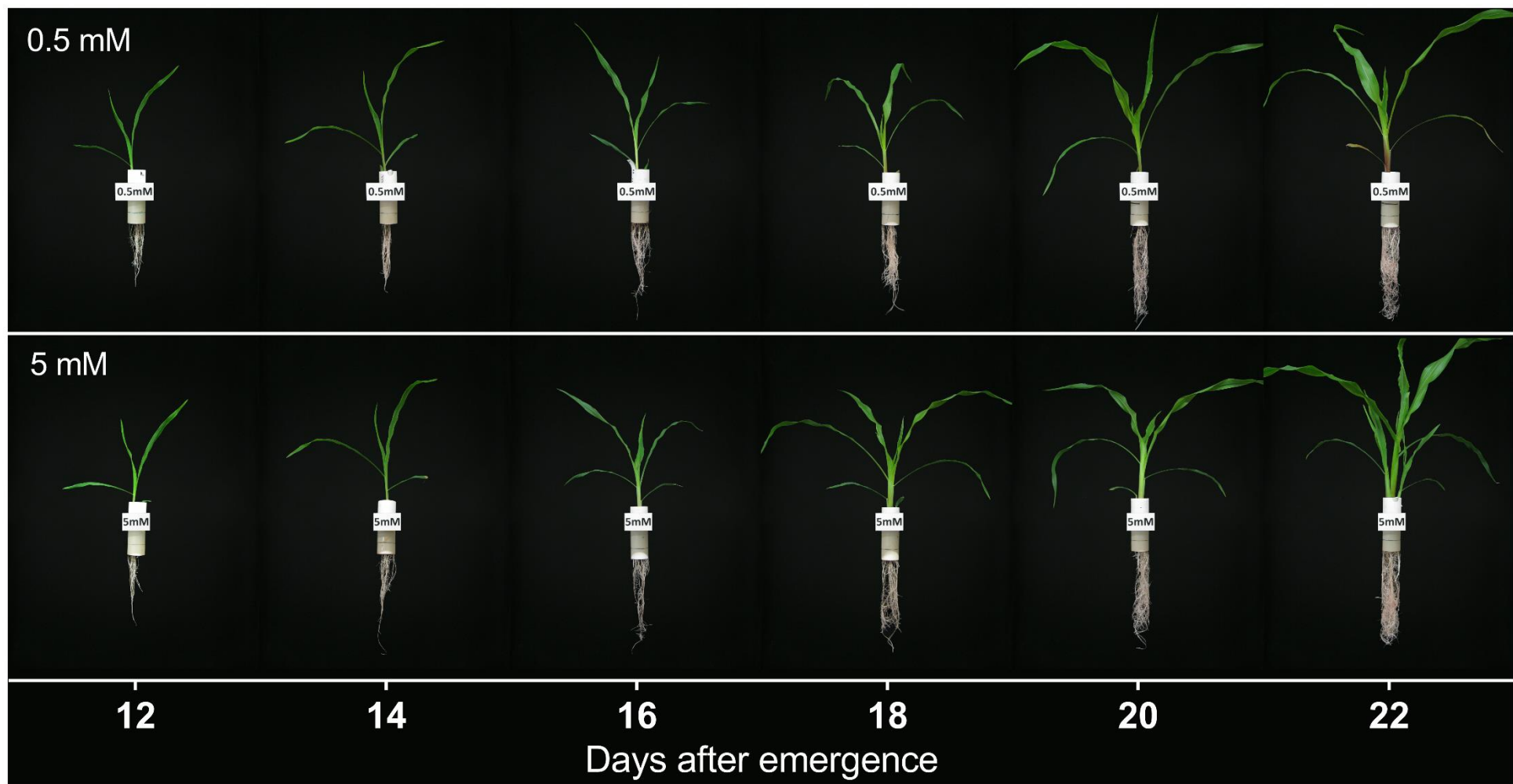
1022 **Supporting Information S2.** Growth of dwarf (*Zea mays)* Gaspe Flint plants. Images are of plants were taken directly after removing from the hydroponic

1023 system.
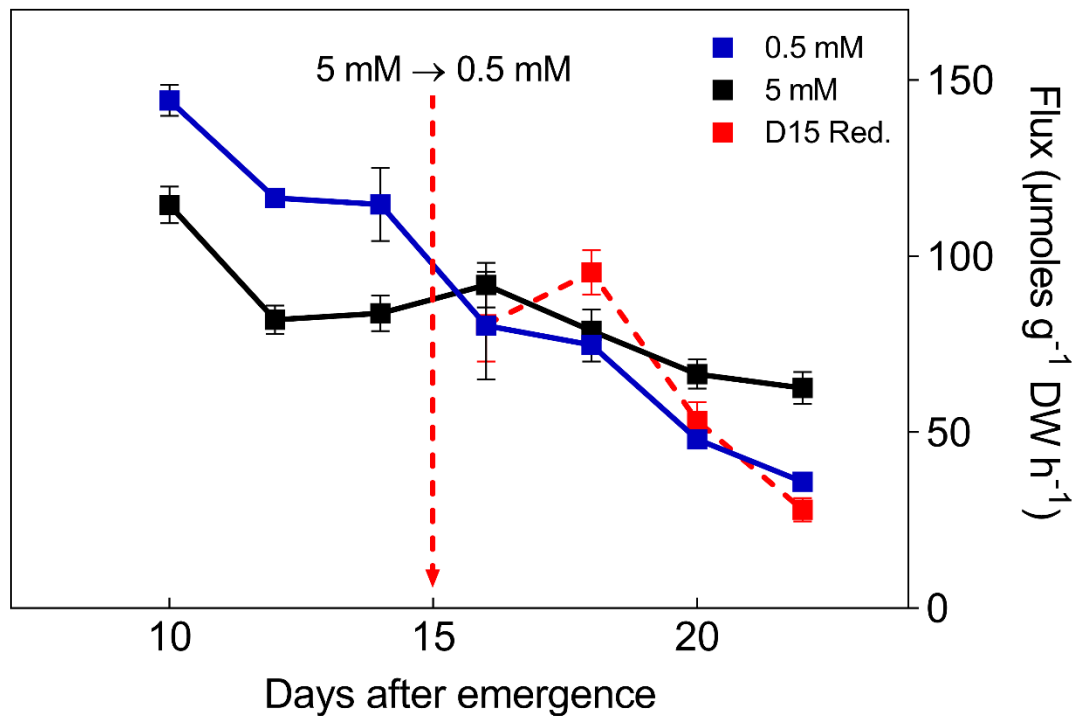
Supporting Information Figure S3. Unidirectional NO_3^- influx into the roots of dwarf (*Zea mays*) Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. NO_3^- flux capacity was measured at 2500 μM. Values are ± SEM (n=4). *Points significantly different between 0.5 mM and 5 mM growth conditions (*P*<0.05). +Points significantly different between 5 mM and D15 Red. growth conditions (*P*<0.05).

1031 **Supporting Information Table S1.** Collection of fitting functions and associated parameters used in the modelling of shoot and root growth and

1032 shoot nitrogen content.

Quantity	Fit function	Parameter 0.5 mM	Parameter 5 mM
Shoot dry weight (g)	$S_o e^{(\mu_s t)}$	$S_o = 0.0128 \pm 0.0017$ $\mu_s = 0.223 \pm 0.008$	$S_o = 0.0106 \pm 0.0016$ $\mu_s = 0.239 \pm 0.009$
Root dry weight (g)	$R_o e^{(\mu_r t)}$	$S_o = 0.0068 \pm 0.00098$ $\mu_s = 0.178 \pm 0.009$	$S_o = 0.00348 \pm 0.00086$ $\mu_s = 0.193 \pm 0.015$
Shoot N content (%DW)	$N_S(w) = \dfrac{a}{b+w^c}$	$a = 5.82 \pm 2.11$ $b = 0.861 \pm 0.634$ $c = 0.702 \pm 0.418$	$a = 16.8 \pm 2.8$ $b = 2.35 \pm 0.56$ $c = 0.509 \pm 0.121$
Root N content (%DW)	$R_s(w) = aw^b$	$a = 5.82 \pm 2.11$ $b = 0.861 \pm 0.634$ $c = 0.702 \pm 0.418$	$a = 5.82 \pm 2.11$ $b = 0.861 \pm 0.634$ $c = 0.702 \pm 0.418$
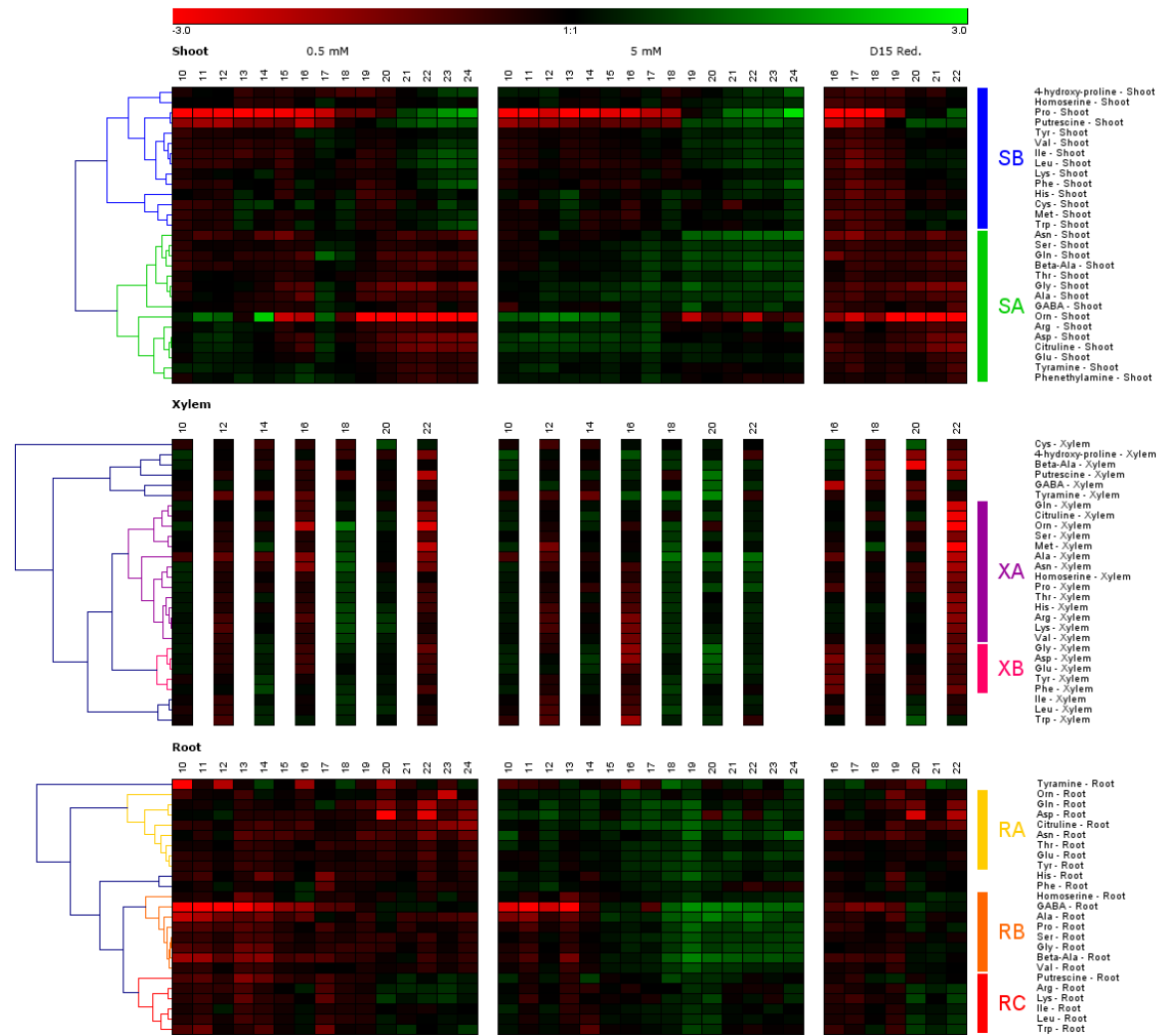
Supporting Information Figure S4. Hierarchical clustering of measured individual free amino acid (AA) concentrations in the root, shoot and xylem sap of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^-, 5 mM NO_3^-, or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red.). Highlighted clusters indicate a high level of correlation.
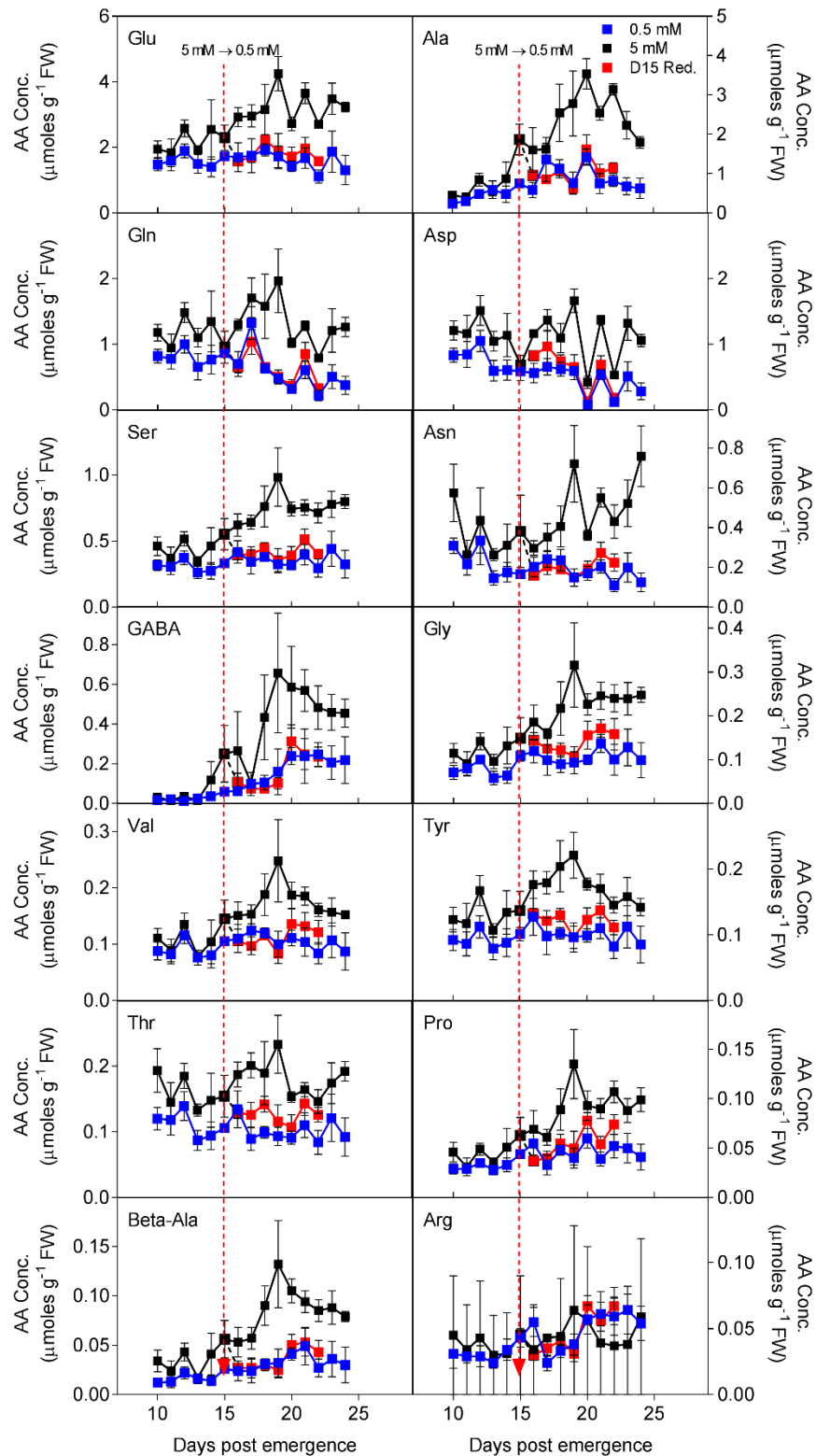
Supporting Information Figure S5. Individual free amino acid concentration (AA) in the root of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. Values are ± SEM (n=4).
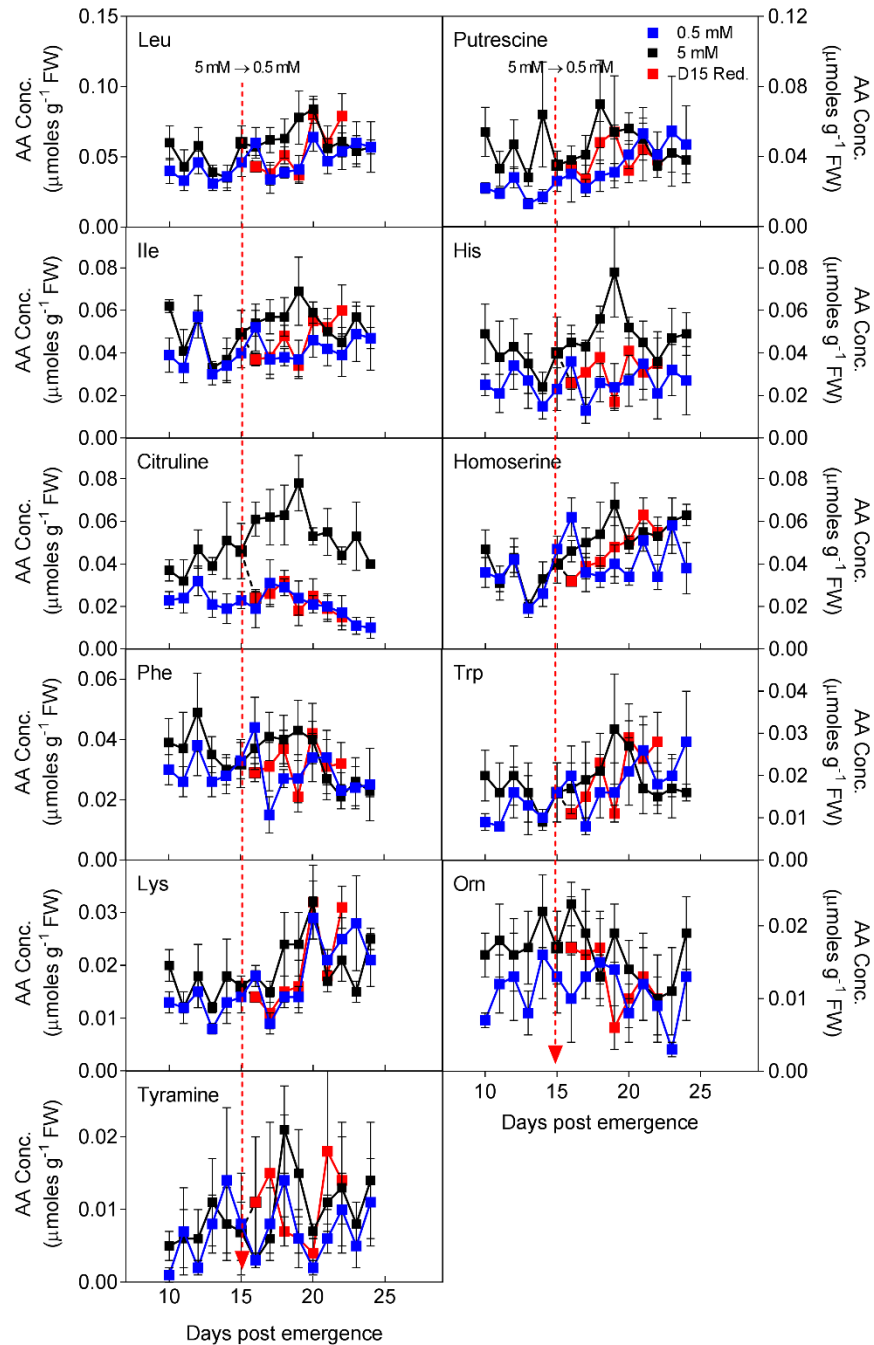
Supporting Information Figure S5 continued. Individual free amino acid concentration (AA) in the root of dwarf (*Zea mays*) Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. Values are $\pm$ SEM (n=4).
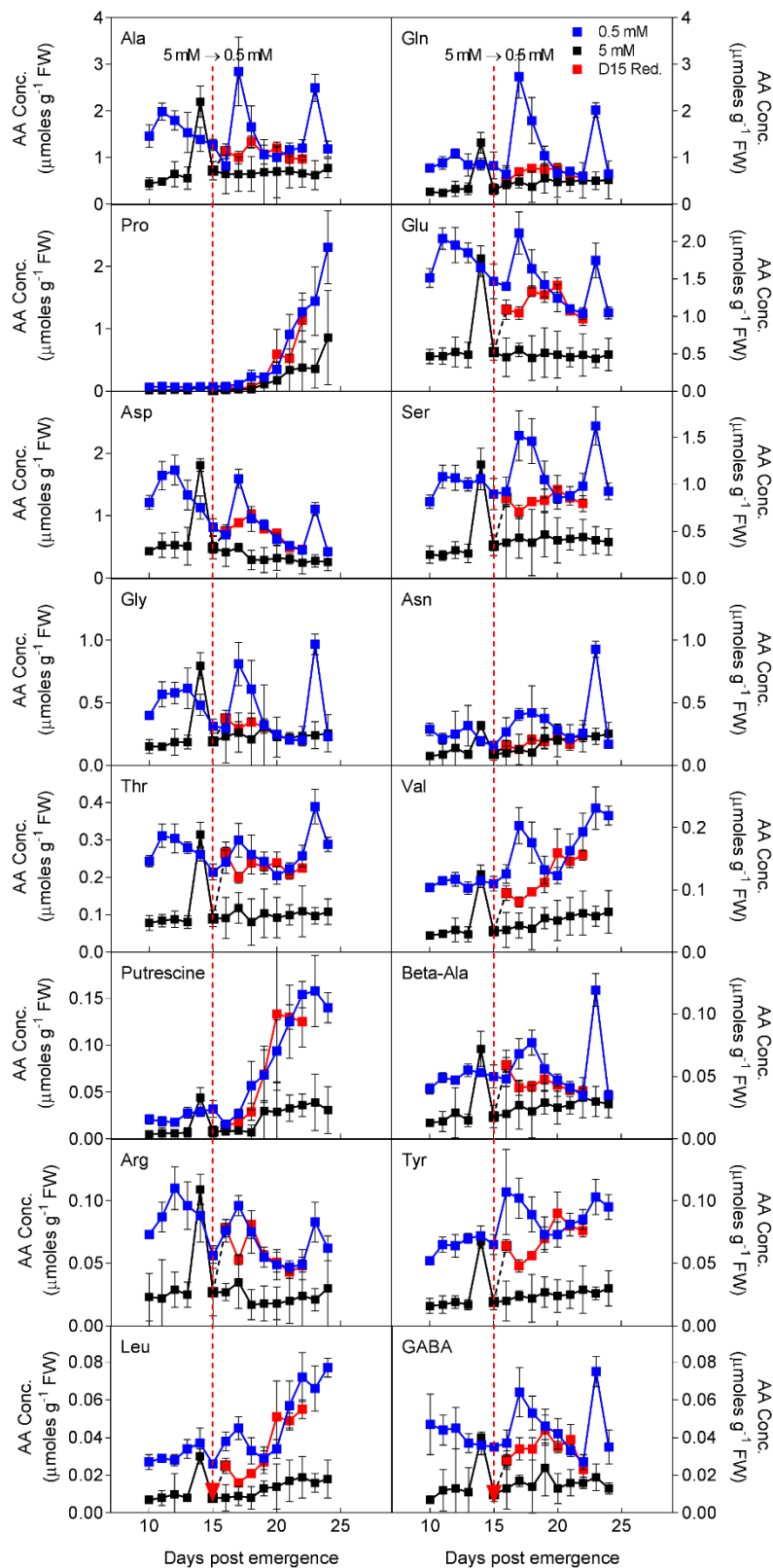
Supporting Information Figure S6. Individual free amino acid concentration (AA) in the shoot of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. Values are ± SEM (n=4).
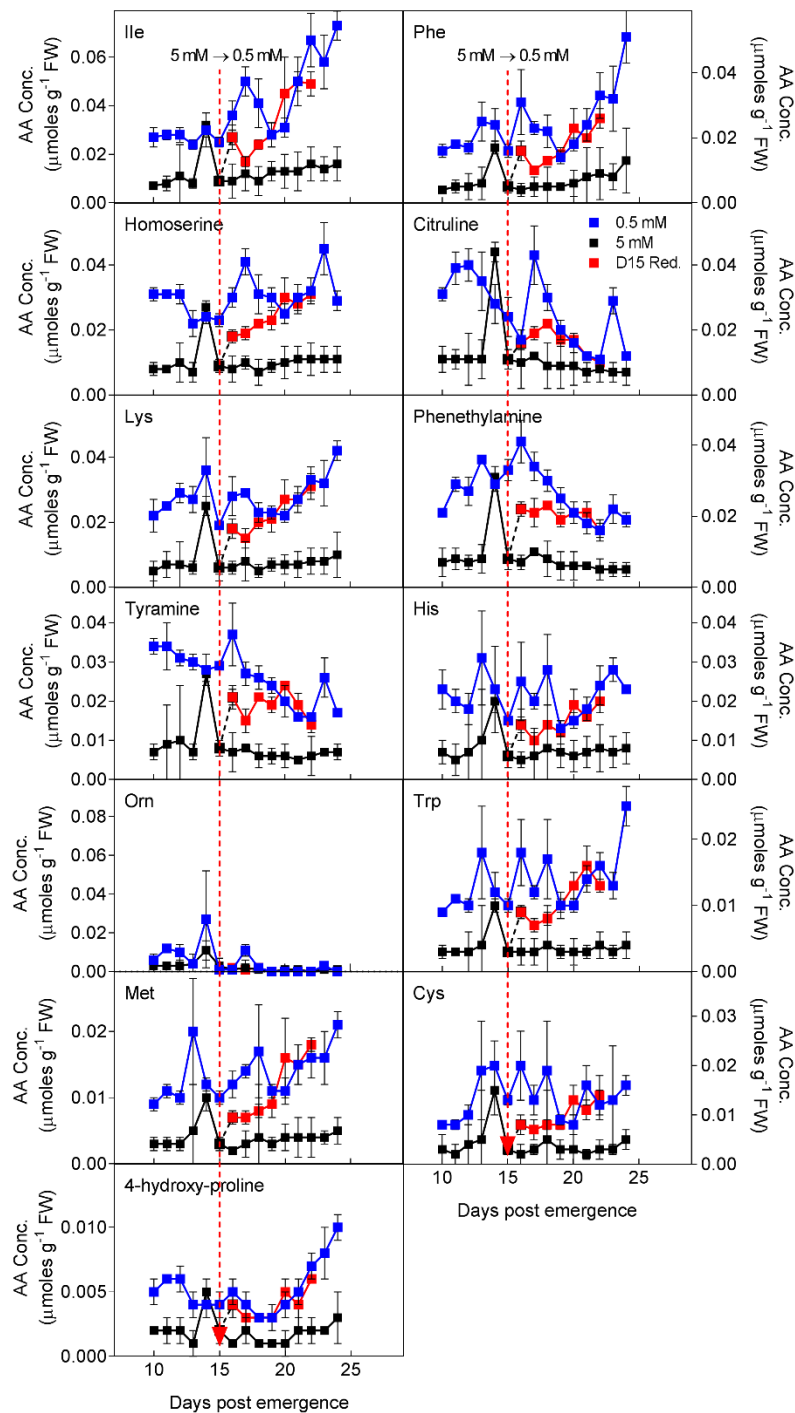
Supporting Information Figure S6 continued. Individual free amino acid concentration

