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NITRATE SENSING BY MAIZE ROOTS: A KEY ROLE FOR NITRIC OXIDE SIGNALING IN THE TRANSITION ZONE

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Table of contents

Riassunto ... **8**

Summary ... **14**

Chapter I - General introduction ... **19**

1. Preface ... **20**

2. Nitrogen use efficiency (NUE) ... **26**

2.1 Nitrogen acquisition by plants ... 27

2.1.1 Nitrate transporters and channels ... 28

2.1.1.1 Nitrate uptake from soil ... 29

2.1.1.2 Nitrate efflux, translocation and allocation in vegetative tissues and seeds, and nitrate-induced stomatal movement ... 31

2.2 Nitrate assimilation through the NR-NiR-GS-GOGAT pathway ... 33

2.3 Nitrate sensing and signaling in plants ... 36

2.3.1 Nitrate sensors ... 37

2.3.2 Nitrate signal transduction ... 39

2.4 Regulation of root growth and development in response to nitrate ... 41

2.5 Nitric oxide as a new regulator of root growth in response to nitrate ... 46

3. Approaches to improve nitrogen use efficiency ... **50**

4. Aim of work ... **55**

5. References ... **56**

5.1 Articles and books ... 56

5.2 Websites cited ... 72

Chapter II - NO homeostasis is a key regulator of early nitrate perception and root elongation in maize* ... **73**

1. Abstract ... **74**

2. Introduction ... **75**

3. Materials and Methods ... **77**

3.1 Maize growth and experimental design ... 77

3.2 Growth of maize seedlings in agar medium ... 79

3.3 Morphological analyses ... 79

3.4 RNA extraction and cDNA synthesis ... 80

3.5 Selection of genes to be evaluated, maize sequences identification and primers design ... 81

3.6 Real time qPCR ... 81

3.7 NO detection ... 82

4. Results ... **83**

4.1 Nitrate exerts specific effects on genes involved in NO homeostatic control 83
4.2 Root growth responds specifically to nitrate availability 84
4.3 NR-dependent NO production after nitrate supply 86
4.4 Genes putatively involved in the control of NO homeostasis are involved in the early response to nitrate 87
4.5 The transcription for genes involved in NO production and scavenging is maximally induced in the transition zone (TZ) of roots after nitrate induction 89
4.6 The nitrate induced root length increase is dependent on a NO signalling pathway 90
4.7 The nitrate-induced NO signalling pathway is a localized effect 92
5. Discussion 94
6. Acknowledgements 100
7. References 101
8. Supplementary data 110
Chapter III - NO signalling is a key component of the root growth response to nitrate in *Zea mays* L. 115
1. Abstract 116
2. Introduction 117
3. NO_3^- has a doubtful role in regulating the growth of primary roots 118
4. The root transition zone 120
5. Nitrate affects root elongation through NO-elicited actions 121
6. Acknowledgements 123
7. References 123
Chapter IV - Immunofluorescence labeling of maize root cells reveals effects on cell wall deposition and PIN1-mediated auxin accumulation in the transition zone in response to nitrate. 131
1. Introduction 132
2. Materials and methods 135
2.1 Plant material, chemicals and experimental layout 135
2.2 Indirect immunofluorescence labeling 135
2.3 Measurements and statistical analysis 136
3. Results 137
3.1 Xyloglucans immunolocalization 137
3.2 Auxin and PIN1 immunolocalization 139
4. Discussion 140
5. References 146

Chapter V - General conclusions 151
References...156

Riassunto

L'attuale modello di produzione agricola di tipo intensivo ha permesso di sfamare negli ultimi cinquanta anni una popolazione mondiale cresciuta in maniera esponenziale, ma, allo stesso tempo, è stato anche la causa dell'insorgere di preoccupanti problemi a livello ambientale, sia a scala locale che globale. Molti di questi problemi sono dovuti all'uso massiccio di fertilizzanti azotati di sintesi che derivano dal processo industriale Haber-Bosch. Mediamente più del 50% dell'azoto fornito come fertilizzante non è assorbito dalle colture, causando una catena di effetti negativi a livello ambientale che hanno come target finale la salute dell'uomo. Il mais (*Zea mays* L.) rappresenta già una delle principali colture agrarie e varie stime attribuiscono a questo cereale un ruolo alimentare fondamentale anche nei prossimi decenni, quando la popolazione mondiale dovrebbe assestarsi, secondo le proiezioni, tra i 9.2 e gli undici miliardi di abitanti. Parallelamente al raggiungimento dell'obiettivo della sicurezza alimentare, l'agricoltura è chiamata a produrre rese crescenti in maniera sempre più sostenibile a livello ambientale. Punto centrale per la ricerca in agricoltura, il miglioramento dell'efficienza di utilizzo dell'azoto (in inglese NUE, Nitrogen Use Efficiency) da parte delle colture agrarie, per garantire produzioni crescenti senza ulteriore accumulo di forme reattive dell'azoto nell'ambiente. Per questo motivo, la comprensione dei meccanismi molecolari e fisiologici che regolano l'adattamento dell'apparato radicale delle colture agrarie alle fluttuazioni delle concentrazioni di azoto nel terreno, rappresenta un obiettivo primario nell'ottica di un progressivo utilizzo di nuove tecnologie finalizzate ad una agricoltura più sostenibile.