(AA) in the shoot of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue

squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM

NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is

indicated by the dotted red arrowed line. Values are ± SEM (n=4).
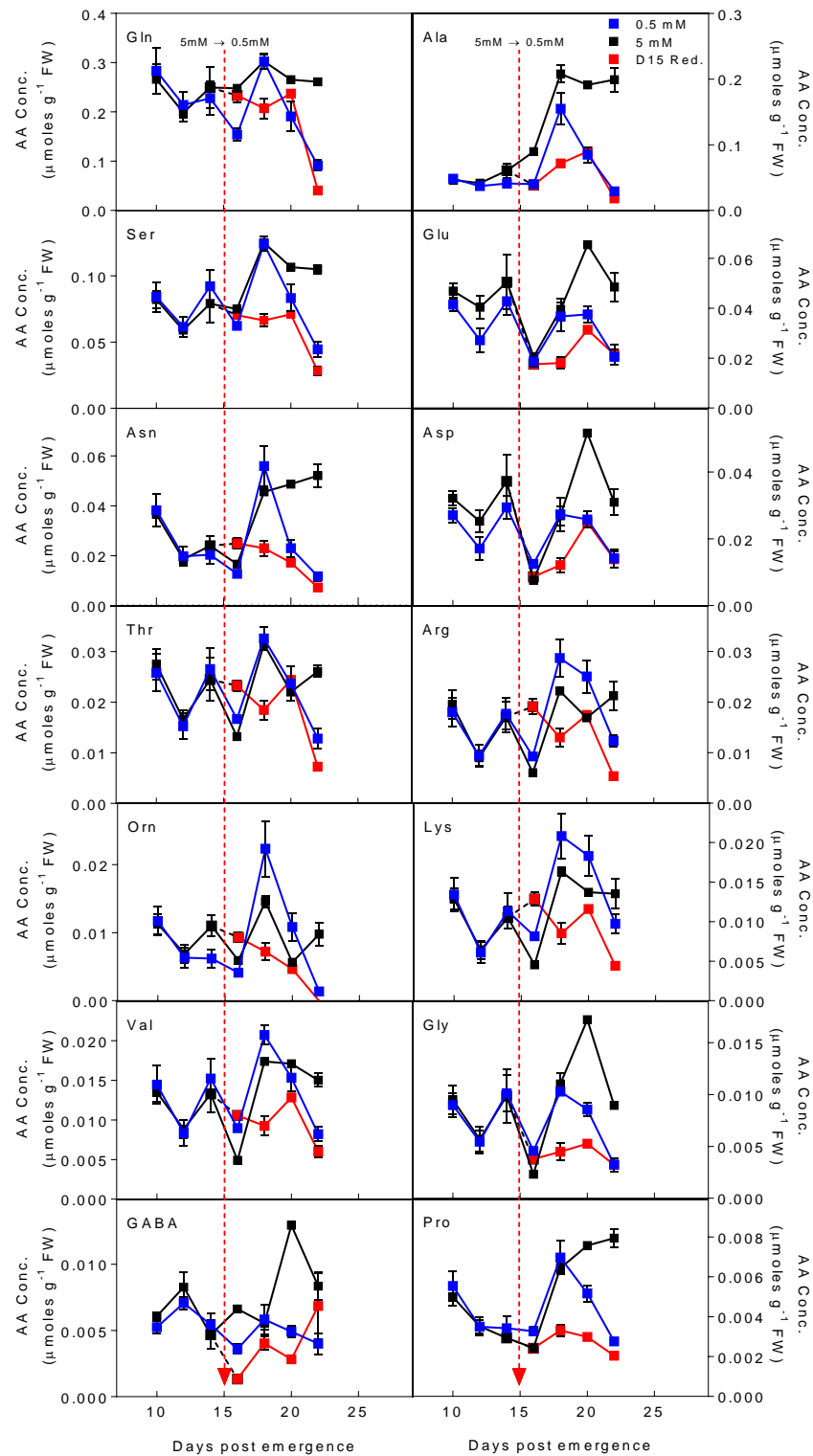
Supporting Information Figure S7. Individual free amino acid concentration (AA) in the xylem sap of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO_3^- (blue squares), 5 mM NO_3^- (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO_3^- at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. Values are $\pm$ SEM (n = between 2 & 6).
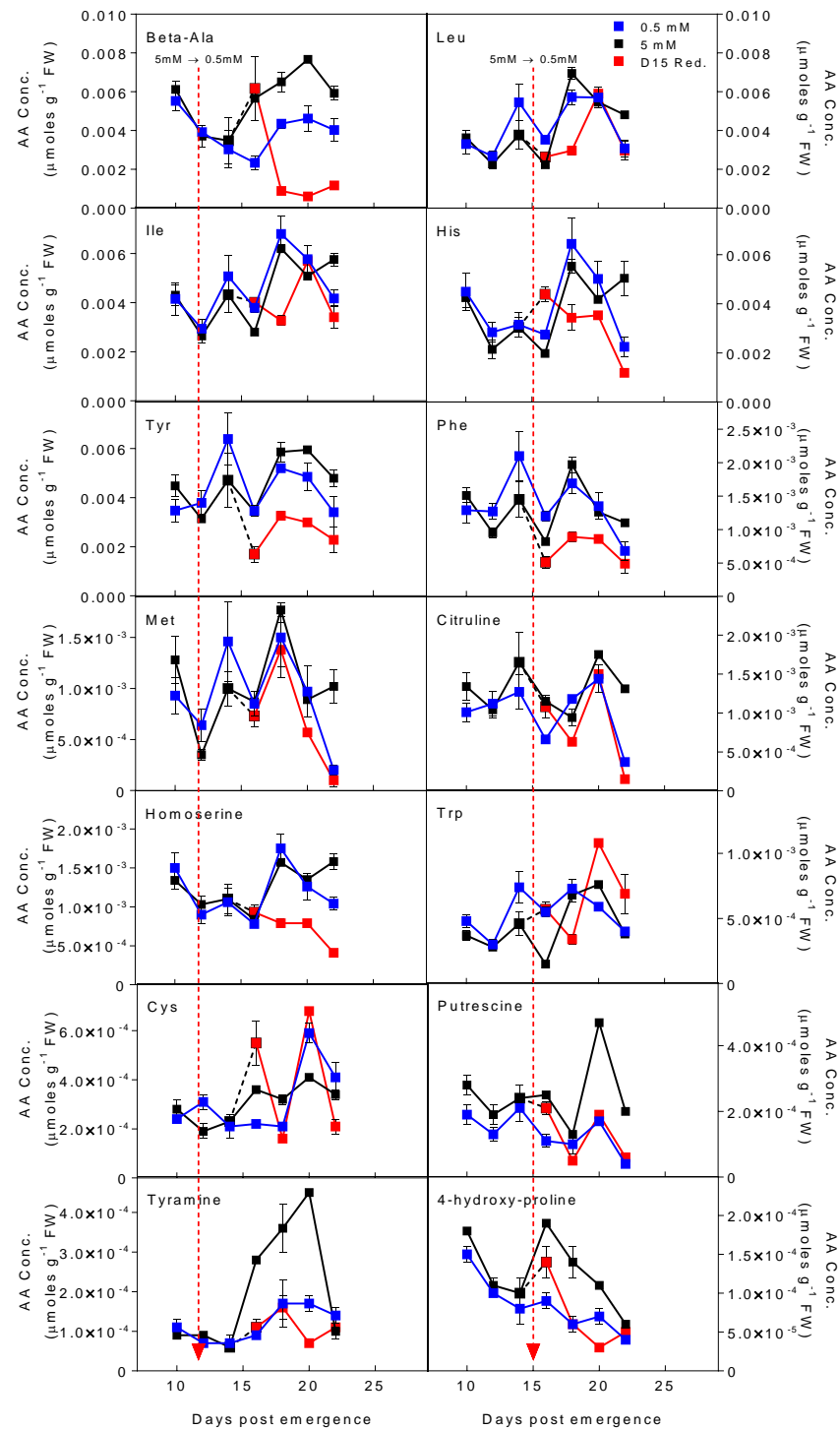
Supporting Information Figure S7 continued. Individual free amino acid concentration (AA) in the xylem sap of dwarf (*Zea mays)* Gaspe Flint plants grown at either 0.5 mM NO$_3^-$ (blue squares), 5 mM NO$_3^-$ (black squares), or plants subject to a reduction from 5 mM to 0.5 mM NO$_3^-$ at 15 DAE (D15 Red. = red squares). The time point of reduction for D15 Red. plants is indicated by the dotted red arrowed line. Values are ± SEM (n = between 2 & 6).

Chapter 3: Dynamics of N response depends on N status in maize plants: Comparison between nitrate induction and steady state

1   **Dynamics of N response depends on N status in maize plants: Comparison between**

2   **nitrate induction and steady state**

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12   **Total word count:**   **5657**

13   Introduction: 1312

14   Materials and Methods: 936

15   Results: 1793

16   Discussion: 1219

17   Conclusion   337

18   Acknowledgments: 60

19   **Figures: 6**

20   **Tables: 1**

21

22   **Supporting Information figures:**   2

23   **Supporting Information tables:**   1

24 **Main Conclusion:**

25 Understanding the nitrate uptake system requires an integrated approach which takes into

26 account not only N availability but N status and N demand.

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28 Australian Centre for Plant Functional Genomics, Waite Research Institute, University of

29 Adelaide, Adelaide, South Australia, 5064, AUSTRALIA

ABSTRACT

As nitrate (NO_3^-) is the principal form of N available to crops in most high-input agricultural soils, understanding the NO_3^- uptake system has attracted research attention globally. A considerable proportion of the literature has focused on NO_3^- supply responses via starvation and re-supply, or switching from different N source(s) such as ammonium. These experiments are widely known as nitrate induction or the Primary Nitrate Response (PNR). In contrast, other attempts have assessed the role that N demand plays on the NO_3^- uptake system and how this changes with growth and developmental stage. To begin to understand how the data between these different experimental models relate we conducted a PNR experiment alongside a separate long term steady-state analysis and compared and contrasted the N responses. By applying different concentrations (0.5 and 5.0 mM) of NO_3^- pre-treatment we observed different plant N status (NO_3^- and amino acid concentrations) prior to starvation with 5 mM grown plants having approximately double the NO_3^- content in root and shoot and 30% lower shoot total free amino acid concentration compared to 0.5 mM plants. Plants were then subject to NO_3^- starvation where different responses to starvation were observed with a rapid decline root and shoot NO_3^- in 5 mM pre-treated plants whilst 0.5 mM plants decreased shoot free amino acids with little change in tissue NO_3^-. When the starved plants were re-induced into their original pre-treated NO_3^- growth condition, we observed both pre-treatment dependent and pre-treatment independent responses, indicating the importance of pre-treatment settings prior to the PNR. The observed putative NRT transcript profiles in response to NO_3^- re-introduction were characteristic of the typical PNR and the maximum induced transcript levels were in some cases similar to the maximum levels seen for plants grown in comparable steady state NO_3^- conditions. This hybrid analysis has provided the basis to begin to bridge the gap and highlights the complexity of the system and the importance of more integrated approaches to understanding the NO_3^- uptake system which take into account not only N availability but N status and N demand.

INTRODUCTION

Nitrogen (N) is a fundamental element for plant growth as a key building block of biological molecules. Since N is essential for plant growth and development, more than 100 million T of N fertilisers are applied annually to crops (Heffer & Prud'homme, 2013). This comes with both a significant environmental and economic cost. (reviewed by Cassman *et al.* (2002)). Cereal crops capture only 40 - 50% of the applied N fertiliser highlighting significant scope for improvement of N fertilisation strategies and development of elite germplasm with improved N uptake characteristics (Peoples *et al.*, 1995; Sylvester-Bradley & Kindred, 2009). As nitrate (NO_3^-) is the principal form of N available to crops in most high-input agricultural soils (Wolt, 1994; Miller *et al.*, 2007), understanding and improving the NO_3^- uptake system has attracted research attention globally.

We know a considerable amount about NO_3^- uptake, signalling and its regulation (Dechorgnat *et al.*, 2011; Gutiérrez, 2012; Wang *et al.*, 2012; Krapp *et al.*, 2014). To cope with variable soil concentrations plants have two NO_3^- uptake systems: a high affinity transport system (HATS) which is active when NO_3^- in the soil is low (< 250 μM); and a low affinity transport system (LATS) which operates at high soil NO_3^- concentration (> 250 μM) (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995; Garnett *et al.*, 2003). More recently however, the HATS has been shown to also be active at high NO_3^- (> 2.5 mM) which has raised questions regarding the roles and activity of each uptake system (Garnett *et al.*, 2013; Holtham *et al.*, Chapter 2). These LATS and HATS uptake systems in Arabidopsis facilitating NO_3^- uptake from the soil have been linked to the NO_3^- transporter (*NRT1*/*NPF* & *NRT2*) genes and their products NRT1.1/NRT1.2 and NRT2.1/NRT2.2/NRT2.4/NRT2.5 respectively (Huang *et al.*, 1996; Okamoto *et al.*, 2003; Li *et al.*, 2007; Tsay *et al.*, 2007; Kiba *et al.*, 2012; Léran *et al.*, 2014; Lezhneva *et al.*, 2014). Transport and storage of NO_3^- within Arabidopsis plants has then been linked to NRT1.11/1.12 (xylem-to-phloem transfer), NRT1.4 (leaf homeostasis), NRT1.5 (root xylem loading), NRT1.6 (seed loading), NRT1.7 (leaf remobilisation), NRT1.8 (xylem unloading), NRT1.9 (root phloem loading), NRT2.7 (embryo storage) (Wang *et al.* (2012);

85 (Hsu & Tsay, 2013). Multiple regulatory pathways and mechanisms have been discovered

86 with evidence suggesting that the NO_3^- uptake system is controlled at the transcriptional,

87 translational and post translational levels (reviewed by Krapp *et al.* (2014)). With all of this

88 information in hand, efforts to improve the NO_3^- uptake or utilisation of NO_3^- through

89 manipulation of transporters, assimilatory enzyme genes or other control points in the N

90 management system have had limited success to date (McAllister *et al.*, 2012).

91 A considerable proportion of the literature attempting to unravel the NO_3^- transport system

92 and its regulation describes experiments growing plants for a period without NO_3^- (starvation)

93 and then analysing the response of the plants immediately following exposure to NO_3^-

94 (induction); named the "primary nitrate response" (PNR) (Medici & Krouk, 2014). The PNR

95 was first described by Gowri *et al.* (1992) and further defined a year later by the same group

96 (Redinbaugh & Campbell, 1993). This response has since been widely used for studying and

97 understanding plant response to NO_3^- availability at the molecular and physiological levels. In

98 the PNR, HATS NO_3^- uptake capacity exhibits strong induction peaking after 6 hrs, followed

99 by repression after a period of sufficient NO_3^- in Arabidopsis, wheat, maize and barley

100 (Minotti *et al.*, 1969; Jackson *et al.*, 1973; Goyal & Huffaker, 1986; Aslam *et al.*, 1993;

101 Henriksen & Spanswick, 1993; Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et al.*,

102 2003). This pattern is consistent with the transcript level response of *NRT2.1* and *NRT2.2* in

103 barley and Arabidopsis (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et al.*, 2003) and

104 subsequent mutant analyses confirmed that these genes were indeed the major drivers of the

105 PNR (Cerezo *et al.*, 2001; Filleur *et al.*, 2001; Orsel *et al.*, 2004; Li *et al.*, 2007). The

106 induction response has been shown to involve up to 1000 genes and has consequently been

107 fruitful for discovery of genes associated with NO_3^- uptake and its regulation (Wang *et al.*,

108 2000; Wang *et al.*, 2003; Scheible *et al.*, 2004; Gutierrez *et al.*, 2007; Medici & Krouk, 2014).

109 A variety of treatments have been used to investigate the PNR (Table 1). The consensus

110 protocol involves three components, namely; germination, pre-treatment and induction. The

germination period involves germinating seed with either ammonium as the sole N source or with no N source (e.g. H_2O with or without $CaSO_4$). Pre-treatment is not employed in all studies but is used by authors as either a final N starvation period where all N is removed from the growth media, or a short pH equilibration period to condition plants to the pH difference of the final NO_3^- induction environment. Finally, the induction period involves exposing the starved plant to NO_3^- and examining the response, which has been explored at a various NO_3^- concentrations ranging from 10 μM to 20 mM (Table 1).

In contrast to the PNR approaches other attempts to understand the NO_3^- uptake system and its regulation have assessed the role that N demand plays on the NO_3^- uptake system and how this varies with growth and developmental stage. Early studies assessed relative growth rates and NO_3^- uptake kinetics using a nitrogen addition technique which gave early insight into N demand effects on the NO_3^- uptake system (Oscarson & Larsson, 1986; Oscarson *et al.*, 1989b; Oscarson *et al.*, 1989a; Mattsson *et al.*, 1991). Malagoli *et al.* (2004) measured uptake capacity of the HATS and LATS in oilseed rape throughout development and combined this analysis with field N data to develop models suggesting that the HATS may play a dominant role in total N uptake over the plant lifecycle. More recently Garnett *et al.* (2013) grew maize under both low and sufficient steady state NO_3^- conditions and demonstrated substantial demand driven variation in NO_3^- uptake across the lifecycle which correlated with the transcript levels of the *ZmNRT2.1, ZmNRT2.2 and ZmNRT2.5*. This was extended by Holtham *et al.* (Chapter 2) where they used the same steady state growth conditions and reduced NO_3^- availability during vegetative growth revealing a rapid response to reduction in nitrogen supply involving tissue NO_3^- concentration, *NRT* transcription and changes in plant organ growth.

With these two quite different approaches to understanding the NO_3^- uptake system and its regulation the question arises: how can we compare and relate the data from these studies to better understand the regulation of the NO_3^- uptake system at different developmental stages

137   in response to different levels of N supply and demand? The PNR has a reproducible response

138   which is measurable at single developmental time points with short experimental timeframes,

139   however, it lacks assessment of N demand responses which vary at different growth stages.

140   Lifecycle studies facilitate integration of N availability and N demand regulated responses,

141   but require long and intensive experiments and produce more complex datasets.

142   Understanding the relationships between these experimental models could provide key insight

143   into the complex regulation network governing the NO_3^- uptake system.

144   In this study we conducted a PNR experiment alongside a separate long term steady-state

145   analysis and compared the N responses. We assessed how different levels of NO_3^- pre-

146   conditioning affects the way plants respond to NO_3^- starvation and induction. We assessed

147   changes in plant N status in response to starvation, induction and steady state at conditions

148   through examining NO_3^- and amino acid (AA) concentration changes in different plant

149   tissues. We examined changes in transcript levels of key NO_3^- inducible genes in response to

150   NO_3^- starvation, induction and steady state at conditions to assess the relationships between

151   the transcriptional responses. Through this combined analysis of the NO_3^- uptake it was

152   revealed that understanding such as complex system requires an integrated approach which

153   takes into account not only N availability but N status and N demand.

154   **MATERIALS AND METHODS**

155   **Plant Growth**

156   Seeds of the dwarf maize (*Zea mays* var. Gaspe Flint) were pre-treated by washing thoroughly

157   with sterile MilliQ water, followed by a 5 min treatment with a combination of Captan®

158   (Farmalinx) and Spinflo® (NuFarm) fungicides at rates of 1.25 g L^{-1} and 2 ml/L^{-1},

159   respectively. Following fungicide treatment the seeds were then thoroughly washed and then

160   imbibed by soaking in sterile MilliQ water for 24 h with continuous aeration. The seeds were

161   then germinated on filter paper moistened with 0.5 mM $CaCl_2$ (3 d at 26°C in the dark). A

162   total of 80 seedlings were transferred to each of six 120 L ebb and flow hydroponic systems

with the fill/drain cycles completed in 15 min. Plants were grown on mesh collars within tubes (300 mm x 50 mm) which kept roots of adjacent plants separate but allowed free access to solution. The hydroponic system was situated in a controlled environment room with 14/10-h 25°C/20°C day/night cycle at a luminous flux density of 500 μmol m^{-2} s^{-1} at canopy level which was maintained throughout the experiment. The nutrient solution was a modified Johnson's solution (Johnson *et al.*, 1957) containing either (in mM) 0.05 NH_4^+-N, 3.05 K, 1.25 Ca, 0.5 Mg, 1.63 S, and 0.5 P for the 0 mM starvation treatment, 0.5 NO_3^--N, 3.05 K, 1.25 Ca, 0.5 Mg, 1.63 S, and 0.5 P for the 0.5 mM NO_3^- treatment or (in mM): 5 NO_3^--N, 3.05 K, 1.25 Ca, 0.5 Mg, 0.5 S, and 0.5 P for the 5 mM NO_3^- treatment. Both treatment solutions contained (in μM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and FeEDDHA). Iron was supplemented twice weekly with the addition of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (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 5.9 and 6.1. Solution NO_3^- was monitored using a NO_3^- electrode (TPS, Springwood, Australia) and maintained within 10% of target concentration. 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. Nutrient solutions were changed every 7 d.

Nitrate determination

Tissue NO_3^- content was determined via a previously published method (Cataldo *et al.*, 1975). Pre-weighed cryogenically fine-ground tissue (20 - 25 mg) was aliquoted into 1.5 ml tubes, 1 ml of deionised H_2O added, and then boiled for 20 min. Samples were then cooled on ice and the supernatant collected after centrifugation for 15 min at 12,000 x g. Supernatant samples were stored at -80°C until required. In 1.5 ml tubes 10 μl of supernatant was then mixed with 40 μl of 5% (w/v) salicyclic acid in concentrated H_2SO_4, mixed, and then incubated at room temperature for 20 min. To this 0.95 ml of 2 N NaOH was then added, mixed well, and incubated at room temperature for 20 min. For each sample 200 μl was transferred to a 96

190   well flat bottom plate (Greiner Bio-One, Vic, Australia) and absorbance was measured at 410

191   nm in a plate reader (POLARstar Optima, BMG Labtech, Germany). To determine NO_3^-

192   concentration 200 µl of KNO_3 standard samples subject to the same reaction (0 –10 mM)

193   were run on each plate and processed the same as the samples above. Nitrate content was

194   expressed as µmol of NO_3^- per g of tissue FW.

195   **Amino acid determination**

196   Tissue AA was determined using liquid chromatography electrospray ionization-mass

197   spectrometry as described by Broughton *et al.* (2011) once the samples had been derivatised

198   following the method of Cohen and Michaud (1993).

199   **Quantitative real time PCR**

200   For steady state plants, on sampling days root material was harvested between 11:00 and

201   13:00 h, whereas induced plants were harvested at 9:00, 9:30, 10:00, 12:00 and 15:00 h

202   (photoperiod started at 6:00). The whole root was excised and snap-frozen in liquid nitrogen

203   and stored at -80°C. Homogenous fine-ground frozen root tissue (100 mg) was added to 1 ml

204   TRIzol-like reagent; containing 38% (v/v) phenol (equilibrated pH 4.3, Sigma-Aldrich,

205   Australia), 11.8% (w/v) guanidine thiocyanate, 7.6% (w/v) ammonium thiocyanate, 3.3%

206   (v/v) sodium acetate (3 M, pH 5), 5% (v/v) glycerol and made up to 100% (v/v) with MQ-

207   H_2O. Extraction of RNA was performed using the method of (Chomczynski, 1993). Extracted

208   RNA was then DNase treated (Ambion, USA), according to the manufacturer's instructions.

209   RNA integrity was checked on a 1.2% (w/v) agarose gel. cDNA synthesis was performed on

210   1 µg of total RNA with oligo(dT)$_{19}$ using SuperScript III reverse transcriptase (Invitrogen,

211   Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR

212   (Q-PCR) was carried out as outlined in Burton *et al.* (2008). In this method, the amount of

213   each amplicon in each cDNA is quantified with respect to a standard curve of the expected

214   amplicon (typically, PCR efficiencies ranged between 0.85 and 1.05). Four control genes

215   (*ZmGaPDh*, *ZmActin*, *ZmTubulin* and *ZmElF1*) were utilised for the calculation of the

216 normalisation factor. Q-PCR normalisation was carried out as detailed in Vandesompele *et al.*

217 (2002) and Burton *et al.* (2004). Q-PCR primers for all *NRTs* were as per Garnett *et al.*

218 (2013), whereas primers for *NRs* and *NiRs* were as per Supplementary Table 1. All running

219 conditions were as per Garnett *et al.* (2013). Q-PCR products were verified by sequencing,

220 agarose gel electrophoresis and melt-curve analysis to confirm a single PCR product was

221 being amplified.

222 **Statistical analyses**

223 The experiment was designed with three independent replicate growth systems for each

224 treatment and plants were randomly harvested. There was no statistical difference for all

225 measured parameters between the replicate systems. All statistical analyses within this study

226 were carried out using two-way analysis of variance (ANOVA) unless otherwise described.

227 **RESULTS**

228 **Biomass**

229 Post germination, plants were grown for 13 d under steady state NO_3^- conditions of 0.5 mM

230 and 5 mM and then subject to 0 mM starvation period for 2 d. On the 15th d, 3 h into the

231 photoperiod, plants were then subject to an induction time course where plants were then re-

232 introduced back into their original NO_3^- treatment (induced) for either 0.5, 1, 3 or 6 h before

233 harvesting. During this induction period some plants were maintained under the 0 mM

234 conditions and harvested at the same time as the induced plants as a control. Prior to

235 starvation 0.5 mM plants had an approximately 30% larger root to shoot ratio than 5 mM

236 plants (*c.* av 0.45 ± 0.01 and 0.32 ± 0.01 for 0.5 mM and 5 mM treatments, respectively, *P* <

237 0.001) due to larger roots (*c.* av 1.237 ± 0.185 g FW/plant and 0.836 ± 0.070 g FW/plant for

238 0.5 mM and 5 mM treatments respectively) as the shoots were approximately the same size.

239 At the end of the starvation period prior to induction, 0.5 mM plants had a 38% larger root to

240 shoot ratio (*c.* av 0.40 ± 0.01 and 0.29 ± 0.01 for 0.5 mM and 5 mM treatments, respectively,

241 *P* < 0.001).

Nitrate

Plants pre-conditioned under 0.5 mM maintained a constant shoot NO_3^- concentration during the NO_3^- starvation period (48 h - 0 h prior to induction), however, root NO_3^- concentration decreased in response to starvation (*c.* 44 to 20 µmoles g^{-1} FW) (Fig. 1a, b). Prior to starvation 5 mM plants had double the NO_3^- concentration in both the root and shoot compared to 0.5 mM plants (Fig. 1). In response to NO_3^- starvation, 5 mM plants exhibited a decrease in NO_3^- concentration of 59 and 52% for root and shoot, respectively (Fig. 1c, d). When LN plants were subject to NO_3^- induction there was a sharp decrease in shoot tissue within 30min compared to the 0 mM control. In root tissue, in response to NO_3^- induction 0.5 mM plants maintained a relatively constant NO_3^- concentration compared to the 0 mM control plants which decreased after 4 hrs into the photoperiod in line with the 1 h post induction point (10:00am) and then maintained at a low level for the remainder of the induction window. Contrasting these results to plants maintained under steady state 0.5 mM (SS(0.5 mM)) conditions the NO_3^- concentrations showed a gradual decline in both root and shoot tissue and NO_3^- concentration was similar to the starved and re-induced plants (Fig. 1a, b). In response to induction 5 mM plants exhibited an increase in the shoot at 30 min and then maintained a steady level for the rest of the induction period comparable to the 0 mM control (Fig. 1c). However, an increase in NO_3^- concentration was observed in roots of 5 mM induced plants compared to the 0 mM control after 3 h (Fig. 1d). Plants maintained under steady state 5 mM conditions maintained a substantially higher NO_3^- concentration compared to the starved and induced plants (Fig. 1 c, d).

Amino Acids

Prior to starvation root total free amino acid concentration (TAA) was similar between 5 mM and 0.5 mM plants whilst shoot TAA was 3 times higher in 0.5 mM plants (Fig. 2). In response to starvation (48 h – 0 h prior to induction) both root and shoot TAA did not change in 5 mM plants, whereas 0.5 mM plants exhibited a decrease in shoot TAA whilst root TAA remained relatively constant around 5 µmoles g^{-1} FW (Figs. 2 a, b). In response to NO_3^-

269   induction the 0.5 mM plants had no difference in root TAA compared to the 0 mM control,

270   both fluctuating around 5 (μmoles g^{-1} FW) in line with SS(0.5 mM) plants. In the shoot

271   however, the 0.5 mM plants showed an increasing trend to 3 h post induction compared to the

272   0 mM control plants which showed a decreasing trend over the induction period (Fig. 2a). For

273   5 mM plants in the shoot there was no observed difference between 5 mM and the 0 mM

274   control, however, both treatments increased TAA within the first 30 min post induction,

275   higher than plants maintained under 5 mM steady state conditions (SS(5 mM)) (Fig. 2c).

276   From 3 h post induction the roots of 5 mM plants showed an increasing trend towards the

277   TAA concentrations observed for the SS(5 mM) plants, whilst 0 mM control plants exhibited

278   a slow decline over the induction period (Fig. 2d).

279   The AA in highest concentration in both root and shoot tissue were glutamine, glutamate,

280   alanine, asparagine and aspartate (Figs. 3, 4). Serine was also high but only in shoot tissue

281   (Fig. S3). In response to NO_3^- starvation, regardless of prior treatment (i.e. 0.5 mM or 5 mM)

282   plants exhibited an increase in root arginine, isoleucine, leucine, histidine, phenylalanine,

283   homoserine, tryptophan, lysine and ornithine concentration (Fig. 3). Another major trend in

284   the root in response to starvation was an increase in concentration for 0.5 mM plants with no

285   change in 5 mM plants (Fig. S2). In the shoot, in response to starvation for 0.5 mM plants we

286   observed a decrease in all AA with the exception of isoleucine, gamma-amino butyric acid,

287   homoserine and ornithine, whereas the 5 mM plants showed little to no change (Fig. 4, S3).

288   One dominant response to NO_3^- induction in the root was increased AA concentration in 5

289   mM plants only (alanine, glutamine and citruline after 6, 3 and 3 h, respectively) (Fig. 3).

290   There were also two other significant changes in the root with arginine in both 0.5 mM and 5

291   mM treatment decreasing in response to NO_3^- induction compared to the 0 mM control, and

292   the other whereby proline decreased in 0.5 mM only (Fig. 3). In the shoot there was one main

293   observed response to NO_3^- induction whereby alanine, citruline and ornithine increased for

294   0.5 mM treated plants only (Fig. 4). Examining the individual AA concentration differences

between the SS(0.5 mM) and SS(5 mM) plants compared to 0.5 mM and 5 mM induced there were two main responses. In root tissue 10 out of the 25 AAs were higher in both 0.5 mM and 5 mM induced plants compared to SS(0.5 mM) and SS(5 mM) (Fig. 3, S2). In shoot tissue 66% of the AAs exhibited lower AA concentration for 0.5 mM induced plants compared to SS(0.5 mM) whilst 5 mM were higher than SS(5 mM) for the same AAs (Fig 4, S3).

Nitrate Reductase and Nitrite Reductase

Transcript levels of both nitrate reductase (NR) and nitrite reductase (NiR) genes were examined as they have been widely characterised for their classic response to NO_3^- induction. All the measured *NRs* and *NiRs* with the exception of *NR027* had a maximum transcript level at 1 h post induction which then declined at 3 h (Fig. 5).The *NR027* gene also increased to 1 h, but peaked at 3 h and didn't decline until 6 h post induction (Fig. 5). Peak transcript levels were higher for *NRs/NiRs* in 0.5 mM compared to 5 mM (*c.* on average 32% higher) with the exception of *NR027* which was equal in both treatments (Fig. 5). For the 0 mM control we observed little change throughout the induction period with the exception of a short peak after 30 min post NO_3^- induction for *NR027* (Fig. 5).

NRT transcript levels

Based on the responses of maize *NRT* transcripts to N supply and demand published by Garnett *et al.* (2013), a subset of putative *NRTs* were selected for examination in this study. For both 0.5 mM and 5 mM plants *ZmNRT3.1A* was most highly represented in the total RNA pool compared to the *NRT2* and *NRT1* genes examined (Fig. 6a, b). This was closely followed by the putative HATS genes *ZmNRT2.1* and *ZmNRT2.2* with approximately 1/10 and 1/2 lower peak transcript levels than *ZmNRT3.1* respectively (Fig. 6c, d, e, f).

In response to starvation (48 h – 0 h prior to induction) the observed changes were much smaller than the induced responses but were significant for many genes. Transcript levels of *ZmNRT1.1A* declined during starvation in both 0.5 mM and 5 mM plants (67% and 80% decrease respectively) (Fig. 6g, h). Both *ZmNRT2.1* and *ZmNRT2.2* exhibited change in

response to starvation for 0.5 mM plants only, although in opposite directions whereby *ZmNRT2.1* increased (22% increase) whilst *ZmNRT2.2* decreased (31% decrease) (Fig. 6c, e). Conversely for *ZmNRT2.5* and *ZmNRT1.1B* we only observed changes for 5 mM plants during the starvation period with an increase in *ZmNRT2.5* (48 fold increase) and a large drop in transcript levels for *ZmNRT1.1B* (84% decrease) (Fig. 6j, l).

Examining the speed of response to NO_3^- induction, most genes responded transcriptionally within 1 h post induction, however, assessing response in comparison to plants maintained under 0 mM control conditions *ZmNRT2.2* and *ZmNRT2.5* responded faster within 30 min in both 0.5 mM and 5 mM. A similar rapid increase was also observed for *ZmNRT1.1B* and *ZmNRT2.1* most pronounced in 5 mM plants. The induction response for the *NRT1s* were less influenced by their pre-treatment compared to the *NRT2s* with the average peak transcript difference between 0.5 mM and 5 mM plants being 19 and 65% for the *NRT1s* and *NRT2s*, respectively. It was observed that the 0 mM control transcript levels were quite variable for some *NRTs* with *ZmNRT1.1A*, *ZmNRT1.5* and *ZmNRT2.5* exhibiting increases throughout the photoperiod.

The maximum transcript level was recorded after 1 h post induction for all measured *NRTs* in both treatments with the exception of *ZmNRT3.1A* for 5 mM plants (Fig. 6). This peak transcript level was higher for all *NRTs* in 0.5 mM compared to 5 mM (*c.* on average 60% higher) with the exception of *ZmNRT1.1B* and *ZmNRT1.5A* which were similar in both treatments (Fig. 6k, l). Plants maintained under SS(0.5 mM) and SS(5 mM) conditions generally maintained relatively low constant levels of expression compared to 0.5 mM and 5 mM induced plants. To further compare induced versus demand driven transcription, the data was contrasted against our parallel study where we assessed *NRT* transcription over the entire vegetative growth period for plants maintained under steady state 5 mM and 0.5 mM conditions (Holtham *et al.*, Chapter 2). In order to draw a comparison, we assessed the maximum transcription level recorded over the vegetative growth period by Holtham *et al.*

347   (Chapter 2) against the maximum expression peak for NO_3^- starved and induced plants. The

348   0.5 mM induced peak was higher than the 5 mM highest steady state vegetative growth

349   transcript level (indicated with dotted red lines in the figures) for *ZmNRT2.1, ZmNRT1.1A*,

350   and *ZmNRT1.1B*, (*c.* 28, 37, and 86% higher, respectively) (Fig. 6c, g, k). For 5 mM plants

351   the induced peak was higher than the 5 mM steady state expression high for *ZmNRT3.1*,

352   *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT2.5* and *ZmNRT1.5* (Fig. 5b, d, f, i, m).

DISCUSSION

Effect of nitrate starvation

355   Plants pre-conditioned at 5 mM had lower shoot TAA but accumulated over 2 fold higher

356   NO_3^- concentrations in both root and shoot tissue compared to 0.5 mM plants. When 5 mM

357   plants were starved, both root and shoot NO_3^- concentration decreased rapidly with no change

358   to TAA levels. It has been widely reported that plants rapidly use NO_3^- from vacuolar stores

359   to meet the needs required for growth in the absence of available NO_3^- (Jackson & Volk,

360   1981; Mackown, 1987; Macduff *et al.*, 1989; van der Leij *et al.*, 1998; Richard-Molard *et al.*,

361   2008). In contrast, plants preconditioned at 0.5 mM showed an inverse N status whereby root

362   and shoot NO_3^- concentration was low and shoot TAA was high. This high concentration of

363   shoot TAA has been reported previously in response to persistent N starvation and described

364   as a product of leaf senescence (via nucleic acid and protein breakdown) facilitating N

365   remobilisation to developing tissues (Schulze *et al.*, 1994; Crafts-Brandner *et al.*, 1998;

366   Masclaux *et al.*, 2000; Hörtensteiner & Feller, 2002). The 0.5mM pre-conditioned plants had

367   a small amount of NO_3^- in the roots therefore to meet growth requirements it appears they

368   utilised the accumulated free AAs in the shoot. This decrease in shoot AAs in response to

369   starvation was also reported by Krapp *et al.* (2011) where they observed a comparable 61%

370   decrease within 3 d post starvation in Arabidopsis.

371   Analysis of the individual AAs revealed both pre-treatment specific and independent

372   responses to starvation. The main pre-treatment independent response to starvation was the

373 increases in root concentrations of arginine, isoleucine, leucine, histidine, phenylalanine,

374 homoserine, tryptophan, lysine and ornithine regardless of NO_3^- pre-treatment (Fig. 3).

375 Concentration changes for these AAs were not complimented by a corresponding shoot

376 decrease so it is unlikely that this is reflective of shoot AAs being transported from shoot to

377 root. It is therefore possible that these AAs were assimilated in the root, an observation which

378 is supported by the decrease in root NO_3^- concentration for both 0.5 mM and 5 mM plants.

379 This root AA increase in response to NO_3^- starvation was also recorded by Krapp *et al.* (2011)

380 in Arabidopsis. By assessing the contribution of AAs to the total N pool, Richard-Molard *et*

381 *al.* (2008) concluded that in their study NO_3^- was most important for the starvation response

382 but highlighted that AAs and protein pools may have a more significant role when NO_3^-

383 reserves are scarce. That statement appears to hold true here where under 0.5 mM NO_3^-

384 conditions the AAs appeared to play a prominent role with large changes in the shoot during

385 starvation.

386 **Effects of nitrate induction**

387 Following NO_3^- induction, plants pre-treated with 0.5 mM were able to maintain root and

388 shoot NO_3^- concentration. These same 0.5 mM plants maintained a relatively constant level of

389 root TAA but demonstrated an increasing trend in shoot TAA. In contrast to this 5 mM plants

390 showed accumulation of NO_3^- and an increase in TAA in the root. In shoot tissue, both 0.5

391 mM and 5 mM treatments exhibited increasing trends for alanine, glutamine, asparagine and

392 glycine in response to NO_3^- induction (Figs. 4, S3). This increase in shoot AA levels for both

393 0.5 mM and 5 mM plants was also observed by Scheible *et al.* (2004) where they reported an

394 increase in the central AAs (glutamine, glutamate, alanine and asparagine) 3 h post induction.

395 Comparing between the 0.5 mM and 5 mM response it was interesting that root NO_3^-

396 concentration did not increase beyond the SS(0.5 mM) and SS(5 mM) NO_3^- concentration

397 levels. With rapid induction of *NR* and *NiR* this may be reflective of rapid reduction and/or its

398 quick translocation to the shoot via the xylem (Marschner & Marschner, 2012).

399   The transcriptional *NRT* induction responses were common between both treatments (i.e. 0.5

400   mM and 5 mM). It was observed that all *NRTs* showed maximum expression within 1 h post

401   induction, followed by a decrease (Fig. 6). This same pattern was reflected in the

402   transcriptional changes of the *NRs* and *NiRs* (Fig. 5). This quick induction and repression

403   profile supports the classic transcriptional primary NO_3^- response that is widely reported

404   (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000a; Okamoto *et al.*, 2003; Medici & Krouk, 2014).

405   Although they had the same characteristic profile, the transcript response was generally 50%

406   higher in plants pre-conditioned at 0.5mM. The PNR induction and suppression patterns have

407   been previously explained by a negative feedback system where N assimilates (particularly

408   glutamate, glutamine, aspartate and asparagine) suppress *NRT* transcription (Zhuo *et al.*,

409   1999; Vidmar *et al.*, 2000b; Gansel *et al.*, 2001; Nazoa *et al.*, 2003; Miller *et al.*, 2008). Here

410   we did not see such strong evidence to support this, with only a slight inverse correlation

411   between *NRT* transcript levels and alanine and glutamine concentration in root and shoot

412   tissue. However, transcript levels were correlated with the difference in root NO_3^-

413   concentration with approximately 50% higher NO_3^- concentration in 5 mM pre-conditioned

414   plants between 30 min to 1 h (Figs. 1d, 6). It has been reported that NO_3^- itself can modify

415   transcript levels of genes involved in the NO_3^- uptake system (Stitt, 1999; Wang *et al.*, 2000;

416   Ho *et al.*, 2009; Vidal *et al.*, 2010). In further support of the link between NO_3^- and *NRT*

417   transcript levels, an inverse correlation has been demonstrated in lifecycle analysis studies of

418   maize with higher NO_3^- concentration correlating with lower *NRT* transcript levels (Garnett *et*

419   *al.*, 2013; Holtham *et al.*, Chapter 2).

420   By comparing the magnitude of the demand driven transcriptional responses with the supply

421   responses observed in the PNR scenario it was thought that it may be possible to determine

422   the putative *NRT* transcription limits of the plant. To assess this we compared the

423   transcriptional peaks that were reached in response to induction, to the maximum

424   transcription level that was measured over the entire vegetative growth period (Holtham *et al.*,

425   Chapter 2). For *ZmNRT1.5*, *ZmNRT2.2* and *ZmNRT3.1* we found that the induced

transcriptional peak was similar to the maximum steady state transcriptional level, indicating that this may be the maximum transcriptional capacity for these genes. This also highlights that for these genes the magnitude of the demand driven transcriptional response to steady state NO_3^- conditions is similar to the induced PNR transcriptional responses what has not been shown before. In contrast to this the steady state maximum transcriptional level was exceeded in the induced response for genes *NRT1.1A*, *NRT1.1B* and *NRT2.1* putatively indicating that there is more transcriptional capacity for these three genes that was not utilised under the steady state 0.5 mM conditions examined in Holtham *et al.* (Chapter 2). One factor which is commonly overlooked within the PNR literature is the response of the non-induced control plants which is typically subtracted from the induced results. These plants are commonly treated with a KCl concentration appropriate to match the ionic change of the NO_3^- induction treatment (Table 1). In this experiment K^+ and Cl^- concentrations in the nutrient solution were made equal between the 0 mM control and the induced NO_3^- treatments with the only difference being the N concentration. With this approach we found that there were marked changes in *NRT* transcript levels (most pronounced for *NRT1.1A*, *NRT1.5* and *NRT2.5*) and AA concentrations throughout the induction period in the 0 mM control. This could be either a diurnal or a stress response, and further investigations are underway.

CONCLUSION

In the soil, plants experience a continual fluctuation of available NO_3^- ranging from micro- to millimolar concentrations due to spatial heterogeneity of N and soil water content (Wolt, 1994; Miller *et al.*, 2007). The PNR is commonly assessed in plants which are established under growth conditions containing zero NO_3^- which is unlikely to reflect how plants are grown in the field (Table 1). Similarly, steady state NO_3^- conditions are unlikely to be encountered in an agricultural setting. The NO_3^- uptake system is complex involving a plethora of interacting internal and external environmental signals which are constantly monitoring NO_3^- supply and demand and determining how the plant should respond (Stitt, 1999; Krapp *et al.*, 2014). With such a fluctuating field environment the challenge is to study

and integrate how plants manage these variable conditions from: different bases of internal N status; different N demand levels and their associated developmental points; different external availability of NO_3^-. By examining NO_3^- and AA concentrations we observed different plant N status prior to starvation by applying different concentrations of NO_3^- pre-conditioning, and subsequently we observed different adaptive responses to starvation. After monitoring the starvation response, plants were then re-introduced back into their original pre-treated NO_3^- growth condition where we observed an array of responses, some which were pre-treatment dependant and some which were pre-treatment independent.

Interesting observations have been made when assessing the response to change in NO_3^- supply from a steady state base (Garnett *et al.*, 2013; Holtham *et al.*, Chapter 2). However, the complexity and importance is not well understood by the broader scientific community due to the focus on the PNR. Understanding how these long term steady state studies relate to the vast PNR literature may be crucial to combining advances made by both research approaches. Here we have presented a hybrid analysis to begin to bridge the gap and have presented data which highlights that understanding the NO_3^- uptake system requires an integrated approach which takes into account not only N availability but N status and N demand.

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FIGURES

Table 1. Highly cited primary nitrate response literature and their experimental growth and treatment conditions. Abbreviations: NR = Not reported, WT =

Wild Type.

Genotype	Germination			Pre-treatment			Induction				Citation
	Treatment	pH	Duration (days)	N supply	pH	Duration	N supply	Control	pH	Duration	
Zea mays L. *var* W64A X W182E	NR	NR	NR	Nitrogen free medium	NR	2 hr	50 mM NH4NO3	NR	NR	8 h	Gowri *et al.* 1992
Zea mays L. var W64A X W182E	0.1 mM CaSO₄	NR	2	50 µg/ml CHX (Cyclohexamine)	NR	1hr	10 mM KNO₃ / 0.01 mM KNO₃ / 0.10 mM KNO₃ / 1 mM KNO₃ / 10 mM KNO₃	None	NR	16 h / 2 h	Redinbaugh & Campbell 1993
Barley (*Herdeum vulgare* L. var CM-72)	0.2 mM CaSO₄	NR	6	Nitrogen free medium	NR	1 day	1 µM NaNO₃ / 1 µM NaNO₂ / 10 µM NaNO₃ / 10 µM NaNO₂	None	NR	24 h	Aslam *et al.* 1993
Barley (*Herdeum vulgare* L. cv Prato)	500 µM CaSO₄	NR	1	300 µM KNO₃ + 200 µM Ca(NO₃)₂ + 100 µM NH₄H₂PO₄ then Nitrogen free medium then 150 µM (NH₄)₂SO₄ or 300 µM Gln or 300 µM Glu or 0 mM	NR	3 / 2 day / 12 hr	100 µM Ca(NO₃)₂	None	NR	12 h	Henriksen & Spanswick 1993
Arabidopsis thaliana (Col)	2mM KNO₃	5.7	2	1 mM Ammonium citrate	NR	3 week	1 mM KNO₃ / 2 mM KNO₄ / 1 mM NH₄KNO₃	None	NR	3 h	Zhou *et al.* 1999

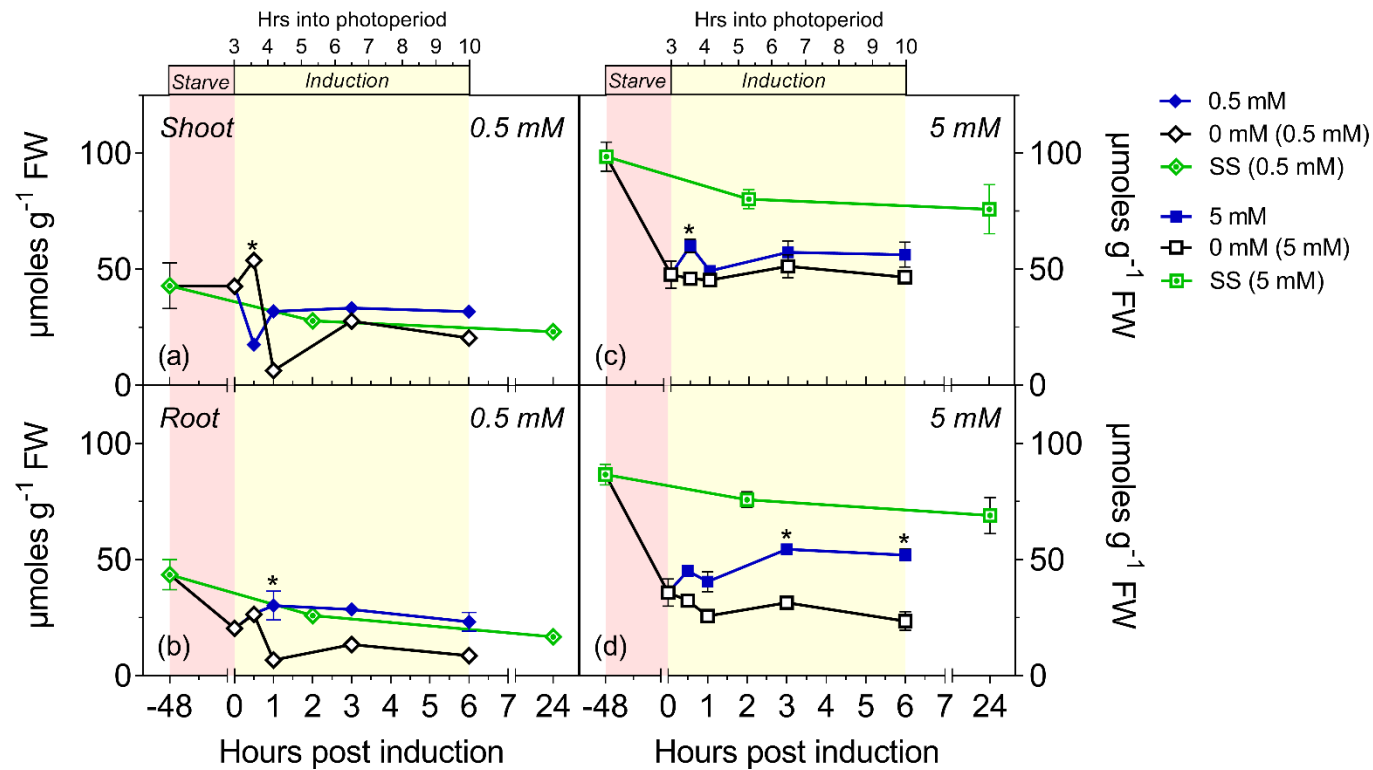**Table 1 continued.** Highly cited primary nitrate response literature and their experimental growth and treatment conditions. Abbreviations: NR = Not
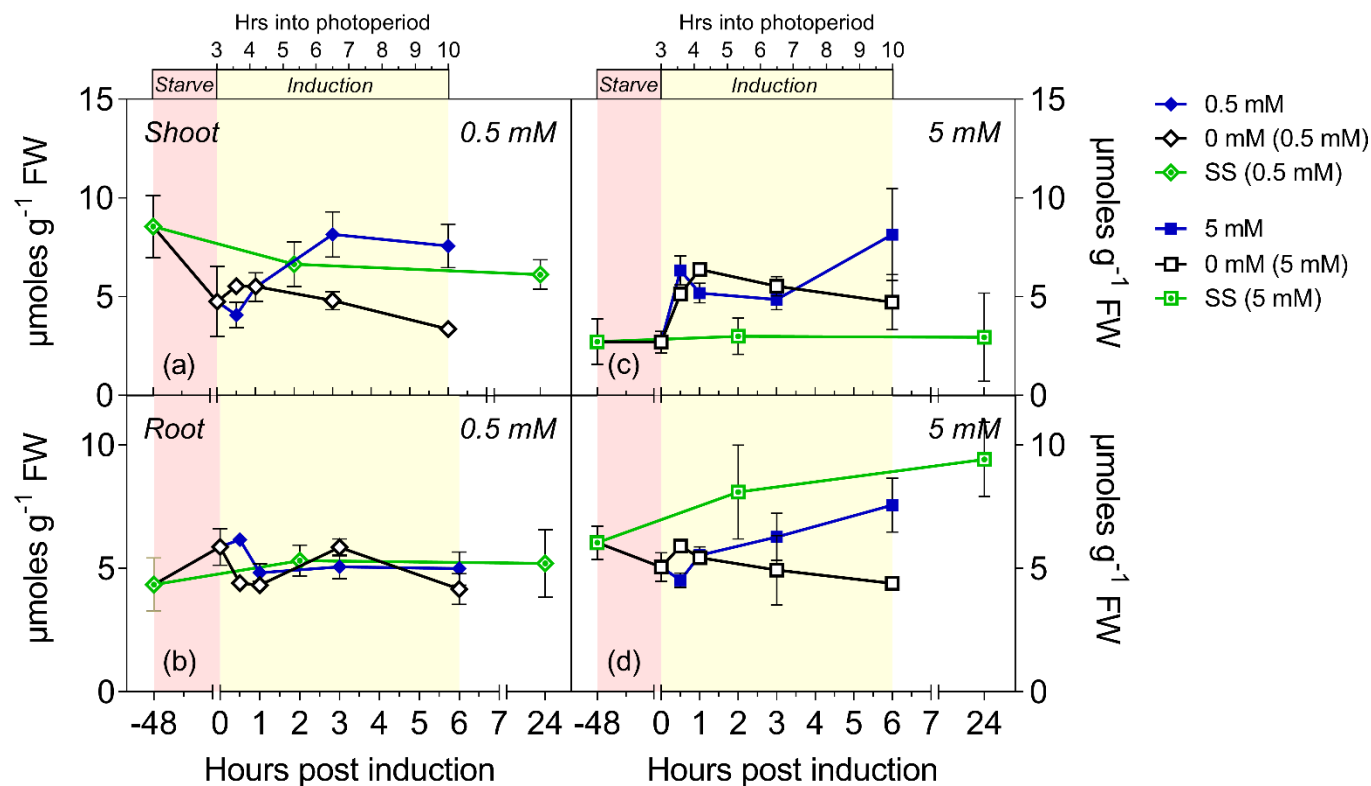
reported, WT = Wild Type