Il corretto sviluppo fisiologico di una coltura agraria non dipende, infatti, solo dalla disponibilità di azoto nel terreno, ma anche dall'efficienza d'intercettazione del nutriente da parte dell'apparato radicale. Le piante generalmente sono in grado di assimilare sia nitrato che ammonio tuttavia, nei suoli agrari ben aerati, la principale fonte di azoto è rappresentata dal nitrato. Negli ultimi anni numerosi lavori hanno evidenziato un ruolo del nitrato come molecola segnale, considerato che moltissimi geni coinvolti nei processi di sviluppo e di crescita della pianta sono regolati da questo anione. Tuttavia molti di questi aspetti rimangono ancora da decifrare nel loro insieme. Anche l'ossido nitrico (NO) si sta ritagliando negli ultimi anni un ruolo sempre più importante nell'ambito dello studio delle risposte delle piante agli stress. Tuttavia, il suo esatto ruolo in risposta agli stress nutrizionali è stato solo

abbozzato. In questo lavoro è stata studiata in dettaglio la produzione di ossido nitrico che si registra nelle radici di mais in presenza di nitrato, concentrandosi inizialmente sullo studio della regolazione dei geni coinvolti nell'omeostasi dell'ossido nitrico. In un secondo momento è stato anche preso in esame il ruolo dell'ossido nitrico come attore chiave nel modulare differenti risposte morfologiche a livello radicale in presenza di nitrato.

Per meglio discriminare eventuali effetti specifici delle varie forme di azoto a livello transcrittomico, l'espressione di un numero di geni in precedenza identificati per essere regolati dall'azoto è stata analizzata in condizioni di disponibilità/carenza sia di nitrato che ammonio. In particolare, il profilo trascrizionale di cinque geni, che specificamente esprimono (i) una nitrato reduttasi citosolica (NR1), (ii) due differenti isoforme di emoglobine di tipo non simbiotico (nsHbs), (iii) una nitrito reduttasi (NiR) e, infine, (iv) un trasportatore ad alta-affinità del nitrato (NRT2.1), ha evidenziato una risposta esclusiva al nitrato. Questi geni non hanno manifestano, infatti, variazioni significative nei livelli di espressione quando è stata utilizzato come unica fonte azotata l'ammonio. Questo primo screening è stato altresì importante perchè ha permesso di focalizzarsi nelle analisi successive solamente su quei geni che rispondono specificatamente al nitrato; gli stessi che hanno dimostrato poi, durante l'avanzamento della ricerca, essere anche quelli attivamente coinvolti nel controllo dell'omeostasi dell'ossido nitrico, e cioè la nitrato reduttasi e l'emoglobina. Questi primi risultati ci hanno permesso quindi di ipotizzare un modello di regolazione molecolare che vede coinvolti questi geni in un'azione coordinata responsabile della sintesi (NR) e rapida inattivazione (nsHb) dell'ossido nitrico, in quanto molecola estremamente reattiva e tossica per la cellula, in risposta al nitrato.

In aggiunta, una serie di analisi morfologiche sulla radice elaborate grazie all'aiuto di uno specifico software (WinRhizo) ha evidenziato come il nitrato agisca anche in maniera specifica e differenziale sullo sviluppo dell'apparato radicale (sono stati presi in esame come indici radicali: (i) la lunghezza totale, (ii) la superficie totale, (iii) il diametro medio delle radici e (iv) il numero di apici laterali).

L'ipotesi che l'ossido nitrico sia prodotto nelle radici in risposta al nitrato è stata vagliata successivamente per mezzo di una serie di misurazioni *in vivo* dell'ossido nitrico, usando come sonda specifica per l'ossido nitrico il composto DAF-2DA e misurando poi la fluorescenza (indice della presenza di ossido nitrico) negli apici radicali sia allo stereo microscopio che al microscopio confocale. I risultati ottenuti hanno confermato pienamente

la teoria che suggerisce come l'ossido nitrico sia specificatamente sintetizzato dall'attività della nitrato reduttasi in presenza di elevate concentrazioni di nitrato. Le osservazioni al microscopio hanno inoltre evidenziato come la fluorescenza, indice della presenza dell'ossido nitrico, fosse massima e concentrata in una specifica area dell'apice radicale, e cioè la zona di transizione, situata tra l'apice meristematico e la zona di allungamento. Infatti successivamente, grazie all'utilizzo di un inibitore dell'attività dell'enzima nitrato reduttasi (tungstato) e del composto cPTIO (uno scavenger dell'ossido nitrico), l'ipotesi di partenza che prevede la sintesi dell'ossido nitrico legata all'attività della nitrato reduttasi in risposta al nitrato, e la rapida inattivazione da parte dell'emoglobina, è stata dimostrata e ulteriormente confermata anche dall'utilizzo di queste sostante in analisi di espressione genica, supportando pienamente l'ipotesi di una azione concertata di questi geni finalizzata alla regolazione omeostatica della sintesi/scavenging dell'ossido nitrico in risposta al nitrato.

Una nuova serie di analisi è stata in seguito condotta nell'ottica di meglio caratterizzare eventuali differenze nei livelli di espressione di questi geni nelle diverse zone che compongono l'apice radicale. A questo proposito, l'espressione dei geni *NR1*, *NiR* e le due *nsHbs*, in risposta al nitrato, è stata analizzata specificatamente in quattro diverse zone della radice: (i) il meristema, (ii) la zona di transizione, (iii) la zona di allungamento e infine (iv) la zona di maturazione. In radici allevate in assenza di nitrato, il livello massimo di accumulo di trascritto per tutti i geni considerati si è concentrato nel meristema. In risposta al nitrato però, la zona di transizione ha registrato invece l'accumulo maggiore. In base a questi ultimi dati e allo studio della letteratura, suggeriamo l'ipotesi che il nitrato agisca nell'attivazione di una via di segnalazione che ha come risultato finale una risposta differenziale a livello morfologico della radice, per meglio rispondere alla presenza dell'anione nel terreno, e di come la percezione e l'attivazione di questa via di signaling avvenga specificatamente nella zona di transizione. Questa ipotesi non deve stupire, considerando la crescente mole di lavori che attribuisce a questa piccolissima zona dell'apice radicale un ruolo fondamentale nella percezione degli stimoli (sia interni sia esterni) e nella immediata traduzione in risposte adattive all'ambiente.

Partendo da queste osservazioni, le analisi successive sono state focalizzate quindi allo studio degli effetti specifici che il nitrato esercita sulla regolazione della crescita della radice primaria, fenomeno che prende avvio a livello cellulare immediatamente a monte della zona di transizione e che gioca un ruolo cruciale nell'adattamento delle radici ai

cambiamenti della disponibilità del nutriente nel suolo. L'analisi condotta ha evidenziato un effetto di stimolazione del nitrato nella crescita ma soprattutto ha confermato una probabile partecipazione dell'ossido nitrico in questo processo di induzione all'allungamento, considerando che l'aggiunta di cPTIO ad una soluzione nutritiva ricca di nitrato riduce la crescita. Parallelamente, l'aggiunta di un donatore di ossido nitrico (SNP) in una soluzione nutritiva questa volta priva di nitrato ha evidenziato coerentemente un effetto di stimolazione alla crescita. L'utilizzo di tungstato infine, che si è manifestato in un forte effetto di inibizione all'allungamento in plantule allevate in presenza della fonte di azoto, suggerisce ulteriolmente un ruolo chiave della nitrato riduttasi nella produzione di ossido nitrico in risposta al nitrato, e di come questo meccanismo di fine-tuning omeostatico abbia degli effetti a livello fenotipico.

Per riassumere, questi dati collettivamente presi indicano come nella radice di mais esista una via di segnalazione in risposta al nitrato mediato dall'attività dell'ossido nitrico, in concerto con la regolazione omeostica garantita dall'azione condivisa della nitrato reduttasi e dell'emoglobina. Tuttavia nuove analisi si rendono necessarie per caratterizzare meglio questa via di signaling e scoprire nuovi attori che partecipano a valle della sintesi di NO in risposta al nitrato, e che intervengono infine nel modulare differenzialmente la morfologia della radice. A questo riguardo, molti studi suggeriscono di concentrare la ricerca sugli effetti dell'ossido nitrico a livello di modificazioni del citoscheletro, così come sulle interconnessioni tra l'ossido nitrico e l'ormone vegetale auxina.

Partendo da queste ultime considerazioni, la parte finale del mio progetto di dottorato è stata finalizzata allo studio degli effetti che il nitrato esplica, specificatamente nella zona di transizione, sulla formazione della parete cellulare (un processo cellulare mediato dall'attività del citoscheletro) e delle emicellulose in particolare (xiloglucani). Inoltre, un'analisi preliminare è stata anche condotta per verificare gli effetti del nitrato sul trasporto polare dell'auxina. I risultati fin qui ottenuti dall'analisi immunochimica, suggeriscono come il nitrato abbia un effetto significativo nella regolazione del trafficking vescicolare degli xiloglucani nella zona di transizione. Infatti, questi costituenti di parete dai nostri primi dati, mostrano un più elevato tasso di sintesi o recycling in risposta all'anione. L'utilizzo dell'inibitore specifico del trafficking vescicolare Brefeldin A (BFA), ha confermato ulteriormente questa ipotesi. Questa ipotesi assegna al nitrato un ruolo di stimolazione del recycling vescicolare degli xiloglucani. Coerentemente con i dati precedenti che hanno