Species	Pre-growth	pH	Age	Pre-treatment	pH	Duration	Treatment	Control	pH	Time	Citation
Barley (*Hordeum vulgare* cv Klondike)	H_2O	NR	3	Nitrogen free nutrient solution	NR	≥4 days	1 mM KNO_3 / 10 mM KNO_3	None	6.2	48 h	Vidmar *et al.* 2000
Arabidopsis thaliana (WT)	10mM $(NH_4)_2$ succinate	NR	10	No pre-treatment	-	-	0.25 mM KNO_3 / 5 mM KNO_3 / 10 mM KNO_3	0.25 mM KCl / 5 mM KCl / 10 mM KCl	NR	2 h	Wang *et al.* 2000
Arabidopsis thaliana (Col)	NR	NR	3-5	0.5 mM NH_4NO_3 then Nitrogen free medium	NR / NR	5 weeks / 1 week	0.5 mM $Ca(NO_3)_2$	None	NR	3-72 h	Okamoto *et al.* 2003
Arabidopsis thaliana (Col)	2.5 mM $(NH_4)_2$ succinate	6.5	10	None	-	-	250 μM KNO_3	250 μM KCl	NR	20 min	Wang *et al.* 2003
Arabidopsis thaliana (Col)	2 mM KNO_3 + 1 mM NH_4NO_3	5.8	7	2 mM KNO_3 + 1 mM NH_4NO_3 or 0.1 mM KNO_3 + 50 μM NH_4NO_3	5.8	2 days	3 mM KNO_3	3 mM KCl	NR	3 h	Scheible *et al.* 2004
Arabidopsis thaliana (Col)	2.5 mM $(NH_4)_2$ succinate	6.5	10	No pre-treatment	-	-	250 μM KNO_3	250 μM KCl	NR	20 min	Wang *et al.* 2004
Arabidopsis thaliana (ecotype Columbia) + mutants	2.5 mM $(NH_4)_2$ succinate	6.5	9	Nitrogen free medium	NR	24hrs	250 μM KNO_3 / 250 μM KNO_2	250 μM KCl	NR	20 min	Wang *et al.* 2007
Arabidopsis thaliana (WT)	12.5 mM $(NH_4)_2$ succinate	6.5	10	12.5 mM $(NH_4)_2$ succinate	5.5	3hrs	25 mM KNO_3 / 0.25 mM KNO_3	25 mM KCl / 0.25 mM KCl	5.5	2 h	Hu *et al.* 2009
Arabidopsis thaliana (Col)	10 mM $(NH_4)_2$ succinate	6	10	No pre-treatment	-	-	0.25 mM KNO_3 / 5 mM KNO_3 / 10 mM KNO_3	0.25 mM KCl / 5 mM KCl / 10 mM KCl	NR	2 h	Wang *et al.* 2000
Arabidopsis thaliana (Col)	12.5 mM $(NH_4)_2$ succinate	6.5	10	12.5 mM $(NH_4)_2$ succinate then fresh 12.5 mM $(NH_4)_2$ succinate	5.5 / 5.5	16hrs / 3 hrs	25mM KNO_3	25mM KCl	5.5	16 h	Ho *et al.* 2009
Arab Col-8 & mutants	6 mM NO_3^- (non-limiting)	NR	21	Nitrogen free medium	NR	5 days	6 mM NO_3^-	None	NR	2 h	Castaings *et al.* 2009
Arabidopsis thaliana (ecotype Columbia) & mutants	4 mM KNO_3	5.8	7	Nitrogen free medium	5.8	2days	3 mM KNO_3	3 mM KCl	NR	3 h	Rubin *et al.* 2009
Various Arabidopsis mutants	2.5 mM $(NH_4)_2$ succinate	6.5	4-5	Nitrogen free medium	NR	1 day	0.2 mM KNO_3 / 1 mM KNO_3 / 5 mM KNO_3 / 10 mM KNO_3 / 20 mM KNO_3	0.2 mM KCl / 1 mM KCl / 5 mM KCl / 10 mM KCl / 20 mM KCl	NR	2 h	Wang *et al.* 2009

Figure 1. Root and shoot NO_3^- concentration for dwarf (*Zea mays)* Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot),

steady state 5 mM (green line hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown

plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM

NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n =

3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

Figure 2. Root and shoot total amino acid (TAA) concentration for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

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Figure 3. Root individual amino acid (AA) concentrations for dwarf (*Zea mays)* Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

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Figure 3 continued. Root individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green line hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

Figure 4. Shoot individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

122

Figure 4 continued. Shoot individual amino acid (AA) concentrations for dwarf (*Zea mays)* Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).
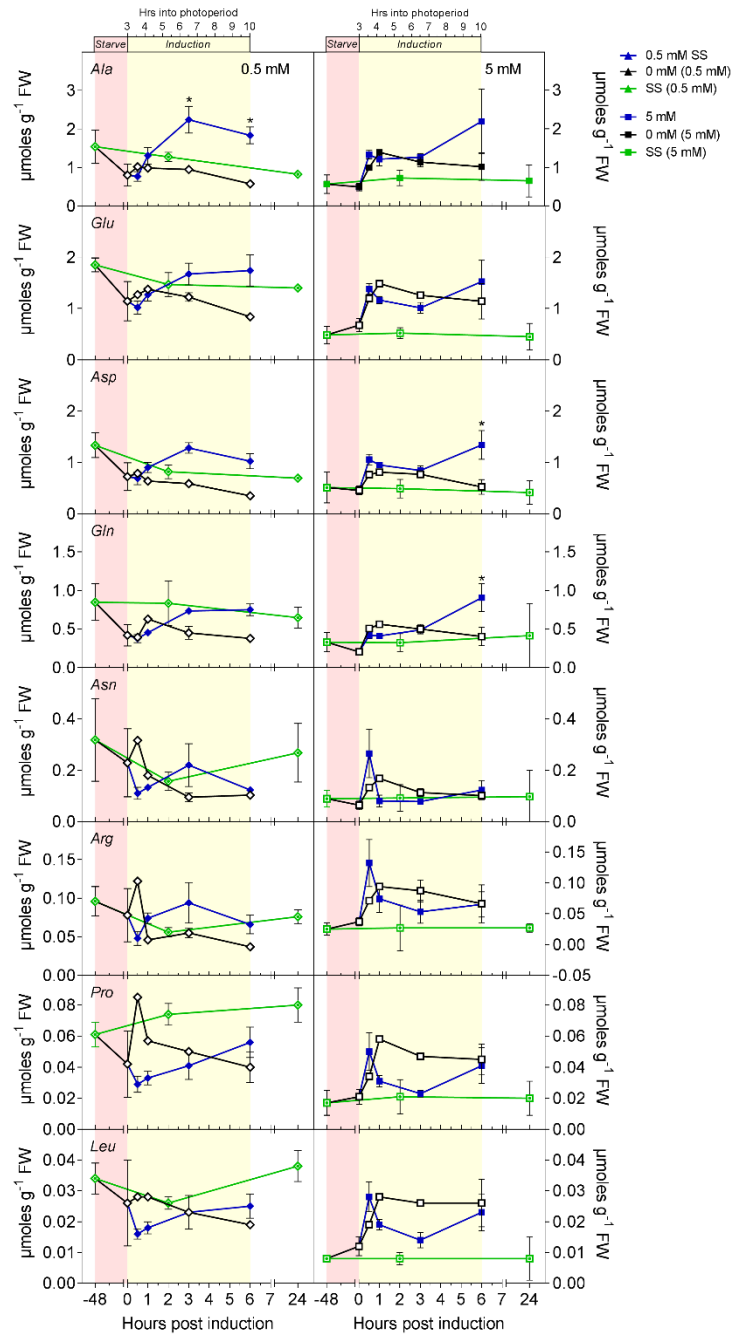
123

Figure 5. Root transcript levels of various putative nitrate (NR) and nitrite (NiR) reductase enzyme genes dwarf (*Zea mays)* Gaspe Flint plants grown under 0.5 mM and subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are $\pm$ SEM (n = 4). Values for NO_3^- starved are $\pm$ SEM (n = 1-3). Values for NO_3^- induced are $\pm$ SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).
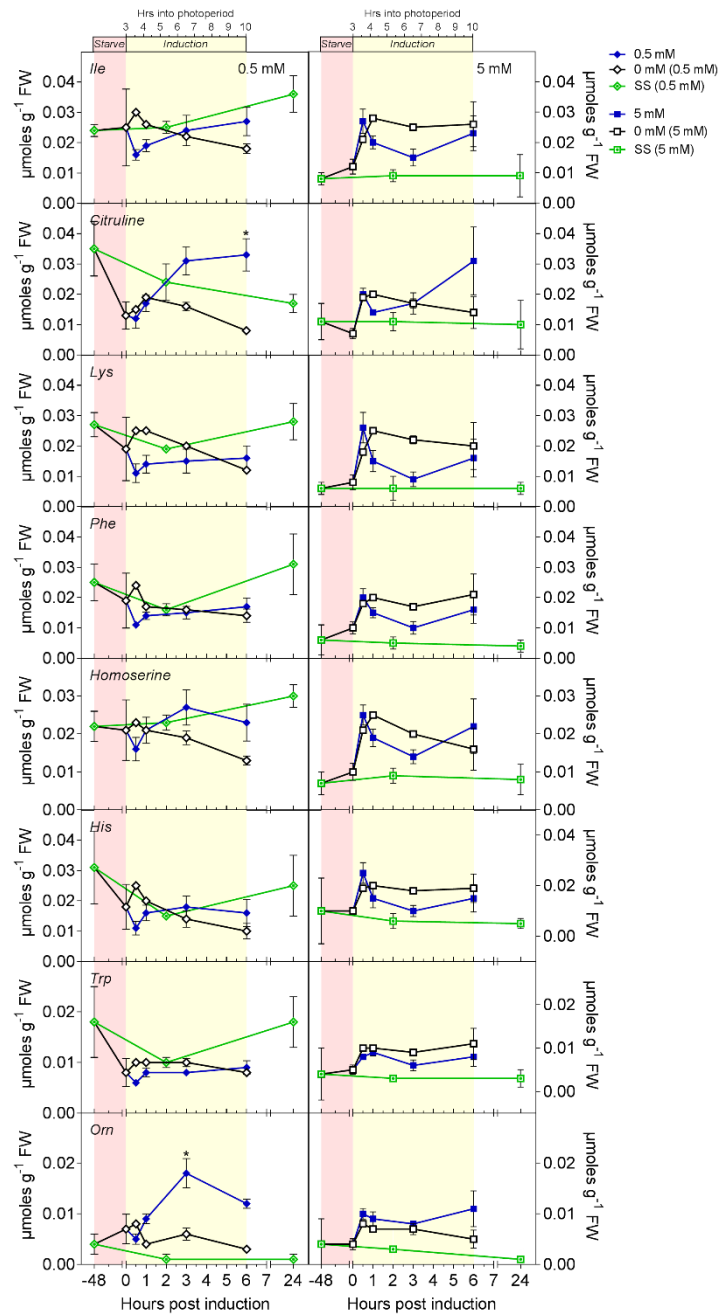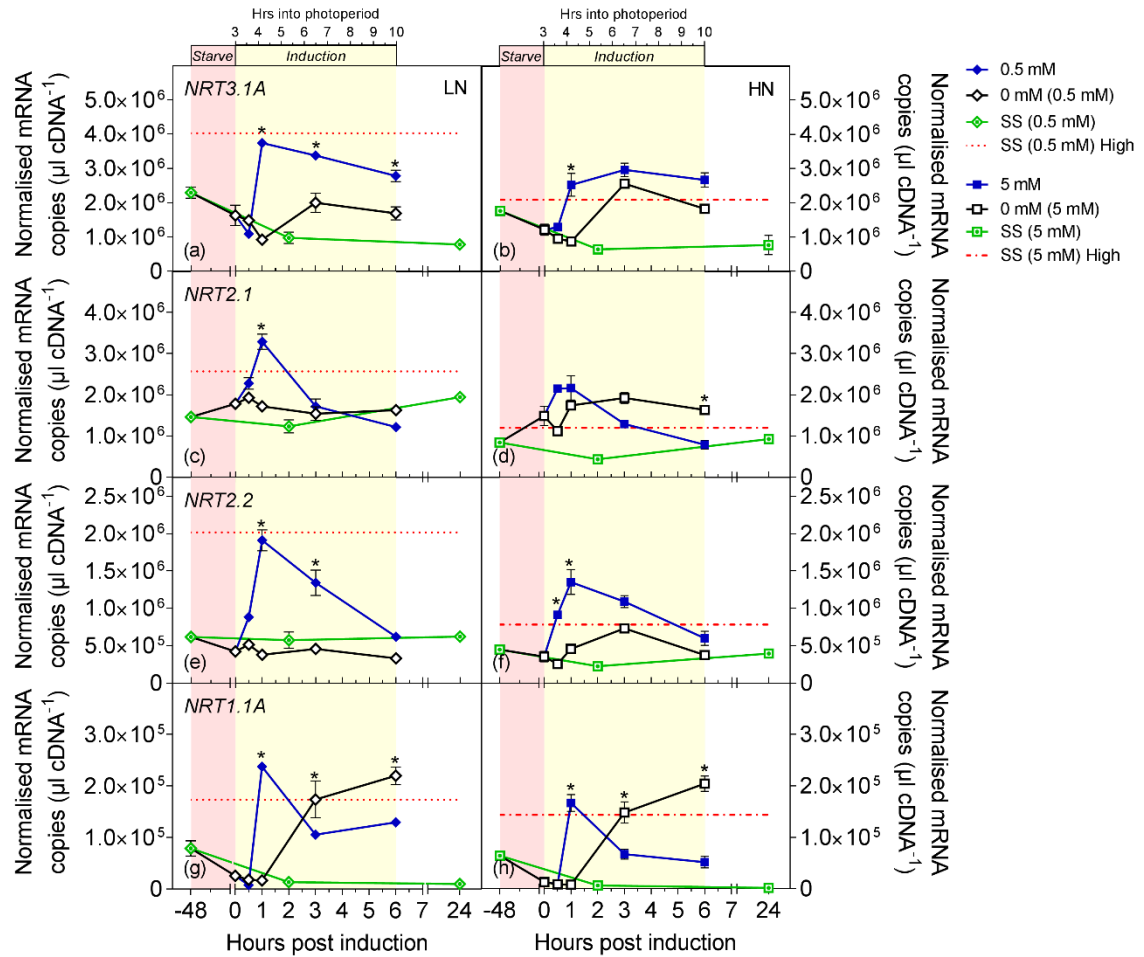
Figure 6. Shoot individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot) (highest transcript reading = red dotted line), steady state 5 mM (green hollow squares with dot) (highest transcript red = dashed and dotted line), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).
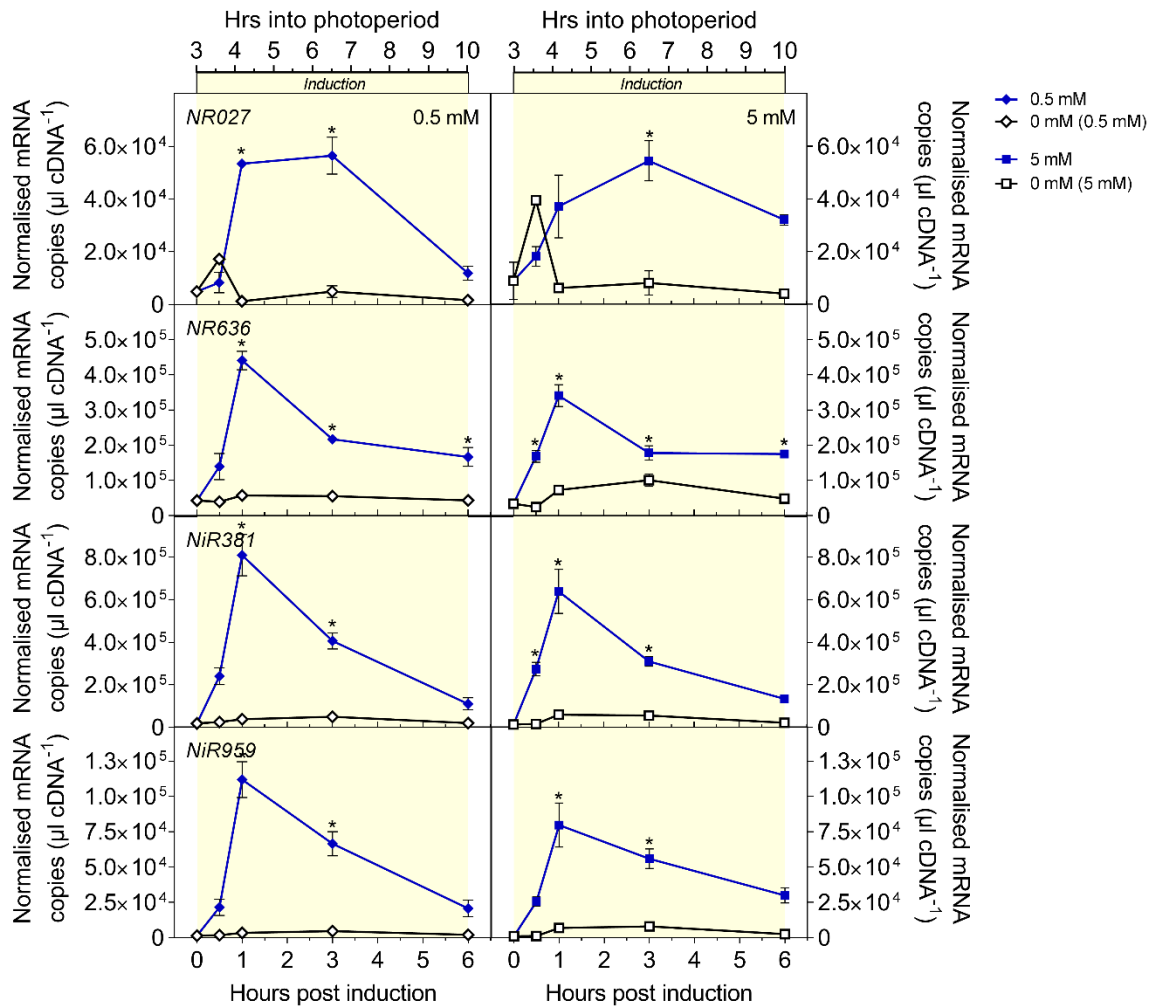
Figure 6 continued. Shoot individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot) (highest transcript reading = red dotted line), steady state 5 mM (green hollow squares with dot) (highest transcript red = dashed and dotted line), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are $\pm$ SEM (n = 4). Values for NO_3^- starved are $\pm$ SEM (n = 1-3). Values for NO_3^- induced are $\pm$ SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants ($P<0.05$).

773 **SUPPLEMENTARY FIGURES**

774 **Supplementary Table S1.** Q-PCR primers for assay of maize gene expression are listed along with the Q-PCR product size (bp).

| Gene	Gene ID	Forward Primer (5'→3')	Reverse Primer (5'→3')	Q-PCR Product size (bp)
ZmNR027	GRMZM2G428027	GCTTTGGCTAACGAATGTC	GCTCGCTACTATTACAACAAG	97
ZmNR636	GRMZM2G568636	GAGGACCACACGGAGATG	CCAACGCTGTACTTCCAC	167
ZmNiR381	GRMZM2G079381	CGTCACCAACAACTCCCAG	GAACCCGCCCACCAGAAG	97
ZmNiR959	GRMZM2G102959	ACCTGCTCTCCTCCTACATC	CCGAACTTGCCGTCCTTG	163

Supplementary Figure 2. Root individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (P<0.05).
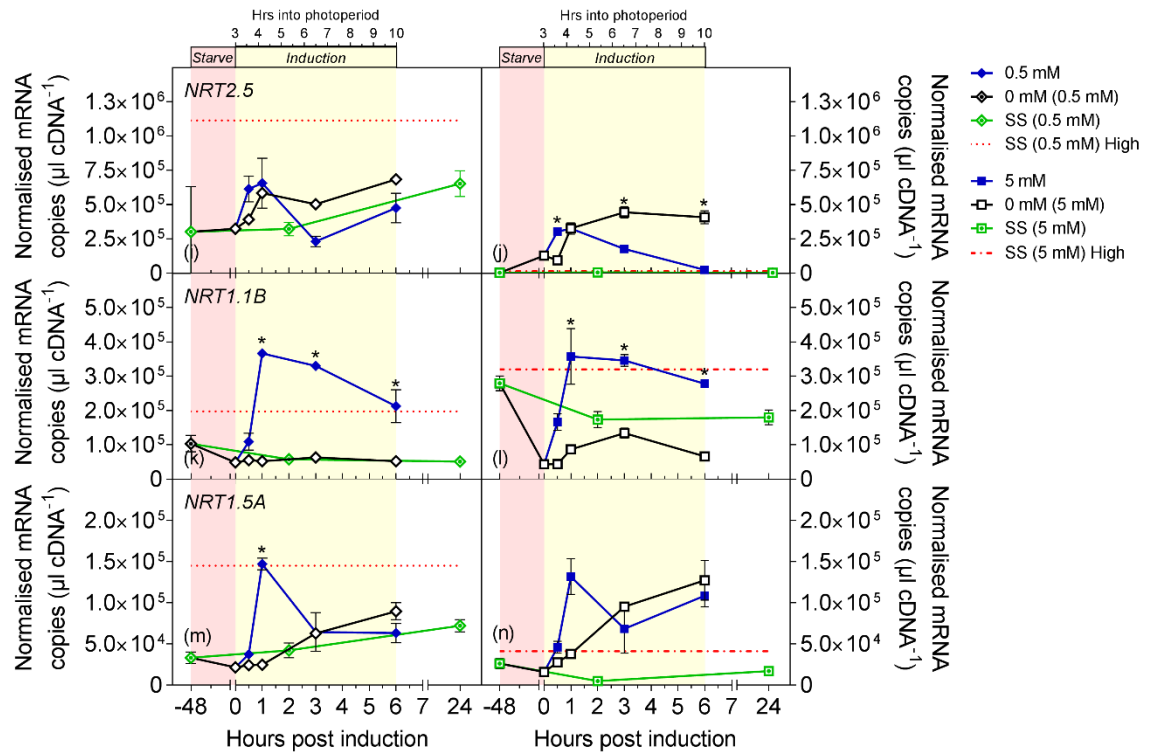
Supplementary Figure 2 continued. Root individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

Supplementary Figure 3. Shoot individual amino acid (AA) concentrations for dwarf (*Zea*

mays) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot),

steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM

NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^-

starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds),

starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM

(n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n

= 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^-
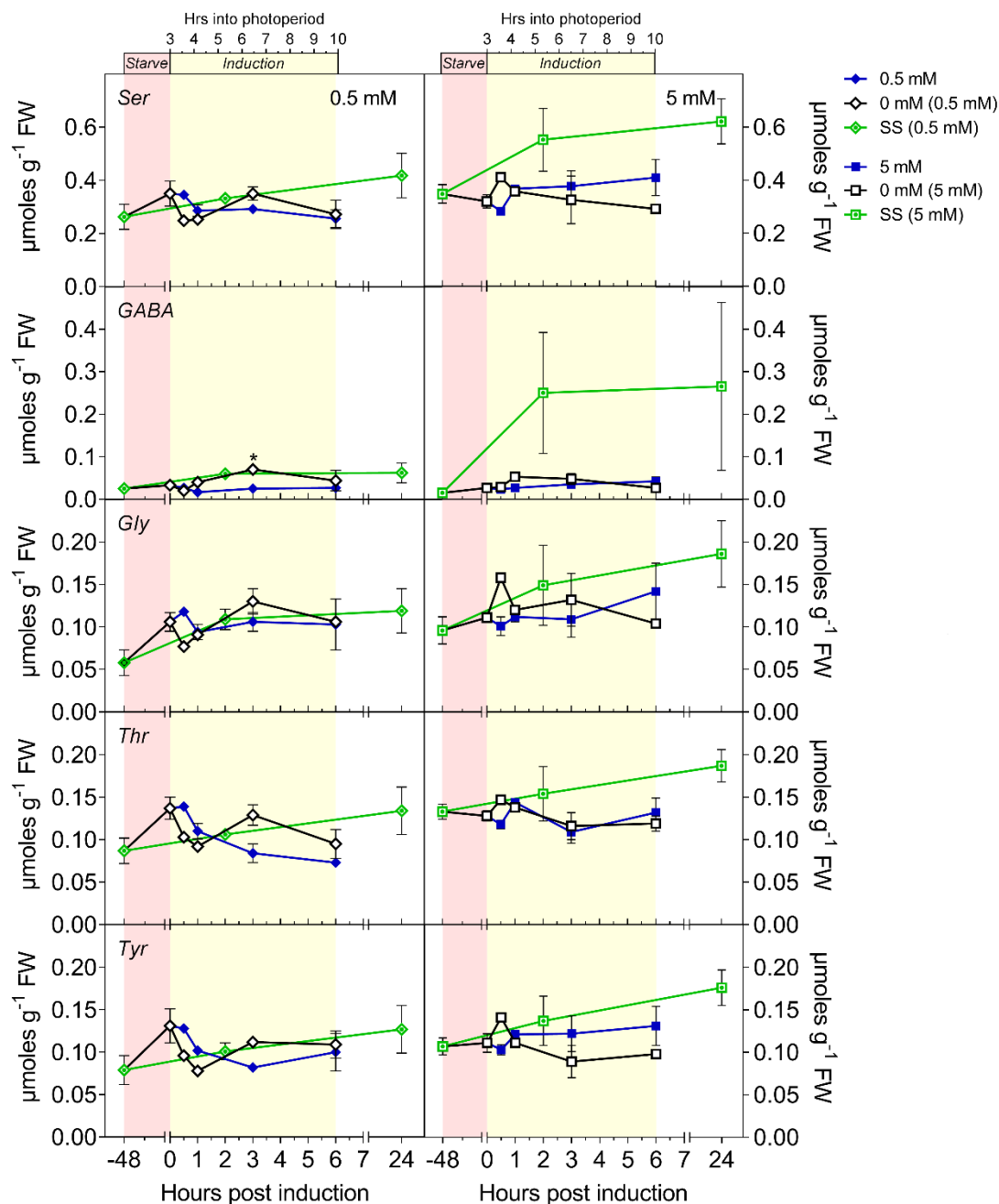
plants ($P<0.05$).