mostrato un effetto di induzione del nitrato nella crescita della radice, un più elevato trafficking di materiale di parete può essere interpretato come un'azione per permettere alle pareti cellulari di distendersi maggiormente e di assecondare un più rapido sviluppo necessario per l'allungamento evocato dal nitrato. Infine, è stato visualizzato anche un effetto del nitrato riguardante il trasporto polare delle auxine mediato dai trasportatori PIN1 nella zona di transizione. In presenza di nitrato infatti, l'auxina si localizza preferibilmente a livello di cross-walls (end-poles), e questo effetto è specificatamente indotto dal nitrato. Questo dato è stato supportato anche dalle osservazioni sull'immunolocalizzazione dei trasportatori PIN1, che hanno mostrato di co-localizzare in risposta al nitrato negli stessi siti di accumulo (end-poles) precedentementi osservati per l'auxina. Nuove analisi sono necessarie per meglio inquadrare il ruolo giocato dall'ossido nitrico all'interno in questi processi cellulari, considerando le numerose interconnessioni che legano NO, auxina e citoscheletro nella regolazione delle risposte adattative della radice all'ambiente esterno.

Summary

Over the past five decades, intensive agriculture has been able to increase the rate of food production more rapidly than that of human population growth but, at the same time, has also given rise to a series of negative environmental consequences, at both local and global level. Most of them are directly due to the large use in agriculture of synthetic ammonia fertilizers industrially produced by the Haber-Bosch process. Over 50% of the applied nitrogen in fact, is lost from the plant-soil system, leading to severe environmental damages and to negative impacts on human health. Maize (*Zea mays* L.) is one of the world's major crops and is also expected to give an important contribution to human nutrition in the next few decades, when world population should exceed 8 billion people and rise to 9.2/11 billion by 2050. To ensure future global food security, increasing crop yields are dramatically needed, however, sustainable ways of crop production are far from being achieved, considering also that further nitrogen accumulation in the environment is expected to be increased in the future without an adequate enhancement of Nitrogen Use Efficiency (NUE) in the main crops. For this reason, the understanding of the molecular events underlying root adaptation to nitrogen fluctuations is a primary goal to develop tools for sustainable agriculture.

Crop plant development in fact, is not only strongly dependent on nitrogen availability in the soil but also on the efficiency of its recruitment by roots. Plants take up and assimilate both nitrate and ammonium, but nitrate is the main source of inorganic nitrogen for plants in aerobic soil conditions typical of most cultivated soils. In addition to its role as a nutrient, nitrate acts as a signaling molecule regulating the expression of the genes involved in growth and developmental processes. However, the mechanisms governing the sensing of nitrate by roots and of the signaling leading to an altered development of roots are still only partially characterized. Nitric oxide (NO) has been recently proposed to be implicated in plant responses to environmental stresses, but its exact role in the response of plants to nutritional stress is still under evaluation. In this work, the role of NO production by maize roots after nitrate perception was investigated by focusing on the regulation of transcription of genes involved in NO homeostasis and by measuring NO production in roots. Moreover, its involvement in the root growth response to nitrate was also investigated.

To better discriminate nitrate-specific effects from those more generally N-dependent, the expression of a number of genes previously identified as being nitrogen-responsive, was evaluated in response to nitrate/ammonium supply and deprivation. The transcriptional response of five genes encoding (i) the cytosolic nitrate reductase NR1, (ii) two different non-symbiotic hemoglobins (nsHbs) isoforms, (iii) a gene encoding nitrite reductase together with (iv) a gene encoding the high-affinity root nitrate transporter (NRT2.1), evidenced a very strong and exclusive nitrate responsiveness in roots. Conversely, no effects were observed when ammonium was supplied as the sole nitrogen source. This first screening allowed the current work to focus later only on genes whose expression seems to depend exclusively on nitrate and to be specifically involved in the control of NO biosynthesis and scavenging. Our results highlight the importance of the coordinate spatio-temporal expression of nitrate reductase and non-symbiotic hemoglobins in controlling the NO homeostasis in the maize root after nitrate provision.

In addition, data obtained by analysing root morphological parameters by the WinRhizo software underlined the same specificity of nitrate, which significantly affected root growth when supplied to N-deprived roots.

To deepen the hypothesis that nitric oxide may be produced by roots as an early signal of nitrate perception, NO *in vivo* detection was carried out. Results obtained using the DAF-2DA probe and stereo- and confocal microscopy evidenced a clear induction of fluorescence after nitrate provision. Very interestingly, the main zone of NO production seemed to be located immediately above the meristematic apex and more precisely to coincide with the root transition zone. The fluorescence detected after nitrate supply was not revealed in the presence of the specific nitrate reductase inhibitor tungstate, giving support to the role of NR in nitric oxide production. Moreover, the addition of the nitric oxide scavengers cPTIO together with nitrate, similarly suppressed the development of fluorescence, confirming the specificity of NO detection by the probe. These results suggest that a NR-dependent NO burst occurred immediately after nitrate supply to roots. The NR-dependent NO production observed after nitrate supply was then further confirmed by the strong induction of *NR1*, *NiR*, and *nsHbs* transcription in the early phases of nitrate perception. In this case also, the transcription was significantly inhibited in response to tungstate and cPTIO addition, endorsing the cooperation between nitrate reductase and haemoglobin activities in the finely tuned control of NO homeostasis.