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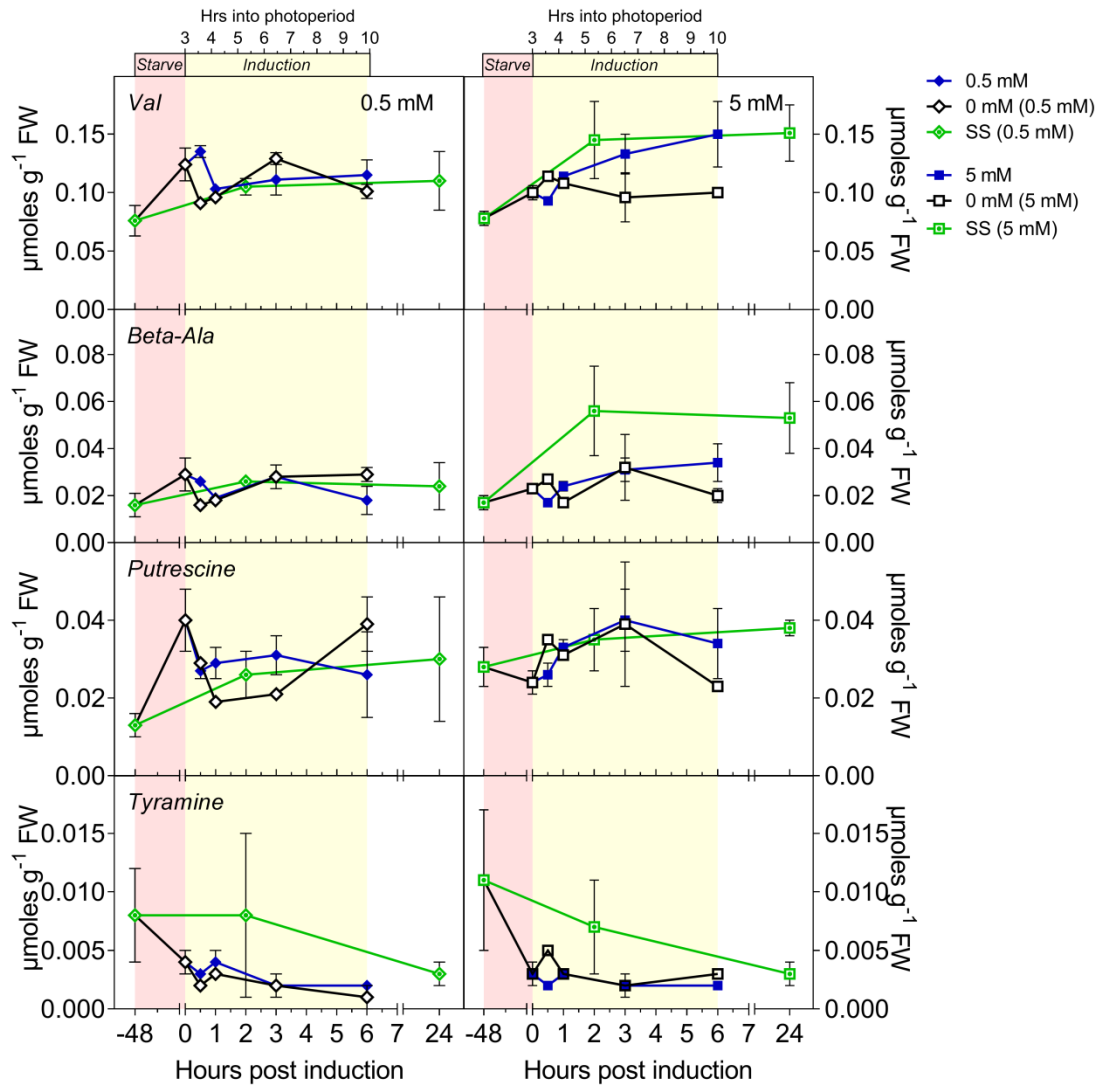**Supplementary Figure 3 continued.** Shoot individual amino acid (AA) concentrations for dwarf (*Zea mays*) Gaspe Flint plants grown under steady state 0.5 mM (green hollow diamonds with dot), steady state 5 mM (green hollow squares with dot), 0.5 mM grown plants subject to 0 mM NO_3^- starvation (black line hollow diamonds), 5 mM grown plants subject to 0 mM NO_3^- starvation (black hollow squares), starved plants induced in 0.5 mM NO_3^- (blue diamonds), starved plants induced in 5 mM NO_3^- (blue squares). Values for steady state NO_3^- are ± SEM (n = 4). Values for NO_3^- starved are ± SEM (n = 1-3). Values for NO_3^- induced are ± SEM (n = 3). *Induced NO_3^- treatment significantly different from corresponding 0 mM control NO_3^- plants (*P*<0.05).

Chapter 4: Discovery of putative cis *NRT* regulation motifs using phylogenomics and co-expression clustering in maize

1 **Discovery of putative cis *NRT* regulation motifs using phylogenomics and co-expression**
2 **clustering in maize**

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Author Contributions:

LH completed the main body of work. UB designed the combined phylogenomics and co-expressed gene promoter analysis approach and provided technical assistance throughout the study. JT participated in the identification of *Setaria italica NRT* homologues and subsequently constructed the *NRT2* family tree. SH, MT, TG and DP supervised the research and participated in the design of the study and interpretation of the data. All authors reads, drafted and approved the manuscript.

Competing Interests

The authors declare that they have no competing interests.

ABSTRACT

Background

In many plant species, transcription of the membrane transporter *NRT2.5* is strongly induced by low nitrogen (N) availability. Understanding the cis-trans regulatory mechanisms responsible for the low N induced transcription of *ZmNRT2.5* could lead to the development of genetic tools for increasing nitrate (NO_3^-) uptake capacity and overall nitrogen use efficiency (NUE) in cereal crops. A combined co-expressed gene and phylogenomics approach was used to examine the promoter of *ZmNRT2.5* to determine functionally and evolutionarily conserved regions.

Results

The *ZmNRT2.5* expression profile in response to 0.5 mM and 2.5 mM NO_3^- was correlated with global gene expression data from a maize lifecycle experiment to determine putatively co-expressed gene cohorts. Six different co-expressed gene cohorts were then examined, exposing seven regions in the *ZmNRT2.5* promoter with potential functional conservation. For the phylogenomics approach the *NRT2* family tree was updated to include foxtail millet (*Setaria italica*) and the promoter regions of *ZmNRT2.5* orthologs were extracted and analysed. The phylogenomic approach revealed five evolutionarily conserved regions which all clustered close to the transcriptional start site.

Searching the conserved regions from both approaches against the PLACE database revealed MYB and bHLH transcription factors sites, strengthening the case for their involvement in regulating N responsive genes. In addition, the results indicated the potential involvement of WRKY, bZIP, nodulin and Dof transcription factors in regulating the low N induced transcription of *ZmNRT2.5*. Several of the identified consensus regions showed no known transcription factor binding sites providing a new resource for the discovery of novel cis-trans regulatory mechanisms.

Conclusions

Combined, the results from this study provide a number of conserved sequences to pursue in further experiments to determine the cis-trans regulatory networks involved in the low NO_3^- induced transcription of *ZmNRT2.5*. Understanding these cis-trans mechanisms could lead to the development of genetic tools for increasing NO_3^- uptake capacity and overall nitrogen use efficiency (NUE) in cereal crops.

KEYWORDS

maize, nitrogen, nitrate, transcription factor, nitrogen use efficiency, NUE, uptake, NRT

BACKGROUND

65 Quantitatively, nitrogen (N) is the mineral nutrient required by plants in the greatest amount

66 (Marschner & Marschner, 2012). As a result in 2012 approximately 120 million tonnes of N

67 fertilizers were applied globally (FAO, 2014). Cereal crops, which supply over half of the

68 world's food energy intake, capture only 40 - 50% of the applied N (Peoples *et al.*, 1995;

69 Sylvester-Bradley & Kindred, 2009) leaving a considerable proportion free in the

70 environment with harmful effects on the ecosphere (Good & Beatty, 2011). With NO_3^- being

71 the predominant form of N available to crops in most high-input agricultural soils (Miller *et*

72 *al.*, 2007), improving the NO_3^- uptake efficiency of cereal crops has the potential to deliver

73 significant environmental and economic gains through improvements in overall cereal

74 nitrogen use efficiency (NUE).

75 In the soil, NO_3^- can vary by four orders of magnitude from micromolar to millimolar (Wolt,

76 1994). To facilitate NO_3^- capture under these variable concentrations, plants have two NO_3^-

77 uptake systems: a high affinity transport system (HATS) when NO_3^- present in the soil is low

78 (< 250μM); and a low affinity transport system (LATS) which operates at high soil NO_3^-

79 concentration (>250 μM) (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995; Garnett *et al.*, 2003).

80 This has been the accepted paradigm for many years, however more recently the HATS has

81 been shown to also be active at high NO_3^- (> 2.5 mM) which has raised questions regarding

82 the contribution of each uptake system (Garnett *et al.*, 2013; Holtham *et al.*, Chapter 2). In

83 Arabidopsis NO_3^- uptake via LATS and HATS activity has been linked to the NO_3^- transporter

84 (NRT/NPF) genes and their related proteins NRT1.1/NRT1.2 and

85 NRT2.1/NRT2.2/NRT2.4/NRT2.5 respectively (Tsay *et al.*, 2007; Kiba *et al.*, 2012; Léran *et*

86 *al.*, 2014; Lezhneva *et al.*, 2014). In plants, transcript levels of *NRT* genes have been shown to

87 respond to numerous internal and external stimuli including: NO_3^- (Wang *et al.*, 2000;

88 Okamoto *et al.*, 2003; Wang *et al.*, 2003; Little *et al.*, 2005; Ho *et al.*, 2009), N metabolites

89 (Glass & Crawford, 1998; Forde & Clarkson, 1999; Gansel *et al.*, 2001; Miller *et al.*, 2007;

90 Gojon *et al.*, 2009), diurnal cues (Lejay *et al.*, 1999; Ono *et al.*, 2000) and developmental

91 stage (Garnett *et al.*, 2013). Due to their role in NO_3^- uptake, understanding how the *NRTs* are

92 transcriptionally controlled is anticipated to be a key factor in developing plants with high

93 NO_3^- uptake characteristics and overall improved NUE.

94 Transcription factors (TFs) act as master switches for regulatory networks by interacting with

95 cis-acting elements and/or with other transcription factors to control gene expression

96 (Guilfoyle, 1997; Spitz & Furlong, 2012; Porto *et al.*, 2014). With such a core role in all

97 aspects of plant function there is evidence that they have played a major role in crop

98 improvement over the years of crop domestication and breeding (Doebley *et al.*, 2006;

99 Kovach *et al.*, 2007; Pourkheirandish & Komatsuda, 2007). Consequently TFs have been

100 suggested as attractive candidates for engineering complex traits such as NO_3^- uptake capacity

101 and NUE (Century *et al.*, 2008). A number of transcription factors have been shown to

102 influence the expression of *NRT* genes in *Arabidopsis* including: MADS box (*NRT2.1*) (Gan

103 et al., 2005), NLP (*NRT2.1* & *NRT2.2*) (Loren Castaings, 2009; Konishi & Yanagisawa,

104 2013a; Konishi & Yanagisawa, 2013b; Liseron-Monfils et al., 2013; Marchive *et al.*, 2013),

105 LBD (*NRT1.1*, *NRT2.1* & *NRT2.2*) (Rubin *et al.*, 2009) and bZIP (*NRT1.1*) (Jonassen *et al.*,

106 2009). With evidence of such strong transcriptional control over *NRTs* there is the potential to

107 exploit key cis-trans regulatory elements to increase functional NRT levels and increase NO_3^-

108 uptake capacity in cereals.

109 To date, identifying NO_3^- specific cis-trans regulatory elements has focused heavily on

110 finding nitrate-responsive cis-elements (NREs) involved in triggering the NO_3^- inducible

111 expression associated with the primary nitrate response (see review Medici and Krouk

112 (2014)). The promoter regions of the nitrate reductase genes (*NIA1* & *NIA2*) have been

113 extensively studied and revealed a number of key cis-elements with the ability to drive NO_3^-

114 induced expression in minimal promoter studies (Hwang *et al.*, 1997; Rastogi *et al.*, 1997;

115 Konishi & Yanagisawa, 2010; Konishi & Yanagisawa, 2011). Using a minimal promoter

116 approach Girin *et al.* (2007) analysed the Arabidopsis *AtNRT2.1* promoter and identified a

150 bp sequence required for the gene's NO_3^- inducible expression and N metabolite repression transcriptional responses. In rice, deletion analysis of the *OsNAR2.1* (*OsNRT3.1*) promoter identified a 311 bp region necessary for the NO_3^- responsive transcriptional activation of the gene (Feng *et al.*, 2011; Yan *et al.*, 2011; Liu *et al.*, 2014). Subsequent motif analysis of that sequence revealed three putative NO_3^- responsive cis-elements which had all previously been associated with the NO_3^- responsiveness of the *NIA* genes in Arabidopsis and Spinach: 5'-GATA-3' (Rastogi *et al.*, 1997; Bi *et al.*, 2005), 5'-A(c/G)TCA-3' (Hwang *et al.*, 1997), and 5'-GACtCTTN10AAG-3' (Konishi & Yanagisawa, 2010; Konishi & Yanagisawa, 2011). Discovery of NREs has provided insight into the cis-trans control mechanisms responsible for the NO_3^- responsive expression of the *NRTs*, however, to date this has not facilitated the discovery of genetic tools for NUE improvement necessitating alternative approaches.

A desirable trait for future cereal crops with improved NUE is the ability to facilitate more efficient N capture under low soil N conditions (Crawford & Glass, 1998; Glass, 2003; Garnett *et al.*, 2009). To enable this, a better understanding of the regulatory mechanisms involved in N uptake under low N conditions is required. Many genes have been shown to be up-regulated during low N conditions (Lian *et al.*, 2006; Bi *et al.*, 2007) or in response to complete N starvation (Scheible *et al.*, 2004; Krapp *et al.*, 2011). Focussing on the *NRTs*, N starvation in rice has been shown to increase *OsNRT2.1* expression (Yin *et al.*, 2014) whilst in maize N limitation leads to higher expression of *ZmNRT1.2*, *ZmNRT2.1*, *ZmNRT2.2*, *ZmNRT2.5* and *ZmNRT1.5A* at various points throughout the lifecycle (Garnett *et al.*, 2013). Interestingly Garnett *et al.* (2013) showed that *ZmNRT2.5* had almost no detectable expression when plants were grown at sufficient NO_3^-, whereas under low NO_3^- conditions the gene had high expression levels. Complementary to this, in Arabidopsis expression of the orthologous gene *AtNRT2.5* is induced by N starvation (Okamoto *et al.*, 2003; Krapp *et al.*, 2011; Lezhneva *et al.*, 2014) and is suppressed by NO_3^- (Okamoto *et al.*, 2003; Orsel *et al.*, 2004). The transcriptional response of this gene to N supply and demand is unique amongst

144 the NRTs and to date no one has examined the genes promoter to understand its

145 transcriptional regulation. Understanding the cis-trans regulatory mechanisms controlling

146 *NRT2.5* could lead to the development of genetic tools for engineering or breeding plants with

147 improved NO_3^- uptake characteristics.

148 For several NREs previously identified via promoter deletion, subsequent bioinformatic

149 analysis has revealed that they are present randomly throughout the Arabidopsis and rice

150 genomes with no enrichment in NO_3^- responsive gene pools (Das *et al.*, 2007; Pathak *et al.*,

151 2009). Consequently in this study, using global expression data generated from the Garnett *et*

152 *al.* (2013) samples, we employed a reverse analysis starting with bioinformatic motif

153 discovery via a combined co-expression and phylogenomics approach with the aim of

154 discovering functionally and evolutionarily conserved cis regulatory motifs associated with

155 the low NO_3^- induced transcription of *ZmNRT2.5*.

156 **RESULTS**

157 **Extension of the NRT2 phylogenetic tree**

158 The phylogenetic relationships of the NRT2 protein family have previously been described by

159 Plett *et al.* (2010), however, since that date the genomic sequence of foxtail millet (*S. italica*)

160 was published (Zhang *et al.*, 2012) warranting an update of the NRT2 protein family tree.

161 Through using a modified reciprocal best hit approach (RBH) approach (Tatusov *et al.*, 1997;

162 Bork *et al.*, 1998; Plett *et al.*, 2010), one distinct *S. italica* homologue was identified for

163 NRT2.5 and found to be most closely related to ZmNRT2.5 and SbNRT2.5 (Fig. 1, Table.

164 S1). In contrast to the NRT2.1/NRT2.2 pairs which are common in other plant species, four *S.*

165 *italica* genes were identified with high sequence similarity to the ZmNRT2.1/NRT2.2

166 sequences (Fig. 1, Table. S1). These *S. italica* genes were all 98.4 - 99.4% identical and

167 located next to each other in pairs on Chromosome 1 (Figs. S1 & S2). Similarly two *S. italica*

168 genes were identified with close similarity to ZmNRT2.3 and SbNRT2.3 whereas all other

169 species only have one orthologous family member (Fig. 1, Table. S1).

Phylogenomic promoter analysis

Global alignment of the 2 kb promoter regions between the NRT2.5 orthologs showed only 40 – 55% identity between the sequences (Fig. S3). The phylogenetic relationships between these promoter regions did not follow the gene ancestral relationships (Fig. 1) with *Brachypodium distachyon* (*B. distachyon*) being more distant than *S. italica* from the *Z. mays* promoter (Fig. S3). Through utilising the MEME analysis tool to interrogate the promoter regions of *NRT2.5* orthologous gene promoters, 14 consensus regions were identified with strong sequence conservation between species (Table. 1, S2). Of those, 9 motifs were found in all species and a further reduced subset of 5 had conserved location close to the transcriptional start site (Table. 1, Figure 2). The *Zmphy1* conserved motif of 5'-CTCGCCG[T/C][C/A][T/C]CCAACCATCG-3' was conserved in all species with the exception of *B. distachyon* (Fig. 2). The *Zmphy2* motif of 5'CCT[C/A]CAA[G/A]GTCAGAGGT[C/T]-3' was highly conserved across all species (Fig. 2). The shorter *Zmphy3* consensus motif of 5'-CCGGCCT[C/T]CCA-3' was also found in all species with conserved location between 233 - 294 bp upstream of the transcriptional start site (Fig. 2). The *Zmphy4* motif revealed a short consensus sequence of 5'-CTCGC[C/A]AACA-3' whilst the *Zmphy8* motif exposed an 8 bp sequence of 5'-AATCTTTA-3' with 100% identity between species (Fig. 2, Table 1).

Co-expression promoter analysis

The NO_3^- controlled transcription of the *ZmNRT2.5* gene results in high transcript levels under low nitrate (0.5 mM) conditions (Fig. S4). Therefore, searching for co-expressed genes was focussed around finding genes with 0.5 mM expression profiles matching the 0.5 mM twin peak pattern (Table 2). This was slightly broadened by also examining 0.5 mM profiles matching only the day 13 to 32 after emergence time period to include genes which may have different regulation during early development and late reproductive growth (Table 2). A more narrow approach was also taken to identify genes which respond similarly to *ZmNRT2.5* under both high and low nitrate conditions as these are most likely to have conserved

141

197 regulation. To identify these genes, correlation in both 0.5 mM and 2.5 mM treatments was

198 required to make up the final cohort for co-expressed gene promoter analysis.

199 A series of preliminary tests were undertaken to assess the capability and output of the

200 MEME analysis tool. Random sequences were generated using the random DNA sequence

201 generator from the sequence manipulation suite (Stothard, 2000). Test motifs were then

202 embedded into these sequences to test MEMEs ability to detect motifs under conditions of:

203 sequence length variance, alternative motif positions, multiple motifs per sequence and fuzzy

204 motifs. The main findings from these preliminary tests identified that: (1) changes to the

205 number of sequences and the length of the sequence significantly affected the ability to detect

206 motifs; (2) larger motifs (>10 bp) need to be contained within a substantial proportion of the

207 query sequences to be detected. As a result both the 1 kb and 2 kb promoter regions upstream

208 of the transcriptional start site for the different sets of co-expressed groups were subject to

209 MEME promoter analysis (Table 2). Input limitations for the MEME platform limits search

210 potential to a maximum of 60 kb which restricted final cohort sequence numbers to a

211 maximum of 60 for 1 kb promoters and 30 for 2 kb promoters. For the "0.5 mM & 2.5 mM"

212 cohort, correlation r-value at 30 and 60 sequences was low so an r-value cut-off of 0.9 was set

213 leaving 10 sequences for promoter analysis. In order to assess the success of the co-

214 expression analysis the groups of co-expressed genes were consolidated, duplicate genes

215 removed, and then subject to a gene ontology (GO) enrichment analysis using AgriGO

216 analysis (Du *et al.*, 2010). The GO analysis assessed whether there was an over-representation

217 of particular functional classes of related genes, indicating the likelihood of conserved control

218 mechanisms. The enriched GO terms featured biological processes (localization and

219 transport), cellular components (membrane) and transport, localisation, and molecular

220 function (transporter activity) (Table 3). The genes with transport (GO:0006810) and

221 transmembrane transport (GO:0055085) included a putative cation antiporter, fatty acid,

222 oligopeptide, APC superfamily (Jack *et al.*, 2000), phosphate, major facilitator superfamily

223 (Pao *et al.*, 1998) and sodium transporters (Table. S3).

224   Results from the promoter analysis of the co-expressed groups identified 16 consensus

225   regions over-represented in the co-expressed gene cohorts (Table. 4, S4). Of that group, 7 met

226   the cut-off criteria: number of sequence "Hits" ≥ 9, E-value < 1.5E+06 and length of the

227   consensus region ≥ 6 bp (Table 4). The *Zmco1.3* consensus sequence revealed a 5'-

228   ACC[A/C]ACC[A/G]-3' motif present in 9 sequences from co-expressed cohort 1 (Fig.2).

229   The *Zmco4.1* consensus region of 5'-CATAGA[A/C/G]AAA[A/T]-3' was conserved in 8

230   sequences from co-expressed cohort 2 (Fig. 3). The *Zmco4.1* region consisting of the 5'-

231   AGC[T/C]AGC[C/T][A/T]-3'sequence was identified in 18 sequences from co-expressed

232   cohort 4 (Fig. 3). From co-expressed cohort 5, two consensus regions were identified, namely

233   *Zmco5.2* and *Zmco5.3*. The *Zmco5.2* region consisted of a 5'-[AG]GCTAGCTAGCT[AT]-

234   3'with the core 5'-GCTAGCTAGCT-3' showing 100% identity for 6 sequences (Fig. 3). The

235   *Zmco5.3* consensus region revealed a 5'-TGCGAGCGAG[AG]-3' sequence that was present

236   in 5 sequences from co-expressed cohort 5 (Fig. 3). Similarly, co-expressed cohort 6 revealed

237   two consensus regions, *Zmco6.2* and *Zmco6.3*.   The *Zmco6.2* region revealed a 5'-

238   TGCAACTGCAA-3' sequence with relatively high homology throughout several sequences

239   within co-expressed cohort 6 (Fig. 3). Finally the *Zmco6.3* consensus region identified a 5'-

240   [TG]CCGCTGC[ACT][GC]T-3' region which was shared amongst a few sequences within

241   cohort 6 (Fig. 3).

242   **PLACE analysis**

243   To determine what published transcription factor binding motifs are associated with the

244   identified consensus regions, the global database of plant cis-acting regulatory DNA elements

245   (PLACE) was interrogated (Higo *et al.*, 1999). PLACE analysis of the consensus motifs

246   identified via both the co-expressed and phylogenomics promoter analysis revealed putative

247   MYB, DOF, NOD, ARR1, OSE, Q element, W box and WRKY transcription factor binding

248   domains (Table 5). The DOFCOREZM site was identified in two separate consensus motif

249   regions and different MYB recognition sites were identified in three separate consensus

250   motifs (Table 5).

DISCUSSION

Gene duplication in foxtail millet

Extending upon the NRT2 phylogenetic tree revealed four *S. italica* peptide sequences homologous to ZmNRT2.1 and ZmNRT2.2. In Arabidopsis, the *AtNRT2.1* and *AtNRT2.2* genes are 90.4% similar and located in tandem on chromosome 1 suggesting they are a product of a duplication event (Orsel *et al.*, 2002). In maize the sequences are even more similar with 99.8% sequence identity due to only one amino acid difference between the two peptide sequences. In contrast to the Arabidopsis genes, the two maize genes are separated by one putative gene and are much further apart (Fig. S2). The foxtail millet genes range from 98.4 to 99.4% identity with six single amino acid variations between the peptide sequences. In addition to the six single amino acid variations there was variability between the beginning of the peptide sequences and Si020228m was missing a 16 amino acid region starting at residue 343 (Fig. S1). The foxtail genes, similar to Arabidopsis and maize, were located close to each other with the Si016891m/Si019202m pair showing equal separation to the Si020228m/Si019373m pair suggesting an additional duplication event (Fig. S2). These duplication events may be similar to the tandem duplication events reported for genes of the C4 pathway in *S.italica* (Monson, 2003; Zhang *et al.*, 2012).

MYB and E-box domains

Through both the phylogenomics and co-expression promoter analysis approaches putative MYB and MYB homolog (MYBPZM) transcription factor binding sites were identified (Table 5). The *Zmco1.3* and *Zmphy1* motifs contained BOXLCOREDCPAL, MYBPZM and PALBOXAPC sites which are known to be MYB and MYB homolog binding elements (Grotewold *et al.*, 1994; Maeda *et al.*, 2005; Prouse & Campbell, 2013). The *Zmco6.2* consensus motif contained a 5'-CANNTG-3' sequence which has been reported as a enhancer box (E-box) element and shown to be involved in circadian rhythms in both mammalian and plant cells (Zhang & Kay, 2010). In plants, these palindromic E-box elements have also been shown to bind with MYB and bHLH transcription factors known to control anthocyanin

278   biosynthesis in *Z. mays* (Hartmann *et al.*, 2005). This is interesting given the link between

279   anthocyanin production and abiotic stress (Chalker-Scott, 1999), especially during nitrogen

280   limitation (Diaz *et al.*, 2006; Peng *et al.*, 2008). In addition the MYB and bHLH transcription

281   factors have been shown to be involved in abscisic acid (ABA) signalling (Abe *et al.*, 2003)

282   and ABA concentration is elevated under N stress (Chapin *et al.*, 1988). MYB and bHLH

283   transcription factors have also directly been shown to be up-regulated under N limitation

284   (Miyake *et al.*, 2003; Lea *et al.*, 2007), severe N stress (Scheible *et al.*, 2004; Bi *et al.*, 2007),

285   and are shown to be induced in the primary NO_3^- response (Wang *et al.*, 2000). In line with

286   the regulation of *NRT2.5* the Myb protein PAP2, a transcriptional regulator of anthocyanin

287   biosynthesis is repressed by NO_3^- and induced by starvation (Scheible *et al.*, 2004). Analysis

288   of the NO_3^- inducible nitrate reductase (*NIA1*) promoter by Wang *et al.* (2010) revealed a cis-

289   regulatory module containing three cis binding elements including MYB and E-box. Together

290   this information strengthens the involvement of MYB and bHLH transcription factors in

291   regulating N responsive genes, and in this case has revealed that they may play a role in

292   regulating the low N induced transcription of *ZmNRT2.5*.

293   **Rich binding elements within the *Zmphy2* & *Zmphy8* consensus regions**

294   A number of 5'-TGAC-3' containing elements were identified in the *Zmphy2* consensus

295   motif. These were identified as W-Box elements which are known to be the binding sites for

296   WRKY transcription factors (Rushton *et al.*, 2010). In Arabidopsis, Bi *et al.* (2007) showed

297   induction of three WRKY transcription factors in response to mild N limitation. A Q-element

298   sequence was also identified in the *Zmphy2* consensus motif which has been shown to be

299   involved in the regulation of the pollen-specific maize *ZM13* gene (Hamilton *et al.*, 1998).

300   This Q-element has also been identified in the promoter region of *tbzF*, a gene encoding a

301   basic leucine zipper-type transcription factor (bZIP) (Hwan Yang *et al.*, 2002). There are

302   examples of bZIP transcription factors being NO_3^- induced (Wang *et al.*, 2001) and regulating

303   genes involved in plant nitrogen nutrition including: nitrate reductase (Jonassen *et al.*, 2008)

304   and genes coordinating root plasticity in response to NO_3^- availability (Tranbarger *et al.*,

2003). The bZIP transcription factor HY5 has been shown to have three putative binding sites in the *AtNRT1.1* promoter region and subsequently shown to act as a positive regulator of NIA2 but as a repressor of *NRT1.1* expression (Jonassen *et al.*, 2009).