To deepen the spatial regulation of NO homeostasis balance, the expression of *NR1*, *NiR* and *nsHbs* genes was also analysed in four different root zones (*i.e.* meristem, transition zone, elongation zone, maturation zone) both in nitrate-depleted and in nitrate-treated seedlings. In N-starved roots, all transcripts evidenced their maximum accumulation at the meristem level. This pattern radically changed when nitrate was furnished to roots with a very significant increase of transcript abundance in the transition zone. As a result, we suggest that nitrate supply could activate its own sensing by stimulating NO production by the transition zone cells, thus initiating a signalling pathway contributing to the physiological adaptation (*e.g.* root growth) to nitrate fluctuations.

Based on the preliminary results showing the preferential localization of NO production at the level of the transition zone, the attention was then focused on nitrate effects on root elongation, which takes place in the zone immediately above and neighbouring the transition zone. Our finding evidenced a strong and specific induction of root elongation of young maize seedlings supplied with 1 mM nitrate and a drastic inhibition in the presence of ammonium, cPTIO, and tungstate. On the contrary, when the negative control ($-NO_3^-$) was supplied with a NO donor (SNP) the root length increased significantly. These results strongly suggest that the NO generated through NR should significantly contribute to the root lengthening noticed after nitrate provision.

To summarize, it would seem that the NO-mediated pathway here described represents an early alert system for external nitrate sensing by root cells, which seem to individually possess the competence to activate this pathway when external nitrate is perceived. Additional experiments are necessary to better understand the functioning of this NO-mediated pathway and to identify the downstream events that link the NO burst with the physiological redirection of root growth. In this regard, it has been reported that NO signaling can alter cell polarity and cytoskeleton-mediated vesicle trafficking processes, thus affecting cell growth and root morphogenesis. This suggests that there should be more downstream effectors of NO action, acting either in parallel or in series with cytoskeletal constituents. Furthermore, since NO and phytohormones auxin act synergically to control diverse aspects of root biology and also considering that lateral root development in response to nitrate is strongly auxin dependent, a role of NO as a coordinator of nitrate and auxin signaling to control the overall root response to the anion cannot be excluded.

In order to try to answer to these last questions, in the final part of my Ph. D. thesis, we focused the attention on studying both cytoskeleton-mediated xyloglucans (a major primary cell wall component) modifications and polar auxin transport in the maize root transition zone cells in response to nitrate. Preliminary data achieved so far by using immunofluorescence labelling indicate that nitrate is able to modify cell wall recycling in the transition zone. Xyloglucans in fact, were very abundant especially in the sample subjected to nitrate treatment, when compared to the negative control, suggesting a higher rate of XGs synthesis /or recycling, in response to the anion in the maize root transition zone. Additionally, Brefeldin A (a chemical which prevents vesicle formation in the exocytosis pathway while allowing endocytosis, resulting in the cytoplasmic accumulation of all recycling molecules) treatment partially failed in removing all XGs from cell walls in +N samples, since a marked immunofluorescence was still visible at cross walls, despite the strong effect of the drug that resulted in the abundance of BFA-compartments also within these cells. These latter data could suggest that nitrate promotes a higher rate of XGs recycling in order to maintain a loosened cell wall structure, thus allowing an extensive and fast cell elongation in response to the anion. Taken together, these data open a fascinating scenario in which nitrate might act in promoting rapid cell elongation of root apex by regulating, in a mechanism as yet unknown, the synthesis or the turn-over (or both) of xyloglucans within root transition cells. Also PIN1-mediated auxin accumulation seems to be interfered in response to nitrate. IAA signal in fact, was strongly localized at the cross wall (end-poles) of transition zone cells only in nitrate-supplied roots, thus suggesting that IAA end-poles labelling was probably due to increased IAA fluxes triggered specifically by nitrate. In support to this hypothesis we also observed that IAA and its transporter PIN1 protein co-localize in NO_3^--treated roots at the cross walls (end-poles), thus providing further, although preliminary, evidences that nitrate in the maize root transition zone is able to increase IAA-fluxes, in a mechanism as yet unknown, that involved also PIN1 proteins. Further immunolabeling data, by also using NO donors and scavengers, will be needed to better understand the coordinated actions of nitric oxide, auxin and cytoskeleton adjustments in tightly regulating root motoric response to nitrate.

Chapter I - General introduction

1. Preface

Intensification of agricultural systems is undoubtedly one of the main global changes occurred in the XX century, which have affected not only the traditional farming but also economy and society throughout the world. In the last five decades overall food production kept pace with the population growth and now modern agriculture feeds more than seven billion people. The breakthrough in crop productivity was extremely intense worldwide, thanks to the novel technological advances introduced during the "Green Revolution". In this scenario, a massive use of artificial nitrogenous fertilizers has played a central role, however, at the same time, environmental concerns about this high application of synthetizing ammonia products have emerged in most of agro-ecosystems. In addition, the capability to maintain long-term intensive agriculture is also debated, taking into account the fact that human population is still increasing and further food production will be consequently necessary. Thus, the shift towards more sustainable agriculture has become a compelling challenge and the breeding of new crops with enhanced nitrogen use efficiency (NUE) a major issue in plant biology.

Over the past five decades, intensive agriculture has been able to increase the rate of food production more rapidly than that of human population growth, as the global population increased from about three billion people in the late 1950s to over seven billion people (according to the United States Census Bureau (USCB), as of 2013, world population is estimated at 7.177 billion). This extraordinary result was mainly due to the enormous growth of global cereal production, considering that cereal species belonging to monocots family provide >50% of human calories (Rich and Watt, 2013). In fact, total grain production is almost tripled since 1961 to 2012, increasing from approximately 880 million tones (Mt) to over 2500 Mt (FAOSTAT, 2012). This trend was not paired with an analogue expansion of the amount of land devoted to arable agriculture that, in the same time, globally has increased by only 9% (Godfray *et al.*, 2010; FAOSTAT, 2012). Among cereals, maize (*Zea mays* L.) is second only to wheat in total area harvested but first in total grain production (872 Mt), to which must be added about 450 Mt of maize harvested for forage and silage (FAOSTAT, 2012). These last few data are sufficient to understand the importance of this monocot in crop production and the consequent need to improve our knowledge on maize biology in

order to ensure, on one hand, higher yields in an increasing world population and, on other hand, reduced environmental costs of maize cultivation.

Intensification of agricultural systems in fact, has also given rise to a series of negative environmental consequences, at both local and global level. Most of them are directly due to the large use in agriculture of synthetic ammonia fertilizers industrially produced by the Haber-Bosch process, in which natural gas (CH_4) is burned to produce hydrogen that react with N_2 under high temperature and very high pressure to form ammonia (NH_3). This process was one of the most important industrial innovations of the 20th century, as it allowed to feed a rapidly growing population and, currently, almost 50% of humanity is still dependent upon the Haber-Bosch process (Erisman *et al*., 2008). Overall, the increase in application of synthetic N fertilizers in the last five decades was dramatic: from about 10 Tg (1 Tg = 1 million tonnes) N/year in the late 1950s to more than 100 Tg N/year in 2008 (Robertson and Vitousek, 2009). The most recent estimates indicate 120-125 Tg of synthetic N applied to agricultural system each year as a more realistic value, and the magnitude of anthropogenic N fixation is so large that it has paired the natural terrestrial sources of reactive nitrogen (N_r) species[1] (Fowler *et al*., 2013), as showed in Fig. 1. The tremendous impact on the environment of this massive application of synthesized N in agro-systems is exacerbated also by considerable greenhouse gases emissions derived from the huge amount of energy required by the Haber-Bosch process: it has estimated as 2.5% of the total world supply (Erisman *et al*., 2008). However, the major problem of excessive fertilizing regimes in intensive agriculture is due to the enhancing losses of N_r to the environment because of the low ability of modern crops to take up efficiently nitrogen from the soil. According to several Authors (Raun and Johnson, 1999; Baligar *et al*., 2001; Robertson and Vitousek, 2009) the Nitrogen Use Efficiency (NUE) in agriculture is very low, with an average of only 30-50% taken up by the plant and the remainder lost from the plant-soil system, which ultimately caused serious pollution to the global biosphere (Good and Beatty, 2011). Thus, the selection of cultivars with enhanced NUE is a fundamental goal for modern agriculture and for cereals in particular, considering that approximately 65% of globally N fertilizers are used for cereal production (Garnett *et al*., 2009).

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[1] Reactive nitrogen (Nr) species include all N species except N_2: inorganic reduced forms (ammonia [NH_3] and ammonium [NH_4^+]), inorganic oxidized forms (nitrogen oxide [NO_x], nitric acid [HNO_3], nitrous oxide [N_2O] and nitrate [NO_3^-]) and organic compounds.