Both the *Zmphy2* and *Zmphy8* consensus motifs identified a 5'-AAAGAT-3' sequence known as an organ-specific element (Stougaard *et al.*, 1990) which are characteristic elements of leghemoglobin and other nodulin gene promoters involved in regulating expression in the infected cells of root nodules (Sandal *et al.*, 1987; Vieweg *et al.*, 2004; Fehlberg *et al.*, 2005). The nodulin gene family has been shown to react to different forms inorganic nitrogen with some genes induced exclusively by ammonium, others induced exclusively by NO_3^-, and still others repressed by one nitrogen source and induced by the other (Patterson *et al.*, 2010). In rice a nodulin gene *OsENOD93-1* has been shown to respond to both N induction and N reduction (Bi *et al.*, 2009). Subsequent overexpression of the gene *OsENOD93-1* produced plants with increased yield, total N and overall better NUE which is now subject to a granted European patent (Kant *et al.*, 2011).

Also common between the *Zmphy2* and *Zmphy8* consensus regions was the 5'-NGATT-3' and 5'-AAAG-3' sequences. The 5'-NGATT-3' sequence has been characterised as an Arabidopsis response regulator (ARR) homologue "ARR1" binding element (Sakai *et al.*, 2000). In N starved plants some of the *ARR* genes have been shown to respond to NO_3^- supplementation, but interestingly *ARR1* was not one of them (Kiba *et al.*, 1999). The 5'-AAAG-3' sequence has been characterised as a cis-binding sequence for Dof proteins which are characterised by their unique DNA binding domain (Yanagisawa & Schmidt, 1999). The Dof proteins are plant specific transcription factors which have been identified in both monocots and dicots and shown to function as both transcriptional activators and repressors involved in a diverse range of biological processes (Yanagisawa, 2004). Maize Dof1 has been shown to activate expression of genes encoding C-metabolising proteins associated with organic acid metabolism (Yanagisawa & Sheen, 1998; Yanagisawa, 2000). Expressing

331 *ZmDof1* in Arabidopsis increased plant nitrogen content by 30%, improved growth under

332 low-N conditions, and increased N metabolites in transgenic potato plants (Yanagisawa *et al.*,

333 2004). Interestingly the 5'-TAAAG-3' sequence found in the *Zmphy8* motif has also been

334 linked to the binding of Dof proteins with the same core 5'-AAAG-3' sequence (Plesch *et al.*,

335 2001). Together this information suggests potential involvement of WRKY, bZIP, nodulin

336 and Dof transcription factors in regulating the low N induced transcription of *ZmNRT2.5*.

337 **Novel motifs**

338 Interestingly none of the consensus regions were identified through both the phylogenomics

339 and co-expressed gene approaches (Fig. 4). The *Zmco1.3* and *Zmphy3* regions were

340 neighbouring but did not overlap (Fig. 4). Out of the 11 top consensus regions identified via

341 the combined phylogenomics and co-expression promoter analysis, six showed no evidence of

342 known transcription factor binding sites (Table. 5). Of note were the palindromic 5'-

343 GCTAGCTAG-3' sequences which were found in both *Zmco4.1* and *Zmco5.2* consensus

344 regions from promoter analysis of two co-expressed cohorts. These sequences may provide a

345 new resource for the discovery of novel cis-trans regulatory mechanisms associated with the

346 low N induced expression of *ZmNRT2.5*.

347 **CONCLUSIONS**

348 Extension of the NRT family to include foxtail millet (*Setaria italica*) has revealed a potential

349 evolutionary duplication event leading to additional copies of *NRT2.1*, *NRT2.2* and *NRT2.3*.

350 Using a combined phylogenomics and co-expression approach a number of evolutionarily and

351 functionally conserved regions were identified in the promoter of *ZmNRT2.5*. Within these

352 regions putative binding sites for MYB, bHLH, WRKY, bZIP, nodulin and Dof transcription

353 factors were identified suggesting their potential involvement in regulating *ZmNRT2.5*. In

354 addition six regions showing no resemblance to known transcription factor binding sites have

355 been identified as a new resource for the discovery of novel cis-trans regulatory mechanisms

356 associated with the low N induced expression of *ZmNRT2.5*. Future bioinformatic work will

357 further explore the evolutionarily conserved motifs to determine whether their conservation

358 extends to dicots. The next step is to test these motifs by applying a minimal promoter study

359 *in planta* (Girin *et al.*, 2007; Konishi & Yanagisawa, 2010; Konishi & Yanagisawa, 2011) to

360 assess whether these elements drive gene expression under low NO_3^- conditions. Following

361 this a yeast-one-hybrid approach could be employed to identify proteins which bind these

362 elements (Lopato *et al.*, 2006; Pyvovarenko & Lopato, 2011) to determine the transcriptional

363 controllers of *ZmNRT2.5*. In this study the 5' flanking region was investigated, however gene

364 regulation is complex and studying other untranslated regions (UTR) could also yield key

365 regulatory elements (Hughes, 2006). For example, future studies could also employ the same

366 approach used herein to explore the 3' flanking region as Konishi and Yanagisawa (2013b)

367 demonstrated that a 345-bp NRE sequence in the 3' UTR of the *A. thaliana* nitrate reductase

368 gene '*NIA1*' plays a dominant role in the genes nitrate-inducible expression. Unravelling the

369 cis-trans control networks governing the low NO_3^- induced transcription of *ZmNRT2.5* could

370 lead to the development of genetic tools for increasing NRT transcription to NO_3^- uptake

371 efficiency and improve overall NUE in cereal crops.

372 **METHODS**

373 **Plant growth and harvesting**

374 Dwarf maize (*Zea mays* L. var Gaspe Flint) was grown in hydroponic systems as described

375 previously (Garnett et al., 2013). Plants were sampled between 5 and 7 h after the start of the

376 light period (06:00). The whole root and the youngest fully emerged leaf blade were excised,

377 snap frozen in liquid N_2 and stored at -80°C.

378 **Microarray**

379 The microarray study was completed by Plett *et al.* (2014 (in review)). In brief, total RNA

380 was extracted (Chomczynski, 1993) from frozen tissue and 10 µg aliquots were prepared for

381 microarray analysis. RNA integrity was checked on a 1.2% (w/v) agarose gel. From these

382 samples mRNA was purified and made into double stranded DNA and labelled with Cy3

383 fluorescent dye using Agilent's Low RNA Input Fluorescent Linear Amplification Kit. The

384 cRNA product was the purified and hybridized overnight to a custom 4x44K Maize Oligo

385 Microarray from Agilent Technologies (Palo Alto, CA) according to Agilent's One-Color

386 Microarray-Based Gene Expression Analysis protocol. The microarray slides were then

387 washed and immediately scanned with Agilent's G2505C DNA Microarray Scanner. Images

388 were subject to rigorous quality control measures and analysis using Agilent's Feature

389 Extraction Software (v10.5.1.1) and GeneData Analyst (v2.2.2). Cy3 median signal intensities

390 were imported into R for further processing, omitting 4825 probes with no and very low

391 fluorescent signals. The intensity values were log(2) transformed and quantile normalized. P-

392 values were adjusted employing the method by Benjamini & Hochberg (1995) to control the

393 false discovery rate (FDR).

394 **Identification of *Setaria italica NRT* homologues**

395 Identification of *NRT2* homologues was based on sequence similarity between the closest

396 related species reported by Plett *et al.* (2010) and the predicted amino acid sequences of

397 *Setaria italica (S. italica)*. This was carried out using the peptide homolog function of

398 Phytozome v7.0 (Goodstein *et al.*, 2012) which uses an all-against-all Smith-Waterman

399 alignment, and was then combined with a modified reciprocal best hit approach (Tatusov *et*

400 *al.*, 1997; Bork *et al.*, 1998; Plett *et al.*, 2010). From the Phytozome alignment, the top hit *S.*

401 *italica* protein sequences were used as queries in subsequent BLAST searches against the

402 Arabidopsis database (reverse BLAST). The sequences that returned the corresponding

403 *AtNRT2* homologue as the best hit were then selected for further evaluation as homologues.

404 The list of homologues was then refined by removal of those candidates not specifically

405 related to the NRT2s of interest via manual inspection of multiple sequence alignments and

406 their corresponding trees. Throughout the analyses all splice variants of all identified

407 homologues accepted for further analysis were used in subsequent rounds of reciprocal best

408 hits and only the one member with the longest protein sequence from each splice variant

409 group was used to build trees.

Multiple sequence alignment and tree building

The protein sequences were aligned by standalone MAFFT v6.846b using the L-INS-I method with associated default parameters (Katoh *et al.*, 2009) and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5 (Tamura *et al.*, 2011). The unrooted tree was generated in MEGA using the neighbour-joining method with the pairwise deletion option for alignment gaps and the equal input correction model (heterogeneous pattern among lineages) for distance computation. Branch lengths are proportional to phylogenetic distance.

Phylogenomic promoter analysis

The promoter regions were retrieved from the Phytozome database v7.0 (Goodstein *et al.*, 2012) using the Biomart tool and restricted to 2 kb upstream of the transcriptional start site. The compiled promoter sequences were then analysed using MEME v4.7.0 with the parameters: zero or one motif per sequence, optimum width = 2 – 30 bp, maximum number of motifs = 30 (Bailey *et al.*, 2006). Motifs were then selected based on manual inspection of the MEME motif output assessing the level of sequence homology in the identified regions. A list of "top motifs" was then compiled and ranked based on the number of sequence "Hits", the length of the consensus regular expression region, E-value and the standard error of the mean (SEM) of the motif start-site. The top 5 were then selected with a conserved location close to the transcriptional start site and low E-values.

Co-expression promoter analysis

Co-expression was determined using a custom software package at the ACPFG which assessed correlation of mean centred data by calculating profile similarity based on Euclidean distance (r-value cut-off 0.9). Gene Ontology analysis was undertaken using AgriGO (Du *et al.*, 2010). A singular enrichment analysis (Complete GO) was undertaken on the co-expressed groups using the 'Zea mays ssp V5a' species and the 'Maize genome V5a transcript ID' reference background. Fisher's exact test statistical method was chosen with the Benjamini-Hochberg (FDR) multi-test adjustment at a significance level of 0.05. From the

437   subsequent co-expressed gene lists, the promoter regions either 1kb or 2 kb upstream of the

438   transcriptional start site were obtained from the Phytozome database v7.0 (Goodstein *et al.*,

439   2012) using the Biomart tool and subject to MEME analysis as described previously.

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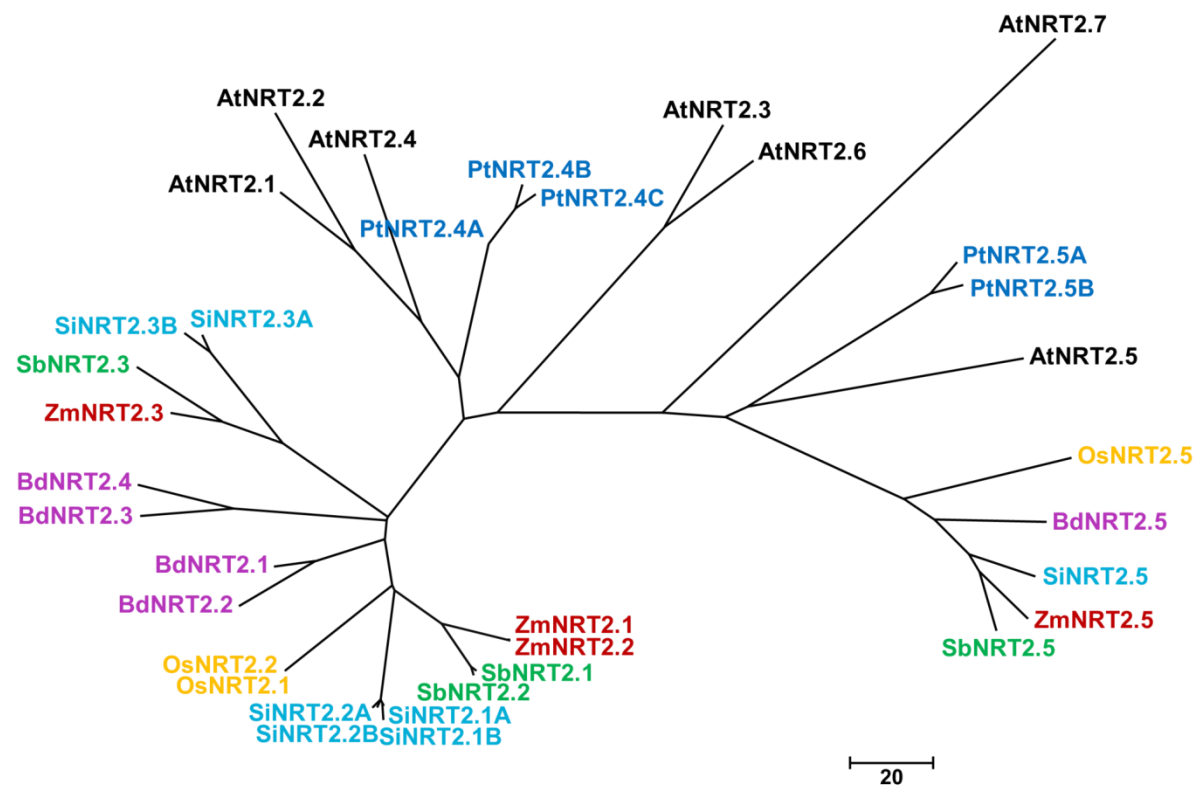
Figure 1. NRT2 family tree. The phylogenomic relationship of the NRT2s reported by Plett *et al.* (2010) was extended to include Foxtail millet (*Setaria italica*) using a modified reciprocal best hit approach. The Unrooted Neighbour-joining tree represents NRT2 transporters in Arabidopsis (black), poplar (dark blue) and 5 grass species: rice (yellow), sorghum (green), maize (red), *Brachypodium* (purple). The scale bar represents a 20 estimated amino acid substitution per residue.

806 **Table 1.** Top motifs discovered via MEME promoter analysis of *ZmNRT2.5* (GRMZM2G455124) orthologous gene promoters. Promoter regions for the

807 orthologous *ZmNRT2.5* genes in rice, sorghum, *Brachypodium* and foxtail millet were examined using a phylogenomics approach. The 2kb promoter regions

808 upstream of the transcriptional start site were analysed using MEME v4.7.0 to identify evolutionarily conserved regions. Top motifs were then selected based

809 on the cut-off criteria: number of sequence "Hits" = 5, E-value < 1.0E-1 and the SEM of the motif start-site < 100. Hits refers to the number of species

810 containing the motif.

Motif Label	Regular expression	Hits	E-value	Motif start site	
				Average	SEM
Zmphy1	G[TC]T[GA][CG]CC[CT]CTC[GA]CCG[TC][CAT][CT]C[CG]AACC[AG]TCG[CAG][GC]	5	4.90E-18	1818	27
Zmphy2	[GT][GA]CCT[CA]CAA[GA]GTCAGAGGT[CT][ACG][GC]A[AC][TA]C[TA]	5	4.50E-13	1598	47
Zmphy3	[CG][CT]G[CGT][GAT][GC][GAC][CG][GCT][CTG][GC][CA][GC][CG]GCCGGCCT[TC]CCA[CT][TC][GC]	5	1.10E-08	1746	25
Zmphy4	C[ACT][GA]CTCGC[CA]AACA[ACT]AA[GAC][CT]C[CG][CG]	5	5.10E-05	1688	34
Zmphy8	[CG][CT][CTA][CG]AAATCTTTA[TA]T[CG][GAC]T[TC][TC][CT]	5	5.50E-01	1793	25
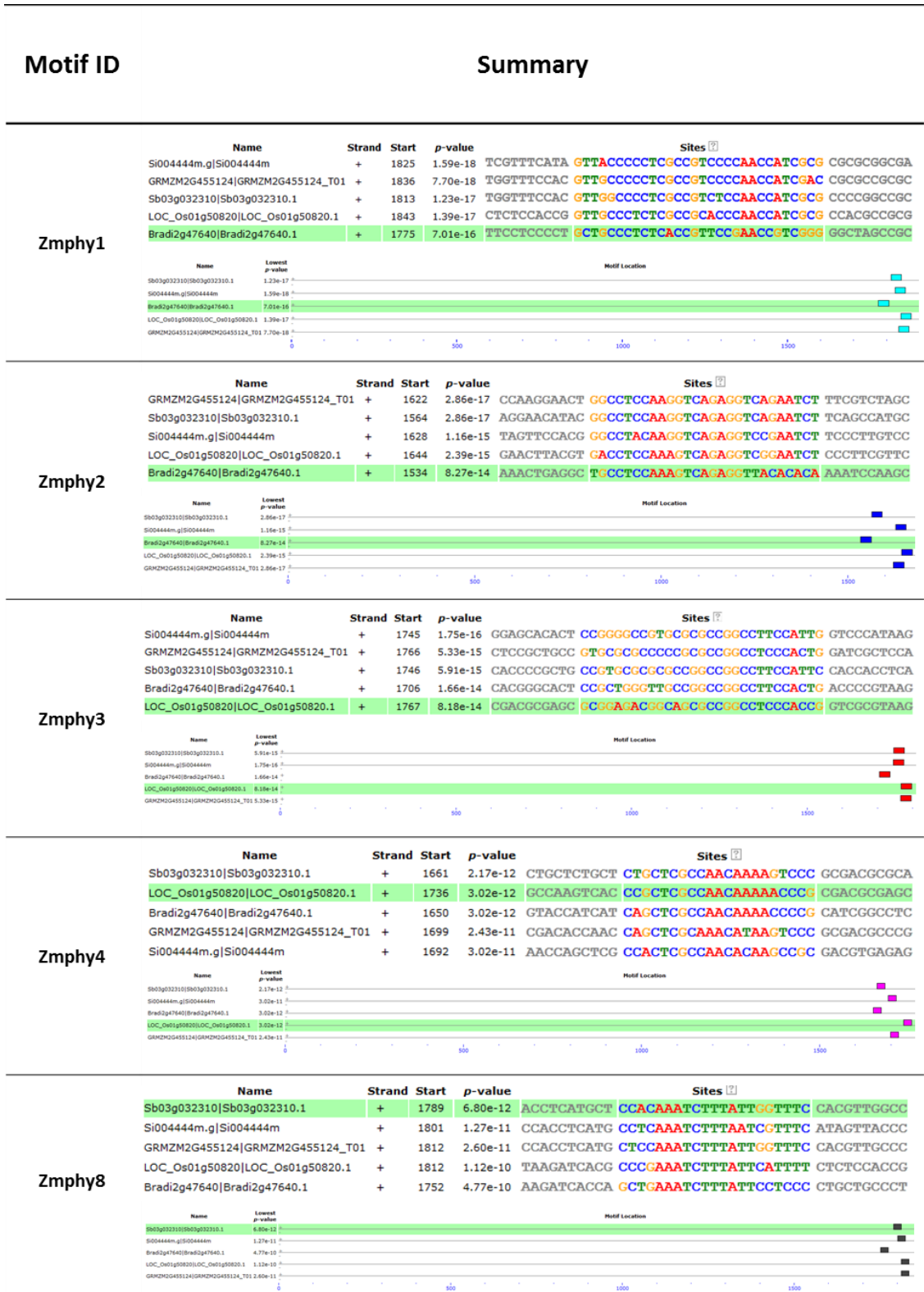
161

Figure 2. Graphical summary of the top identified evolutionarily conserved regions discovered via MEME promoter analysis of *NRT2.5* orthologous gene promoters. The 2kb promoter regions upstream of the transcriptional start site we analysed using MEME v4.7.0. Top motifs were then selected based on the cut-off criteria: number of sequence "Hits" = 5, E-value < 1.0E-1 and the SEM of the motif start-site < 100. Hits refers to the number of species containing the motif.

817   **Table 2.** Summary of the different correlation searches used to select *ZmNRT2.5* (GRMZM2G455124) co-expressed sequences for promoter analysis, and

818   the number of motifs generated using MEME v4.7.0 promoter analysis.

Cohort ID	Treatment correlation	Time points	Number of sequences	r-value	Promoter length	Motifs
1	0.5 mM & 2.5 mM	All	10	> 0.9	1kb	4
2	0.5 mM & 2.5 mM	All	10	> 0.9	2kb	2
3	0.5 mM only	13 - 32	60	> 0.982	1kb	1
4	0.5 mM only	13 - 32	30	> 0.990	2kb	2
5	0.5 mM only	All	60	> 0.941	1kb	3
6	0.5 mM only	All	30	> 0.955	2kb	4

819 **Table 3.** GO enrichment analysis summary. The *ZmNRT2.5* (GRMZM2G455124) co-expressed cohorts were compiled and subject to AgriGO gene ontology

820 (GO) singular enrichment analysis revealing several enriched GO terms.

GO Term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0006810	P	transport	18	3841	0.00028	0.0056
GO:0051234	P	establishment of localization	18	3841	0.00028	0.0056
GO:0051179	P	localization	18	3887	0.00032	0.0056
GO:0055085	P	transmembrane transport	9	1420	0.0016	0.021
GO:0022804	F	active transmembrane transporter activity	7	825	0.0011	0.029
GO:0015291	F	secondary active transmembrane transporter activity	5	421	0.0014	0.029
GO:0005215	F	transporter activity	12	2411	0.0021	0.029
GO:0008324	F	cation transmembrane transporter activity	7	996	0.0031	0.032
GO:0016758	F	transferase activity, transferring hexosyl groups	5	542	0.004	0.033
GO:0022857	F	transmembrane transporter activity	9	1672	0.0047	0.033
GO:0016021	C	integral to membrane	16	2156	2.80E-06	1.50E-05
GO:0031224	C	intrinsic to membrane	16	2183	3.20E-06	1.50E-05
GO:0044425	C	membrane part	17	2684	1.00E-05	3.10E-05
GO:0016020	C	membrane	24	5307	3.30E-05	7.40E-05

821 **Table 4.** Top motifs discovered via the MEME promoter analysis of genes putatively co-expressed with *ZmNRT2.5* (GRMZM2G455124). The 1kb or 2kb

822 promoter regions upstream of the transcriptional start site we analysed using MEME v4.7.0. Top motifs were then selected based on the cut-off criteria:

823 number of sequence "Hits" ≥ 9, E-value < 1.5E+06 and length of the consensus region ≥ 6 bp. Hits refers to the number of co-expressed gene containing the

824 motif.

Search ID	Motif ID	Regular expression	Hits	E-value	Consensus length (bp)
1	Zmco1.3	ACC[AC]ACC[AG]	10	1.80E+05	7
2	Zmco2.1	CATAGA[ACG]AAA[AT]	10	1.20E+06	6
4	Zmco4.1	AGC[TC]AGC[CT][AT][GA][CG][CT]	19	3.50E-02	7
5	Zmco5.2	[AG]GCTAGCTAGCT[AT][GAC]C	11	2.70E-01	11
5	Zmco5.3	TGCGAGCGAG[AG]	9	8.80E+04	10
6	Zmco6.2	[TG]CCGCTGC[ACT][GC]T[GT]C[TGC][CG]	16	1.40E+04	7
6	Zmco6.3	TGCAACTGCAA	11	1.30E+06	11

Motif ID	Summary

Zmco1.3

Name	Strand	Start	p-value	Sites
GRMZM2G455124	+	694	1.53e-05	CGACAGCGAC ACCAACCA GCTCGCAAAC
GRMZM2G447691	-	697	1.53e-05	ACCCAAGGCA ACCAACCA TGTGCTTCAG
GRMZM5G825515	+	734	1.53e-05	CAATGAACCT ACCAACCA CGAGCATATT
GRMZM2G152028	-	456	1.53e-05	AAAAGAAACG ACCAACCA AGTTAGAAAC
GRMZM2G152127	-	143	1.53e-05	TGCTTGAGCG ACCAACCA ACGTGTTCGA
GRMZM2G024211	+	893	3.06e-05	AACTACTCGC ACCCACCA GAAACCTACC
GRMZM2G397675	-	878	3.06e-05	GGTGAGCAGA ACCCACCA AACTGAGGAG
GRMZM2G413853	+	849	6.15e-05	CGCCACCCAC ACCCACCG CCTCACGCAC
GRMZM2G113252	+	200	6.15e-05	AATCGGCTGC ACCCACCG GACACCGGCA
GRMZM5G877259	-	121	9.19e-05	TGAGATAAGC ACTCACCA CTCAGAGAGA

Zmco2.1

Name	Strand	Start	p-value	Sites
GRMZM2G024211	+	14	1.07e-06	ATAACTTCTT CATAGAGAAAA AAAAAATACA
GRMZM2G455124	-	194	1.07e-06	CAAGGCTAGT CATAGACAAAA TTTTTTGGGT
GRMZM2G447691	+	1274	1.07e-06	AACTCTGGTT CATAGAGAAAA AGGGGTCGCA
GRMZM5G825515	+	1767	1.07e-06	AATTCAAATT CATAGAAAAAT AGAGACGTTG
GRMZM2G152028	-	1412	2.14e-06	TAATAAAAAA CATAGAAAAGA CAAAGGAAGG
GRMZM2G113252	+	850	2.68e-06	TTGTCTTTCT CATACAAAAAA ATCTTATTAA
GRMZM2G397675	-	463	2.68e-06	GGGTAGACAA CATAGAAAATA ATAGACAAAA
GRMZM2G413853	+	1105	3.52e-06	TAACTAAGTT TATAGAAAAAA TACACCAACA
GRMZM2G152127	+	1489	1.28e-05	TGCCGTGCCA CATAAACAAAT CCGTCGCCGT
GRMZM5G877259	-	513	1.83e-05	TCAGCGAATA CAGAGATAAAT TTATTCAGCT

Zmco4.1

Name	Strand	Start	p-value	Sites
GRMZM2G152028	-	106	7.89e-08	AGCCAGAGCC AGCCAGCCAGCC CCCGACCGCC
GRMZM2G062151	+	1903	1.28e-07	TACACTAGCT AGCTAGCTAGCC ACAGCCAGCT
GRMZM2G020148	-	1949	1.28e-07	CTAGCTAGCT AGCTAGCTAGCC GAGAGGATCT
GRMZM2G455124	-	1187	1.28e-07	CTGGGGCTGG AGCTAGCTAGCC GCTGTTTCAA
GRMZM2G046750	-	1554	1.28e-07	AAAACAAGCT AGCTAGCTAGCC AATTTCCATT
GRMZM5G800488	+	479	3.91e-07	AAGAGCTCCT AGCCAGCTAGGC TTTGCCTGCC
GRMZM2G111521	+	1830	5.68e-07	CGGGAGCAAC AGCCAGCCAACC ACCTCTCGTT
GRMZM2G059637	+	1927	5.68e-07	TCACGGCAAA AGCTAGCCAGCG CACAGGACCT
GRMZM2G174834	-	1292	7.41e-07	TAGTAGTAGT AGCTAGCTAACC TCGTTTCATT
GRMZM2G031120	-	1681	8.97e-07	ACGATGAGCT AGCTAGCTAGCT GACGATGCTA
GRMZM2G003930	-	927	8.97e-07	GGGGAGGCCA AGCTAGCCAAGC CAAGGCAGGC
GRMZM2G131378	-	336	1.39e-06	GTTTGACCAA AGCCAGCCTGGC GCACCGGACA
GRMZM2G174107	-	192	1.69e-06	CCGTAGCTTG AGCTAGCTAGGG GTAGGGTAAT
GRMZM2G077553	+	1881	2.85e-06	CCATTTTAAT AGCTAGCCTAGC CTCGTCCCAC
GRMZM2G166176	-	1898	3.18e-06	TGGGTGGGCT AGCTAGCCTGGT GTGATCGGCG
GRMZM2G043117	+	1775	3.77e-06	GCAAGAGCAG AGCCAGCCTGGG AACAAGACAA
GRMZM2G466309	+	1207	5.14e-06	AACGGGCAAC AGCCAGCCCGCT GGAAATCGCA
GRMZM2G065640	+	1964	5.62e-06	CACACGCTCT CGCCAGCCAACC GAAGCCACCG
GRMZM2G033820	+	1916	6.33e-06	ATGCAGCAAT AGCCACCCAGCT GGTGGCGGTC

Figure 3. Graphical summary of the top identified putative functionally conserved regions discovered via the MEME promoter analysis of *ZmNRT2.5* (GRMZM2G455124) co-expressed cohorts. The 1kb or 2kb promoter regions upstream of the transcriptional start site we analysed using MEME v4.7.0. Top motifs were then selected based on the cut-off criteria: number of sequence "Hits" ≥ 9, E-value $< 1.5E+06$ and length of the consensus region ≥ 6 bp. Hits refers to the number of co-expressed gene containing the motif.

Name	Strand	Start	*p*-value	Sites
GRMZM2G062151	+	899	1.63e-09	GCATTACACT AGCTAGCTAGCTAGC CACAGCCAGC
GRMZM2G031120	+	681	1.63e-09	TAGCATCGTC AGCTAGCTAGCTAGC TCATCGTTAG
GRMZM2G020148	-	950	1.63e-09	TGTCCTAGCT AGCTAGCTAGCTAGC CGAGAGGATC
GRMZM2G046750	-	555	1.63e-09	CGCAAAAACA AGCTAGCTAGCTAGC CAATTTCCAT
GRMZM5G858417	-	962	1.98e-08	CGGCCGGGCT AGCTAGCTAGCTCCT CGGCGCACCA
GRMZM2G455124	+	187	5.95e-08	TTGAAACAGC GGCTAGCTAGCTCCA GCCCCAGTGG
GRMZM2G003930	+	249	1.15e-07	CGAGATTGAG AGCTCGCTCGCTCGC TCGGTGTCTT
GRMZM2G166176	+	902	1.15e-07	GATCACACCA GGCTAGCTAGCCCAC CCACAGTCGT
GRMZM2G059637	+	927	2.65e-07	TCACGGCAAA AGCTAGCCAGCGCAC AGGACCTGCG
GRMZM2G174834	+	292	3.64e-07	AATGAAACGA GGTTAGCTAGCTACT ACTACTAAAC
GRMZM2G397675	+	343	4.61e-07	TTAACGAGTC AGCTCGCAAGCTAAA CGAGCTATCA

Zmco5.2

Name	Strand	Start	*p*-value	Sites
GRMZM2G043117	-	897	1.98e-07	CTTCGTATAC TGCGAGCGAGA TTGGTGAGCA
GRMZM2G086430	+	14	1.98e-07	AGCTTGCAAT TGCGAGCGAGA TACCACCGTT
GRMZM2G397675	-	897	1.98e-07	CTTTGTATAC TGCGAGCGAGA CTGGTGAGCA
GRMZM2G466309	+	987	3.75e-07	TGTGTTCCTG TGCGAGCGAGG CA
GRMZM2G455124	-	364	5.73e-07	TGTTTGGCCA TGGGAGCGAGA GAGACAGCGT
GRMZM2G149619	+	660	1.02e-06	TTGATATTTT TGTGAGCGAGA CCTTTGAAGT
GRMZM2G111521	+	63	1.37e-06	TGCTCACGGC GGCGAGCGAGA GAACGAGAAA
GRMZM2G046750	+	705	1.37e-06	TTTTGACTAA TGCGCGCGAGA GAAGATAAAA
GRMZM2G319454	+	803	1.94e-06	TTAGAATTTT TGAGAGCGAGG ATGGTATTTT

Zmco5.3

Name	Strand	Start	*p*-value	Sites
GRMZM2G455124	+	1758	1.85e-09	TGAAGAGCGC TCCGCTGCCGTGCGC GCCCCCGCGC
GRMZM2G024312	+	435	5.51e-08	ACGAGCAACT GCCGCTGCAGTGGGC GGAGGCGGTG
GRMZM2G481843	+	1937	7.43e-08	TGATGCTGGT GCCGCTGCTGTGGTC GACGAAGCGG
GRMZM2G132704	+	319	9.31e-08	GAGACGGTGA GCCGCTGCTCTTCGC TTCTCGCCAC
GRMZM2G103357	+	55	1.09e-07	AAATATAATA TCCGCTGCACTGCCT GTATCGACCA
GRMZM2G035595	-	1666	5.39e-07	GTAATTTGAC GCTGCTGCAGTGCTG CGTGATCGCC
GRMZM5G854655	+	1856	9.76e-07	CTTGGCTTTG TCCACTGCACTTCTG CTGCGGTATA
GRMZM2G106836	+	946	1.42e-06	TCTGGGCTTG TCCTCTGCACTGCAC CACACAGCTA
GRMZM2G024211	+	1771	2.39e-06	AGAACTTGGG TCCGCGGCTCCGCGC ATCCATACAT
GRMZM2G415359	+	75	2.83e-06	ATCCAGACGG TCCGGTGACGTGCCC GGACGGTCCG
GRMZM2G047875	-	1657	3.31e-06	CGTCTACGTG TCCGCGGCCGTTGCG GCAGGCAGGC
GRMZM2G176542	+	686	4.20e-06	GTACGGGTAT GATGCTGCTGTGCGC TCGCTGGAGG
GRMZM2G415327	-	1729	7.01e-06	GTGGCACACT GGCACTGCCGTGCCT AGAGAAGCGC
GRMZM2G149442	-	1793	7.51e-06	TCAACCCATT TCCGTTGCTGTTTTG GTGCCGGTTA
GRMZM2G007615	-	1725	1.44e-05	CAGGGCAGGA TCCTTTGCCGTTTTC TCGTCCAATA
GRMZM2G047815	+	41	2.86e-05	TATCTGAAAA TCCACTGAACAGCTT TCCAATGCTC

Zmco6.2

Name	Strand	Start	*p*-value	Sites
GRMZM2G106836	-	1649	2.50e-07	ACTGCCTGCC TGCAACTGCAA GGTCTCCTGA
GRMZM2G007615	+	1035	2.50e-07	TTTTGCCTGT TGCAACTGCAA CTGTAGAAAG
GRMZM2G047875	+	1520	4.99e-07	GCGCGTGGGG TGCAAGTGCAA TTGTGCAATT
GRMZM2G024312	-	617	4.99e-07	GAACGCCGTG TGCAAGTGCAA AATGTGCACT
GRMZM2G481843	-	1197	1.72e-06	TTAGTCATCT TGCAATTGCAA AGCACTTATT
GRMZM2G455124	+	547	2.68e-06	TTTATTATCG TCCAACTGCAA ACTGTTCCAC
GRMZM5G854655	+	1159	3.76e-06	CATCAAGAGA TGCAAATGCAC CCGCTGAAAA
GRMZM2G176542	+	186	5.52e-06	ATTGGCTGAC TGCACCTGCGA CTGTATCCAT
GRMZM2G415327	+	1176	7.33e-06	GGGGGAGAGG TGCATCTGCGA TCAGGAGAGC
GRMZM2G149442	+	536	7.33e-06	AACCGCCAAG TGCCAATGCAA CGTCACTGTT
GRMZM2G024211	+	1353	8.76e-06	TTCCTAGCCG TGCAACTGGAC AGGCCAGAGG

Zmco6.3

831 **Figure 3 continued.** Graphical summary of the top identified putative functionally conserved

832 regions discovered via the MEME promoter analysis of *ZmNRT2.5* (GRMZM2G455124) co-

833 expressed cohorts. The 1kb or 2kb promoter regions upstream of the transcriptional start site

834 we analysed using MEME v4.7.0. Top motifs were then selected based on the cut-off criteria:

835 number of sequence "Hits" ≥ 9, E-value < 1.5E+06 and length of the consensus region ≥ 6 bp.

836 Hits refers to the number of co-expressed gene containing the motif.

167

837 **Table 5.** Results from interrogating the Plant Cis-acting Regulatory DNA Elements Database

838 (PLACE). Searching the consensus regions identified via the phylogenomics and co-

839 expressed gene promoter analysis of *ZmNRT2.5* orthologous and co-expressed cohort gene

840 promoters respectively, revealed several transcription factor binding motifs from previously

841 published reports.

Motif ID	Factor or Site name	Strand	Start bp	Sequence
Zmco1.3	BOXLCOREDCPAL	+	1	ACCWWCC
	MYBPZM	+	2	CCWACC
Zmco2.1	None			
Zmco4.1/5.2	None			
Zmco5.3	None			
Zmco6.2	EBOXBNNAPA	+	3	CANNTG
	EBOXBNNAPA	-	3	CANNTG
	MYB2CONSENSUSAT	+	3	YAACKG
	MYBCORE	-	3	CANNTG
	MYB2CONSENSUSAT	+	3	CANNTG
	MYB2CONSENSUSAT	-	3	CANNTG
Zmco6.3	None			
Zmphy1	MYBPZM	+	11	CCWACC
	PALBOXAPC	+	5	CCGTCC
Zmphy2	ARR1AT	-	17	NGATT
	DOFCOREZM	-	20	AAAG
	NODCON1GM	-	18	AAAGAT
	OSE1ROOTNODULE	-	18	AAAGAT
	QELEMENTZMZM13	+	3	AGGTCA
	QELEMENTZMZM13	+	10	AGGTCA
	WBOXNTCHN48	-	4	CTGACY
	WBOXNTCHN48	-	11	CTGACY
	WBOXNTERF3	-	4	TGACY
	WBOXNTERF3	-	11	TGACY
	WRKY71OS	-	5	TGAC
	WRKY71OS	-	12	TGAC
Zmphy3	None			
Zmphy4	None			
Zmphy8	ARR1AT	-	2	NGATT
	DOFCOREZM	-	5	AAAG
	NODCON1GM	-	3	AAAGAT
	OSE1ROOTNODULE	-	3	AAAGAT
	TAAGSTKST1	-	5	TAAAG
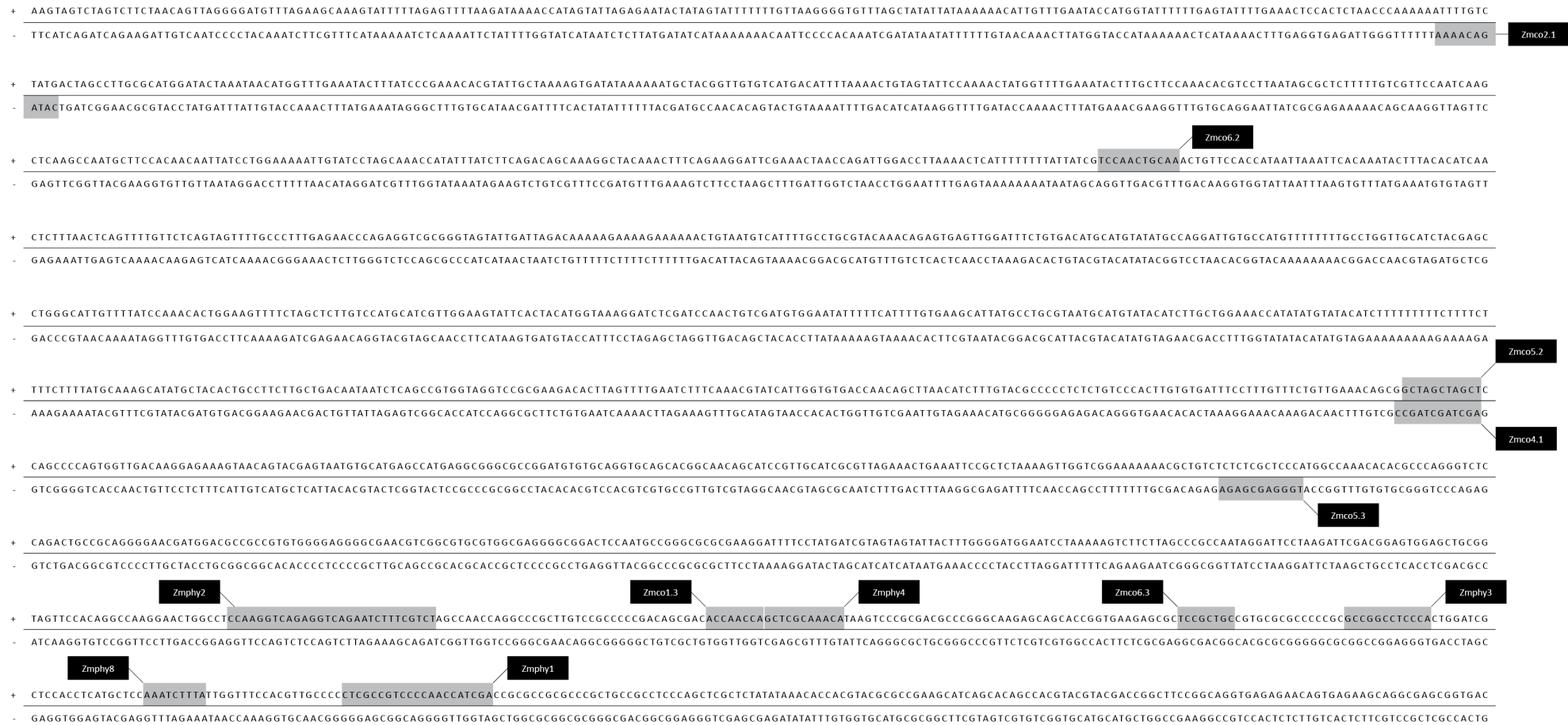
842   **Figure 4.** *ZmNRT2.5* (GRMZM2G455124) 2 kb promoter region with the identified putative functionally and evolutionarily conserved regions.

844 **Supplementary Table 1.** Summary of the gene identifiers and new *NRT* nomenclature for *NRT1, 2* and *3* genes in Arabidopsis, poplar, rice, maize, sorghum,

845 *Brachypodium* and foxtail millet.