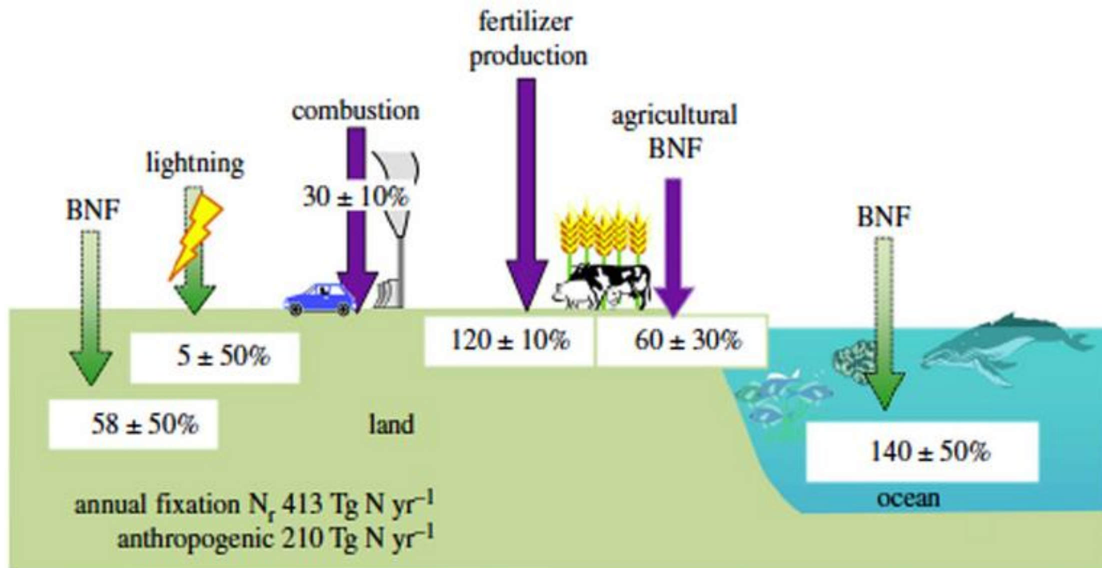

Figure 1. Global nitrogen fixation, natural and anthropogenic in both oxidized and reduced forms through combustion, biological fixation, lightning and fertilizer and industrial production through the Haber–Bosch process for 2010. The arrows indicate a transfer from the atmospheric N_2 reservoir to terrestrial and marine ecosystems, regardless of the subsequent fate of the N_r. Green arrows represent natural sources, purple arrows represent anthropogenic sources, from Fowler *et al*., 2013.

Nitrogen (N) is one of the most abundant elements in Earth's atmosphere, hydrosphere and biosphere; however, it is also the least available for living beings, since it is present predominately in its molecular un-reactive form N_2 (99% of the total amount of N in nature) that is reduced to ammonium compounds only by a few species of specialized microorganisms through the biological nitrogen fixation (Fowler *et al*., 2013). Before the widespread diffusion of intensive agricultural systems, the rate of Reactive nitrogen (N_r) formation was approximately counterbalanced by the rate of denitrification (Robertson and Vitousek, 2009) but presently, as mentioned before, anthropogenic sources of newly created N_r has paired the global amount of natural terrestrial sources (Fowler *et al*., 2013) leading to an important change in the global nitrogen cycle (Galloway *et al*., 2013). N_r are highly mobile and the negative effects on environment are numerous and magnified by the "nitrogen cascade", in which a single N_r atom can trigger a cascade of negative environmental impacts in sequence (Galloway *et al*., 2013), and with time, considering that the lifetime of N_r in terrestrial ecosystems and in the oceans is estimated a few decades (Fowler *et al*., 2013). As nitrogen is a major nutrient, massive variations in its supply deeply influence the productivity of ecosystems and change the competition between species, causing a widespread loss of biodiversity, to which must be added a multitude of effects in the atmosphere (being N_r species precursor of tropospheric ozone), in freshwater and marine

systems, and finally on human health (Erisman *et al*., 2013; Fowler *et al*., 2013; Galloway *et al*., 2013). In addition, N_r accumulation in the environment also contributes to the radiative forcing of climate change (Robertson and Vitousek, 2009; Cameron *et al*., 2013). A comprehensive illustration of the “nitrogen cascade” is represented in Table 1 and explained in more detail in Box 1.

In 2030, world population should exceed 8 billion people and rise to 9.2/11 billion by 2050 (Parry and Hawkesford, 2010). This population growth means at least to expand crop production of the 50% by 2030 and double by 2050 to meet projected demands, also because of a progressive shift towards diets with higher proportion of meat (Godfray *et al*., 2010; Tilman *et al*., 2011). To ensure future global food security, increasing crop yields are dramatically needed, however, sustainable ways of crop production are far from being achieved, considering also that further N_r accumulation in the environment is expected to be increased in the next decades without an adequate enhancement of NUE in the main crops (Fowler *et al*., 2013). At the same time that demand for food is growing, crop production is being limited by expanding urbanisation, land degradation (due to erosion and salinization), non-food uses of crops (mainly for bioenergy production) and climate change (Parry and Hawkseford, 2010; Lobell *et al*., 2011). Thus, a call for a second “Green Revolution”, which would allow increasing crop yields in a more sustainable way, is dramatically needed. However, traditional breeding strategies to enhance NUE in crops have experienced a plateau, and this indicates that new solutions, which should be able to increase yields while decreasing N application, are no longer delayed (McAllister *et al*., 2012). A possible way out of this impasse can be the successful application of biotechnology to crop breeding (Godfray *et al*., 2010; Parry and Hawkseford, 2010), but this requires a deeper understanding of the physiological, genetic and molecular mechanisms regulating NUE in plants.