Arabidopsis thaliana		*Populas tricocharpa*		*Orayza sativa*		*Zea mays*		*Sorghum bicolor*		*Brachypodium distachon*		*Setaria italica*	
Symbol	TAIR ID	Symbol	JGI ID	Symbol	MSU ID	Symbol	ID	Symbol	JGI ID	Symbol	ID	Symbol	ID
AtNRT2.1	AT1G08090											SiNRT2.1A	Si016891m
AtNRT2.2	AT1G08100	PtNRT2.1	POPTR_0009s01420.1			ZmNRT2.1	GRMZM2G010251_T01	SbNRT2.1	Sb04g001000.1	BdNRT2.1	Bradi3g01270.1	SiNRT2.1B	Si020228m
AtNRT2.3	AT5G60780	PtNRT2.2	POPTR_0143s00200.1	OsNRT2.1	LOC_Os02g02190.1	ZmNRT2.2	GRMZM2G010280_T01	SbNRT2.2	Sb04g000990.1	BdNRT2.2	Bradi3g01250.1	SiNRT2.2A	Si019202m
AtNRT2.4	AT5G60770	PtNRT2.3	POPTR_0009s01410.1	OsNRT2.2	LOC_Os02g02170.1	ZmNRT2.3	GRMZM2G163866_T01	SbNRT2.3	Sb04g000970.1	BdNRT2.3	Bradi3g01280.1	SiNRT2.2B	Si019373m
AtNRT2.6	AT3G45060									BdNRT2.4	Bradi3g01290.1	SiNRT2.3A	Si01945m
												SiNRT2.3B	Si016894m
AtNRT2.5	AT1G12940	PtNRT2.5A	POPTR_0015s09290.1	OsNRT2.5	LOC_Os01g50820.1	ZmNRT2.5	GRMZM2G455124_T01	SbNRT2.5	Sb03g032310.1	BdNRT2.5	Bradi2g47640.1	SiNRT2.5	Si004444m
		PtNRT2.5B	POPTR_0015s09310.1										
AtNRT2.7	AT5G14570	none	none	none	none	none	none	none	none	none	none	none	none

```
CLUSTAL O(1.2.1) multiple sequence alignment

Si020228m      -MDMERGAPGSSLHGMTGREPTFAFSTEDATAASKFDLPVDSEHKAKTIRLFSFANPHMR
Si019373m      ----ERGAPGSSLHGMTGREPTFAFSTEDATAASKFDLPVDSEHKAKTIRLFSFANPHMR
Si016891m      -MDMERGAPGSSLHGMTGREPTFAFSTEDATAASKFDLPVDSEHKAKTIRLFSFANPHMR
Si019202m      MADGERGAPGSSLHGMTGREPTFAFSTEDATAASKFDLPVDSEHKAKTIRLFSFANPHMR
               ************************************************************

Si020228m      TFHLSWISFFTCFVSTFAAAPLVPIIRDNLNLTKADIGNAGVASVSGSIFSRLAMGAVCD
Si019373m      TFHLSWISFFTCFVSTFAAAPLVPIIRDNLNLTKADIGNAGVASVSGSIFSRLAMGAVCD
Si016891m      TFHLSWISFFTCFVSTFAAAPLVPIIRDNLNLTKADIGNAGVASVSGSIFSRLAMGAVCD
Si019202m      TFHLSWISFFTCFVSTFAAAPLVPIIRDNLNLTKADIGNAGVASVSGSIFSRLAMGAVCD
               ************************************************************

Si020228m      LLGPRYGCAFLIMLAAPTVFCMAIIDDAAGYIVVRFLIGFSLATFVSCQYWMSTMFNSKI
Si019373m      LLGPRYGCAFLIMLAAPTVFCMAIIDDAAGYIVVRFLIGFSLATFVSCQYWMSTMFNSKI
Si016891m      LLGPRYGCAFLIMLAAPTVFCMAIIDDAAGYIVVRFLIGFSLATFVSCQYWMSTMFNSKI
Si019202m      LLGPRYGCAFLIMLAAPTVFCMAIIDDAAGYIVVRFLIGFSLATFVSCQYWMSTMFNSKI
               ************************************************************

Si020228m      IGTVNGLAAGWGNMGGGATQLIMPLVYDIIRKCGATPFTAWRLAYFVPGSLHIVMGILVL
Si019373m      IGTVNGLAAGWGNMGGGATQLIMPLVYDIIRKCGATPFTAWRLAYFVPGSLHIVMGILVL
Si016891m      IGTVNGLAAGWGNMGGGATQLIMPLVYDIIRKCGATPFTAWRLAYFVPGSLHIVMGILVL
Si019202m      IGTVNGLAAGWGNMGGGATQLIMPLVYDIIRKCGATPFTAWRLAYFVPGSLHIVMGILVL
               ************************************************************

Si020228m      TMGQDLPDGNLRSLQKKGDANKDKFSKVMWYAITNYRTWIFVLLYGYCMGVELTTDNVIA
Si019373m      TMGQDLPDGNLRSLQKKGDANKDKFSKVMWYAITNYRTWVFVLLYGYCMGVELTTDNVIA
Si016891m      TMGQDLPDGNLRSLQKKGDANKDKFSKVMWYAITNYRTWIFVLLYGYCMGVELTTDNVIA
Si019202m      TMGQDLPDGNLRSLQKKGDANKDKFSKVMWYAITNYRTWIFVLLYGYCMGVELTTDNVIA
               ******************************************.*****************

Si020228m      EYYFDHFNLDLRVAGIIAACFGMANIVARPLGGILSDVGARYWGIPQ-----------A
Si019373m      EYYFDHFNLDLRVAGIIAACFGMANIVARPLGGILSDVGARYWGMRARLWNIWILQTAGG
Si016891m      EYYFDHFNLDLRVAGIIAACFGMANIVARPLGGILSDVGARYWGMRARLWNIWILQTAGG
Si019202m      EYYFDHFNLDLRVAGIIAACFGMANIVARPLGGILSDVGARYWGMRARLWNIWILQTAGG
               *******************************************.         .

Si020228m      AFCLWLGRATTLPASITAMVLFSFCAQAACGAIFGVTPFISRRSLGIISGMTGAGGNFGA
Si019373m      AFCLWLGRATTLPASITAMVLFSFCAQAACGAIFGVTPFISRRSLGIISGMTGAGGNFGA
Si016891m      AFCLWLGRATTLPASITAMVLFSFCAQAACGAIFGVTPFISRRSLGIISGMTGAGGNFGA
Si019202m      AFCLWLGRATTLPASITAMVLFSFCAQAACGAIFGVTPFISRRSLGIISGMTGAGGNFGA
               ************************************************************

Si020228m      GLTQLLFFTSSKYSTGMGLEYMGIMIMACTLPVVFVHFPQWGSMLFPANAGAVEEHYYSS
Si019373m      GLTQLLFFTSSKYSTGMGLEYMGIMIMACTLPVVFVHFPQWGSMLFPANAGAVEEHYYSS
Si016891m      GLTQLLFFTSSKYSTGMGLEYMGIMIMACTLPVVFVHFPQWGSMLFPANAGAVEEHYYSS
Si019202m      GLTQLLFFTSSKYSTGMGLEYMGIMIMACTLPVVFVHFPQWGSMLFPANAGAVEEHYYSS
               ************************************************************

Si020228m      EWNEEEKSKGLHSASLKFAENCRSERGKRNVIQATSSTQPNNTPEHV*
Si019373m      EWNEEEKSKGLHSASLKFAENCRSERGRRNVIQATSNTQPNNTPENV*
Si016891m      EWNEEEKSKGLHSASLKFAENCRSERGKRNVIQATSSTQPNNTPEHV*
Si019202m      EWNEEEKSKGLHSASLKFAENCRSERGKRNVIQATSSTQPNNTPEN--
               ***************************:********.********.
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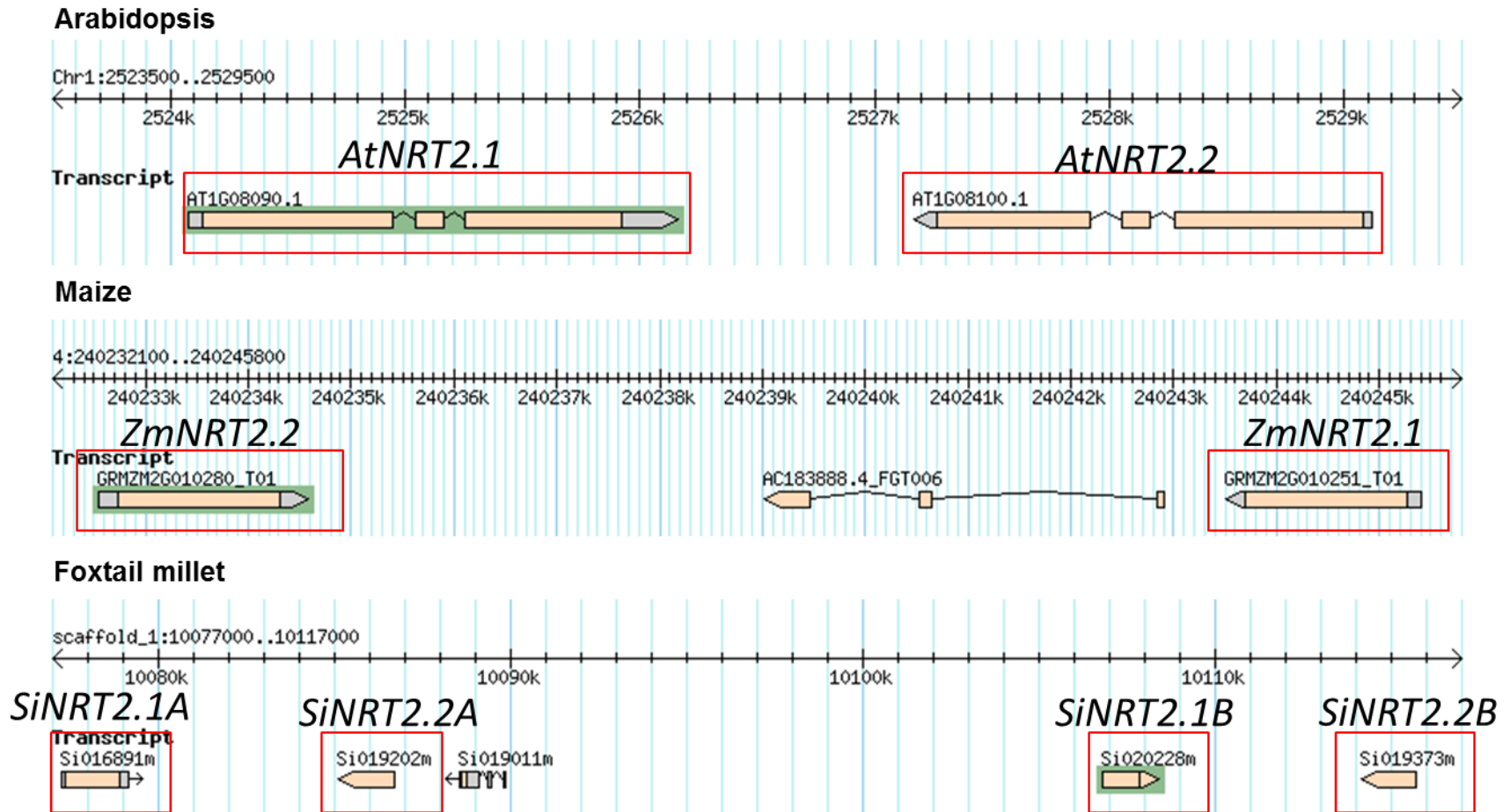
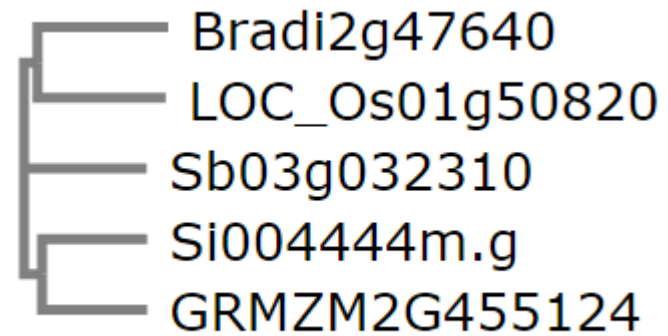|	Si020228m	Si019373	Si016891m	Si019202m
Si020228m	100			
Si019373m	98.43	100		
Si016891m	99.22	99.24	100	
Si019202m	98.64	99.43	99.43	100

846 **Supplementary Figure 1.** Relationship between the 4 identified ZmNRT2.5 orthologous

847 Foxtail millet peptide sequences. Alignment generated using the clustal omega tool via

848 EMBL-EBI (Sievers *et al*. 2011).

Supplementary Figure 2. Genome location of the *NRT2.1/NRT2.2* orthologous genes in Arabidopsis, maize and foxtail millet. Sourced from Phytozome v9.1 Gbrowse environment.

851 **Supplementary Figure 3.** Relationship between the 2kb promoter regions of the 5 identified *ZmNRT2.5* orthologous genes. Alignment generated using the

852 clustal omega tool via EMBL-EBI (Sievers *et al.* 2011).

853   **Supplementary Table 2.** Additional motifs discovered via MEME promoter analysis of *ZmNRT2.5* (GRMZM2G455124) orthologous gene promoters.

854   Promoter regions for the orthologous *ZmNRT2.5* genes in rice, sorghum, *Brachypodium* and foxtail millet were examined using a phylogenomics approach.

855   The 2kb promoter regions upstream of the transcriptional start site were analysed using MEME v4.7.0 to identify evolutionarily conserved regions. These

856   motifs did not meet the cut-off criteria (number of sequence "Hits" = 5, E-value < 1.0E-1 and the SEM of the motif start-site < 100) but were still found to

857   have a level of conservation. Hits refers to the number of species containing the motif

Motif Label	Regular expression	Hits	E-value	Motif start site	
				Average	SEM
Motif 5	GG[CT]AG[TC]TCC[AG][CG][AT][GC][GC]CC[AG][AC]G[GC][AG]	5	6.60E-05	1621	139
Motif 6	[CG][GAT][CG][GC]C[CGT][CGA][AGC][AG][GA][CG][ACT][TA][CG]AG[CT][AG][CG][AC][GT][CG][CT]ACG[TG][AG]C	5	6.40E-03	1869	109
Motif 7	CAGCATCCGTTGCATCG[TC]GTTAGAA	3	1.00E-01	919	729
Motif 9	[AC]A[AG]G[TAG][AC]TT[CT][TCG]T[TAC][GC]C[TAC]CGC[CG]AA[TAC]A[GA][GT]AT[TC]C[CG]	4	5.60E+00	1687	114
Motif 10	A[GC]G[AC]TCT[CT][CG]A[AGT][CT][CT]A	5	1.30E+04	755	345
Motif 11	[CG][AG][CT][CTA]GG[ACGTC]G[CA][CAGTA]GGAG[CA]	5	1.70E+04	1549	326
Motif 12	CAGCTC[CG][AT]C[TA]C	4	1.50E+05	1420	253
Motif 13	GCC[AG][CT]GCCCGCT[GT]CCGC	2	1.60E+05	1732	194
Motif 14	C[TAC]CCAC[CA]TCAT	4	2.10E+05	1662	252
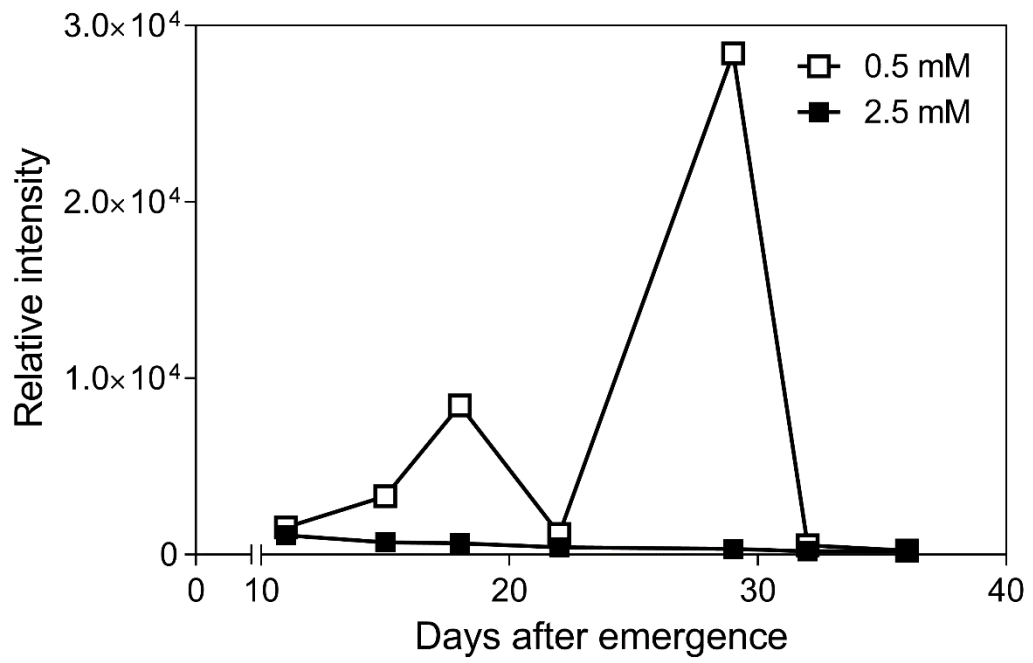
858 **Supplementary Figure 4.** *ZmNRT2.5* (GRMZM2G455124) expression profile across the

859 Maize lifecycle as measured by custom 44k array. See methods.

Supplementary Table 3. Functional annotation of *ZmNRT2.5* co-expressed genes with the labelled GO terms transmembrane transport (GO:0055085) or transport (GO:0006810). Functional annotation was sourced using Phytozome v9.1 with reference to Panther, Pfam, KEGG and KOG annotations (Kanehisa & Goto, 2000; Tatusov *et al.*, 2003; Thomas *et al.*, 2003; Finn *et al.*, 2014).

Gene ID	GO accession	Functional annotation
GRMZM2G047875	GO:0055085 // GO:0006810	Predicted K+/H+-antiporter
GRMZM2G074103	GO:0055085 // GO:0006810	Long-chain acyl-CoA transporter, ABC superfamily (involved in peroxisome organization and biogenesis)
GRMZM2G166176	GO:0055085 // GO:0006810	Acyltransferase
GRMZM5G825515	GO:0055085 // GO:0006810	OPT oligopeptide transporter protein // ISP4 LIKE PROTEIN
GRMZM2G176542	GO:0055085 // GO:0006810	Lipase // Predicted lipase/calmodulin-binding heat-shock protein
GRMZM5G858417	GO:0055085 // GO:0006810	Permease family // SODIUM-DEPENDENT VITAMIN C TRANSPORTER // Xanthine/uracil transporters
GRMZM2G174107	GO:0055085 // GO:0006810	Triose-phosphate Transporter family // EamA-like transporter family // SOLUTE CARRIER FAMILY 35 // Glucose-6-phosphate/phosphate and phosphoenolpyruvate/phosphate antiporter
GRMZM2G086430	GO:0055085 // GO:0006810	Major Facilitator Superfamily // MAJOR FACILITATOR SUPERFAMILY DOMAIN-CONTAINING PROTEIN-RELATED // Predicted transporter/transmembrane protein
GRMZM2G046750	GO:0055085 // GO:0006810	None
GRMZM2G415327	GO:0006810	RHO FAMILY GTPASE // Ras-related small GTPase, Rho type
GRMZM2G035595	GO:0006810	None
GRMZM2G173878	GO:0006810	Ras family // GTPase Rab2, small G protein superfamily
GRMZM2G103357	GO:0006810	SCAMP family // SECRETORY CARRIER-ASSOCIATED MEMBRANE PROTEIN (SCAMP) // Secretory carrier membrane protein
GRMZM2G149619	GO:0006810	Sodium Bile acid symporter family // SODIUM-BILE ACID COTRANSPORTER // Na+-bile acid cotransporter
GRMZM2G065640	GO:0006810	Protease inhibitor/seed storage/LTP family
GRMZM2G180625	GO:0006810	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE
GRMZM2G038780	GO:0006810	Domain of unknown function (DUF588) // OS05G0245300 PROTEIN // NITRATE, FROMATE, IRON DEHYDROGENASE

863 **Supplementary Table 4.** Additional motifs discovered via the MEME promoter analysis of genes putatively co-expressed with *ZmNRT2.5*

864 (GRMZM2G455124). The 1kb or 2kb promoter regions upstream of the transcriptional start site we analysed using MEME v4.7.0. These motifs did not meet

865 the cut-off criteria (number of sequence "Hits" ≥ 9, E-value < 1.5E+06 and length of the consensus region ≥ 6 bp) but were still found to be reasonably

866 conserved. Hits refers to the number of co-expressed gene containing the motif.

867

Search ID	Motif ID	Regular expression	Hits	E-value	Consensus length
1	Zmco1.1	[TA][GA]A[GA]A[AT]AGAGA	10	4.40E+03	5
1	Zmco1.2	T[CG]CA[TC][GC][AGT]GCCA	10	9.00E+03	4
1	Zmco1.4	A[AC][AC]T[ATG]T[GT]AGAG	8	9.40E+05	4
2	Zmco2.2	GA[AT]G[TG][CT][GA]AGC[TA][GT][CT]T[CT][GT]TCA	6	3.80E+06	3
3	Zmco3.1	GA[AG]C[GCT][AT]T[CG][AT][AG]GCGGGC[AG][CA]CG	4	2.40E+05	3
4	Zmco4.2	T[TGA][AC]G[TG]G[CT][AG]TG[TC]TTGG	16	5.50E+04	4
5	Zmco5.1	[CA][GC][CG][TG]CCG[CA][CT][TG]CCG[AC]G	13	1.80E+03	3
6	Zmco6.1	C[TC]CCCTC[CT][ATC][CG][TGA][CG]C[GCA][GACT][AC][GC][TG]CC	9	9.60E+01	5
6	Zmco6.4	TTG[TG]ATGC[TG]CC	16	2.50E+06	4

Chapter 5: General discussion

Improving NUE in cereals is an important goal for ensuring economical and environmentally sustainable food production into the future (Tilman *et al.*, 2002; Hirel *et al.*, 2011). The low N uptake capacity of cereal crops appears to be an attractive target for improving NUpE and overall NUE (Garnett *et al.*, 2009). With NO_3^- being the main N form available to cereal crops (Miller *et al.*, 2007), understanding the NO_3^- uptake system and its control may aid in the development of plants with improved NUpE. Recently Garnett *et al.* (2013) raised questions regarding the accepted paradigm describing the contributions of the HATS and LATS uptake systems to total NO_3^- uptake. Utilising a lifecycle analysis approach the same group moved closer towards unravelling the ambiguity around the role of NO_3^- and its assimilates in signalling N status and regulating the NO_3^- uptake system (Garnett *et al.*, 2013). With much of the literature focused on PNR starvation and resupply experiments, integrating and relating this data to longer term analysis approaches was highlighted as an important step for leveraging the existing body of knowledge around the NO_3^- uptake system. Significant evidence was presented to suggest the existence of strong transcriptional control mechanisms governing NO_3^- uptake. Subsequently this was flagged as a key opportunity for generating plants with improved NUpE through identifying and modifying *NRT* cis-trans regulatory mechanisms. As such the research described in this dissertation used dwarf maize (*Zea mays* L. var. Gaspe Flint) to:

i)   clarify the contribution of the HATS and LATS to total NO_3^- uptake in cereals

ii)   gain further insight into the roles of NO_3^- and its assimilates in signalling N status and regulating the NO_3^- uptake system

iii)   to understand how data from the PNR literature relates to longer term lifecycle analysis studies

iv)   identify novel *NRT* cis-trans regulatory elements

5.1 Advances in knowledge from this thesis

The scope of this thesis covered three different approaches to understanding the NO_3^- uptake system. The key findings are subsequently discussed herein.

5.1.1 The HATS – a main contributor to total nitrate uptake

In Chapter 2 it was observed that total NO_3^- uptake capacity was dominated by the activity of the HATS for plants subjected to both limited and sufficient NO_3^- availability. This supports our previous work (Garnett *et al.*, 2013) further demonstrating that; under sufficient NO_3^- growth conditions (≥ 2.5 mM) the HATS appear to contribute a major portion of total NO_3^- uptake capacity (c. in this study on average ~ 65% of total uptake capacity for plants grown at 5 mM NO_3^-) where previously the LATS were thought to predominate (Siddiqi *et al.*, 1990; Kronzucker *et al.*, 1995; Garnett *et al.*, 2003; Malagoli *et al.*, 2004). By reducing NO_3^- availability during vegetative growth it was also shown that HATS uptake capacity increased in response to the change in N supply whilst LATS decreased. As the NRT2 transporters have been shown to be responsible for HATS activity (Huang *et al.*, 1996; Okamoto *et al.*, 2003; Li *et al.*, 2007; Tsay *et al.*, 2007; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014), this new information supports a wider role for the HATS which may focus efforts for improving NUpE by targeting attention to the NRT2s.

5.1.2 *NRT* levels fluctuate daily in response to N demand

Previous work in our lab highlighted the significant variation of *NRT* transcript levels across the lifecycle under steady state NO_3^- conditions (Garnett *et al.*, 2013). This work demonstrated two distinct peaks in the transcript levels of a number of putative *NRTs* which were correlated with growth and its associated N demand. In Chapter 2, a focus on the vegetative growth period and a finer time resolution revealed substantially greater and more dynamic variation over time. This included examples of transcriptional change in excess of 3-fold between consecutive daily measurements, highlighting the responsiveness of *NRT* transcription to N demand under steady state NO_3^- conditions. Revealing this dynamic temporal variability in *NRT* transcript levels equips future researchers to better focus their

efforts on understanding the signalling mechanisms controlling *NRT* transcription. Without an acute understanding of this, studies which do not take this variability into account may be misinterpreted.

5.1.3 *NRT* changes in response to decreasing nitrate availability

As discussed previously, the majority of the literature is focussed around NO_3^- starvation and resupply experiments (Medici & Krouk, 2014). This provides insights into how plants respond to NO_3^- exposure and the genes involved with this signalling process. An alternative approach to complement this is to understand how plants respond to change in NO_3^- availability from a controlled steady state base level. In Chapter 2 plants were grown under steady state sufficient NO_3^- and then reduced to limited NO_3 conditions to assess plants adaptive changes. In response to reducing NO_3^- availability a rapid transcriptional increase of *ZmNT3.1A*, *ZmNRT2.5* and *ZmNRT1.5A* was observed followed by an increase in *ZmNRT2.1* and *ZmNRT2.2* transcription a day later. Due to the published functional roles of orthologous genes in Arabidopsis, rice and barley and the observed changes in NO_3^- concentration throughout the plant in Chapter 2, this may reflect an order of responses whereby plants: first increase root-to-shoot NO_3^- transfer to maintain shoot growth, then increase the uptake system to increase NO_3^- uptake. Understanding this series of responses provides key insight into the regulation and plasticity of the NO_3^- uptake system in response to changes in NO_3^- availability.

5.1.4 Nitrate may be the key signalling molecule for the HATS

The regulation of NO_3^- uptake by plant N status has been widely reported (Cooper & Clarkson, 1989; Imsande & Touraine, 1994; Forde, 2002; Miller *et al.*, 2008; Gojon *et al.*, 2009). These studies have highlighted both tissue concentration of NO_3^- itself, or down-stream assimilates such as amino acids being potential signals of N status and regulators of the NO_3^- uptake system. Previous work by Garnett *et al.* (2013) indicated that both of these options were plausible, but insufficient time resolution meant that they were unable to correlate NO_3^- or assimilates directly with the observed changes in the NO_3^- uptake system. Through

supplying exogenous amino acids, previous studies have proposed a negative feedback system whereby certain amino acids (specifically glutamate, glutamine, aspartate and asparagine) suppress *NRT* transcription and uptake capacity at high levels (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000; Gansel *et al.*, 2001; Nazoa *et al.*, 2003; Miller *et al.*, 2008). Under the conditions of this study we did not see evidence to support this inverse correlation between *NRT* transcript levels, NO_3^- uptake capacity and amino acid concentrations. However the individual treatment profiles for root, shoot and xylem sap NO_3^- concentration compared to HATS NO_3^- uptake capacity showed a much stronger correlation. In addition low tissue NO_3^- concentration aligned with up regulation of *NRT* transcription indicating a potential internal NO_3^- threshold and providing more supporting evidence to suggest that nitrate itself may be the main signalling molecule regulating the NO_3^- uptake system.

5.1.5 The energy cost of nitrate uptake may be important

Chapter 2 demonstrated an inverse correlation between root growth rate and NO_3^- uptake capacity. Both HATS and LATS NO_3^- uptake are active transport mechanisms and the energy cost of N acquisition has been reported to be as high as 60% of total root respiration (Veen, 1981; Van der Werf *et al.*, 1988). It is speculated that this may support the numerous cost/benefit models that have been proposed examining the carbon and energy cost of N acquisition (Veen, 1981; Chapin *et al.*, 1987; Van der Werf *et al.*, 1988; Fisher *et al.*, 2010). As a result it is hypothesized that the observed reduction in NO_3^- uptake capacity corresponding with increased root growth observed in Chapter 2, may be a reflection of limited energy resources and a trade-off between energy investment in NO_3^- uptake and increased root growth to increase total N capture area. This may be an important consideration for the development of cereal crops with increased NUpE as there may be energy restrictions that need to be considered when manipulating the uptake system. This is an interesting topic for further investigation.

5.1.6 A new model

Previously Garnett *et al.* (2013) described a two-component model of NO_3^- uptake capacity

regulation, with transcription providing long-term regulation and short-term uptake capacity

regulated via the post-translational control of existing transport capacity. The results from

Chapter 2 further support this model in part with the observed maximum NO_3^- uptake capacity

preceding maximum *NRT* expression. However, the Garnett *et al.* (2013) model did not

consider a NO_3^- limited growth condition severe enough to induce growth changes. In that

study plasticity within the NO_3^- uptake system was able to meet N demand and no difference

in growth or yield was observed, however, in Chapter 2 a significant increase was observed in

root growth and decrease in shoot growth. Based on the growth, *NRT* transcript and NO_3^-

uptake capacity changes a new model is proposed involving a "decision cycle", whereby

plants first harness the plasticity within the uptake system via the two-component model and

then if unable to meet N demand will shut down N uptake and divert energy to invest in root

growth. Further investigation of the validity of this model may provide new insight into the

NO_3^- uptake system and its regulation.

5.1.7 Understanding a complex system requires complex approaches

Interesting observations have been made when assessing the response to change in NO_3^-

supply from a steady state base (Garnett *et al.*, 2013). However, the complexity and

importance is not well aligned with data from the broader scientific community due to the

focus on PNR studies. As previously highlighted, understanding how long term steady state

studies relate to the vast primary nitrate response literature may be crucial to combining

advances made by both research approaches. The hybrid analysis in Chapter 3 showed that

plants exhibited different adaptive responses to starvation depending on plant N status (NO_3^-

and amino acid concentrations) prior to starvation. A direct comparison was able to be drawn

between the *NRT* transcript levels induced by steady state demand experiments in contrast to

the PNR revealing a putative transcriptional limit for some *NRT*s. The observed changes in

amino acid levels and *NRT* transcripts in the non-induced control plants highlighted the

importance of appropriate control monitoring. Overall the array of responses observed in response to NO_3^- induction, some which were pre-treatment dependent and some which were pre-treatment independent, highlighted the importance of pre-treatment settings prior to the PNR.

5.1.8 *NRT2.5* cis-trans regulatory motifs

In Chapter 4 through using a combined phylogenomics and co-expression approach a number of evolutionarily and functionally conserved regions were revealed within the *ZmNRT2.5* promoter. Within these regions putative binding sites for MYB, bHLH, WRKY, bZIP, nodulin and Dof transcription factors were identified suggesting their potential involvement in regulating *ZmNRT2.5*. As discussed in Chapter 4, there is existing evidence to suggest a role for these TFs in regulating N responses, and there are examples showing that overexpressing some of these TFs have led to increased NUE in Arabidopsis and rice (Yanagisawa *et al.*, 2004; Kant *et al.*, 2011). This provides new information which could be used to explain the regulation of *ZmNRT2.5* and used towards the development of cereals with improved NUpE. Also through the combined phylogenomics and co-expressed gene promoter analysis approach in Chapter 4, six regions showing no evidence of known TF binding sites were identified. These regions are an exciting new resource for the discovery of novel cis-trans regulatory mechanisms associated with the low N induced expression of *ZmNRT2.5*. Future investigations into the trans factors which bind these regions could lead to the development of genetic tools for increasing NUpE and improving overall NUE in cereal crops.

5.2 Future directions

Following the research presented and discussed in this thesis there are some important next steps to realising the impact of this work:

5.2.1 Completing the loop – Phloem sap measurements

The experiment in Chapter 2 provided new insight into the uptake and movement of NO_3^- and its assimilates from root-to-shoot via the xylem. With the observed changes in growth and NO_3^- uptake capacity it is likely that signals eliciting these changes could be coming from the shoot to the root. The literature supporting negative feedback regulation from N assimilates is focused on shoot-to-root signals via the phloem (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000; Gansel *et al.*, 2001; Nazoa *et al.*, 2003; Miller *et al.*, 2008). Perhaps this is why the inverse correlation between amino acid concentration and *NRT* expression was not observed in Chapter 2 and Chapter 3. Consequently understanding the movement of NO_3^- and its assimilates in the phloem is a key next step to understanding regulation of the NO_3^- uptake system.

5.2.2 Relating transcripts to functional protein

It is well known that transcript levels do not equate to functional protein levels (Gygi *et al.*, 1999; Maier *et al.*, 2009). It was reviewed earlier that most NRT2 proteins must exist within a complex with NAR2 to facilitate NO_3^- transport (Orsel *et al.*, 2006; Orsel *et al.*, 2007; Yong *et al.*, 2010; Kotur *et al.*, 2012). There is also evidence to suggest that the level of AtNRT2.1 protein is independent of transcript levels or changes in NO_3^- uptake capacity, and that NRT2s may be long lived proteins (Wirth *et al.*, 2007). There are currently no commercially or publically available antibodies for the ZmNRTs. During this thesis attempts have been made to extract proteins and identify signature peptides for protein quantification via mass spectrometry (Gerber *et al.*, 2003; Kirkpatrick *et al.*, 2005). If this methodology is found to be viable for detecting differentiating the *NRT*s, or specific antibodies can be raised, the relationship between *NRT* transcript levels, plasma membrane NRT protein, changes in NO_3^- uptake capacity and their changes in response to N supply and demand can be understood. In

178 addition samples could be separated into plasma membrane, ER/Golgi and cytoplasm

179 fractions to gain insight into the trafficking of these proteins and the process of complex

180 formation. This approach has been successful for understanding how iron homeostasis is

181 controlled via trafficking and degradation of IRON-REGULATED TRANSPORTER 1

182 (IRT1) (Shin *et al.*, 2013; Barberon *et al.*, 2014). Creating this methodology is crucial for

183 unravelling the association between *NRT2* transcript levels, functional protein levels, and

184 NO_3^- uptake to understand the relative importance of transcriptional and post translational

185 machinery in regulating NO_3^- uptake.

186 **5.2.3 Investigating the energy cost of nitrate uptake.**

187 The speculation of energy costs restricting plant investment in NO_3^- uptake and growth

188 discussed in Chapter 5 based on the results in Chapter 2 requires further investigation. There

189 are many studies proposing cost/benefit models by examining the carbon and energy cost of N

190 acquisition (Veen, 1981; Chapin *et al.*, 1987; Van der Werf *et al.*, 1988; Fisher *et al.*, 2010).

191 Despite this, to date no one has experimentally demonstrated the energy limitations and their

192 effect on NO_3^- uptake capacity. A clearer understanding of the ATP energy cost of N

193 acquisition and the energy availability of plants under N stress is required to further assess

194 this. Overall it is important to explore this further as any energy restrictions may need to be

195 considered when modifying the uptake system for the development of cereal cops with

196 increased NUpE.

197 **5.2.4 Transcriptomics**

198 Key time points were revealed in Chapter 2 where multiple components of the N system

199 (uptake capacity, NO_3^- pools, amino acids, *NRT* transcripts) were rapidly changing. In

200 addition, reducing NO_3^- availability provided significant insight into how plants respond to

201 changes in N supply and demand, and highlighted a number of key time points in the series of

202 responses where transcriptional and physiological changes in the NO_3^- uptake system were

203 prominent. There is a significant opportunity to extend on this study through investigating

204 global gene expression across the datasets to identify key genes that may be involved in

205 regulating the NO_3^- uptake system. These genes could be key leads for generating crops with

206 improved NUpE.

5.2.5 The generation of cereal *NRT* mutants

208 To date, a key tool for understanding the NO_3^- uptake system in Arabidopsis has been the use

209 of *NRT* mutants (Liu *et al.*, 1999; Cerezo *et al.*, 2001; Li *et al.*, 2007; Almagro *et al.*, 2008;

210 Wang & Tsay, 2011; Hsu & Tsay, 2013). As highlighted earlier, due to the dichotomy

211 between dicots and cereals this level of functional characterisation also needs to be performed

212 in cereals to understand the role of these proteins. Efforts were made during this thesis to

213 produce dwarf maize (*Zea mays* L. var. Gaspe Flint) *NRT* mutants using siRNA (Tang *et al.*,

214 2003; Baulcombe, 2004), however the tissue culture methods still require optimising to

215 produce fertile regenerates in our facility. The constructs and transformation vectors are

216 prepared and ready for future efforts to produce these *NRT* dwarf maize mutants. Having

217 mutants to include in future experiments is important for understanding the functional roles of

218 the NRTs in cereal NO_3^- uptake.

5.2.6 Extending the comparative study

220 The aim of Chapter 3 was to understand how long term steady state studies relate to the

221 primary nitrate response literature. The data presented in Chapter 3 provided some initial

222 insight, however due to experimental constraints, the misalignment of sampling time points

223 between plants subject to a PNR approach and the steady state treated plants made a complete

224 comparison difficult. Measurement of NO_3^- uptake capacity and total N is required to

225 effectively link the Chapter 2 observations to the end NO_3^- uptake effects. In addition,

226 extending sampling to a full 24 h time period is necessary to understand whether the observed

227 changes in the 0 mM NO_3^- control plants were due to N starvation or were diurnally regulated.

228 As a result an extension of this study to provide better sampling alignment and a longer

229 sampling window would inform future experiments in this field, and help to leverage the data

230 from these different experimental approaches towards understanding the NO_3^- uptake system

231 in cereals.

232 **5.2.7 Continuing the cis-trans regulation discovery**

233 In Chapter 4, six putative functionally and evolutionarily conserved regions were identified in

234 the promoter of *ZmNRT2.5* with no evidence of known transcription factor binding sites.

235 These sequences are an attractive new resource for the discovery of novel cis-trans regulatory

236 mechanisms associated with the low N induced expression of *ZmNRT2.5*. The next step is to

237 test these motifs by applying a minimal promoter study *in planta* to assess whether these

238 elements drive gene expression under low NO_3^- conditions. Following this, a yeast-one-

239 hybrid approach could be employed to identify proteins which bind these elements to

240 determine the transcriptional controllers of *ZmNRT2.5*.

5.3 Summary

Understanding the intricacies of the NO_3^- uptake system is essential for improving NUpE and overall NUE in cereal crops. The findings in this thesis have identified key time points for future transcriptome analysis, and revealed putative cis-elements as new leads for discovering novel cis-trans regulatory elements associated with the regulation of NO_3^- uptake in maize. Ultimately, further research may lead to the identification of key regulatory genes as candidates for the improvement of NUpE and overall N use efficiency in cereal crops. The information contained within this thesis has provided new information into the complexities of the NO_3^- uptake system, moving the scientific community forward to the improvement of NUpE and overall NUE in cereal crops.

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