BOX 1. Nitrogen losses from the soil/plant system and effects on environment and human health

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The N cycle is complex in natural ecosystems as well as in agriculture and includes several connections between air, water and soil (Fig. 2). Regarding crop systems, what makes N different is the absence of an available N pools in most soils: unlike other elements, there is no potentially available N in the rocks and, thus, N must come outside the plant-soil system by fertilization to re-equilibrate the annual N loss via harvest. However, as discussed before, most of the N added to crops does not reach its ultimate target and the N surplus is lost to the aqueous and atmospheric environments where it becomes a serious pollutant. Among the reactive N species, the leaching of very soluble compound such as nitrate (NO_3^-) represents a major risk to human health and a threat to the environment. Contamination of drinking water by an excess amount of NO_3^- can cause methemoglobinemia in babies and has also been linked to cancer and heart disease (Grizzetti *et al*., 2011). In this scenario, the same Author reports that about 20% of the European lives in areas where NO_3^- concentrations exceed the recommended level of 11.3 mg/L. In addition, NO_3^- in concert with ammonia (NH_3) and ammonium (NH_4^+) that enter rivers or lakes contributes to eutrophication, a natural process in which increasing N_r levels lead to surface water hypoxia and to the release of toxic compounds due to explosive growth of phytoplankton and algal species. This in turn dramatically impacts higher trophic level organisms and the rest of aquatic ecosystem (Grizzetti *et al*., 2011, and references therein). In addition to solution losses, N_r can also been lost to the atmosphere as different kinds of polluting N-containing gaseous, such as nitrous oxide (N_20), nitric oxide (NO) and nitrogen dioxide (NO_2), collectively known as NO_x, and ammonia (NH_3). Nitrous oxide is a very strong greenhouse gas with nearly 300 times the global warming potential per unit weight of carbon dioxide (CO_2) and has also implicated in stratospheric ozone depletion (Mulvaney *et al*., 2009). The concentration of N_20 in the troposphere has increased from 270 parts per billion (ppv) in the preindustrial era to around 320 ppv today, contributing about 6% to the global greenhouse gas forcing that drives climate change (Forster *et al*., 2007; Good and Beatty, 2011). About 80% of this N_20 is associated with intensive agriculture and the most part (>50%) with N-fertilized soils (Robertson, 2004). NO_x emissions in the atmosphere (of which about one-quarter of the total is from agriculture) are directly linked to the generation of secondary pollutants (such as tropospheric ozone and other photochemical oxidants and aerosols) that are responsible for severe damage to human health and to vegetation on one hand (Erisman *et al*., 2013; Fowler *et al*., 2013) and for N_r deposition on environment by acid rain on the other hand (Robertson and Vitousek, 2009; Gallowey *et al*., 2013). Finally, the loss of N_r from the soil/plant system due to ammonia volatilization is returned to the earth's surface through wet deposition (acid rain) or dry deposition (i.e. attached to particulate matter), causing acidification and eutrophication of natural ecosystem (Cameron *et al*., 2013). Unlike NO_x, a very large fraction of global NH_3 emissions are from agricultural sources, including N fertilizer application (about a quarter of the total; Galloway *et al*., 2004). To conclude, the global N cycle has been largely modified by human activities and the obvious benefits for food security has been accomplished by a great number of negative effects on biosphere and human health. Thus, there is a compelling need to reduce the use of N fertilizers in most of agro-ecosystems and newly developed crops with greater nitrogen use efficiency (NUE) can be a key solution to increase the sustainability of intensive agriculture and minimize the impacts on environment.

Table 1. Illustrating the nitrogen cascade: a possible life cycle of a nitrogen atom following fixation in the Haber–Bosch process to NH_3 and its pathway through terrestrial and marine ecosystems and the atmosphere before returning to the atmospheric N_2 reservoir. The single N atom contributes en route to eutrophication and acidification of terrestrial and marine ecosystems, and to human health and climate effects, modified from Fowler *et al*., 2013.

transformation	pathway	environmental effects
N_2 fixation: Haber-Bosch process $N_2 \rightarrow NH_3$	industry	energy intensive process, production of CO2 plus all the consequences of the N_r as it cascades through soils, the atmosphere and aqueous phases
N fertilizer on crops	agricultural lands	provision of food for human consumption
NH_4 nitrified to $\rightarrow NO_3^-$ NO in soil $\rightarrow$ atmosphere oxidation of NO $\rightarrow NO_2^- \rightarrow HNO_3$	NO emission from soil to atmosphere and ozone production during volatile organic compound degradation	ozone effects on vegetation and human health
aerosol formation: $HNO_3 \rightarrow NO_3^-$	in atmosphere	planetary albedo, human health
wet + dry deposition NO_3^- to soil $\rightarrow$vegetation $NO_3^- \rightarrow$ R-NH_2	removal from atmosphere and transfer to plant biomass	eutrophication acidification
consumption by herbivores (excreted as urea) R-$NH_2 \rightarrow CO(NH_2)_2$	plant biomass $\rightarrow$ animal protein $\rightarrow$ excreted and returned to soil	eutrophication
urea converted to NH_3 in soil and released to atmosphere	soil to atmosphere flux of NH_3	eutrophication
NH_3/ NH_4^+ uptake by vegetation	removal from atmosphere by dry deposition to vegetation	eutrophication
decomposition R-NH3 $\rightarrow NH_4^+$	vegetation to soil	eutrophication
NH_4^+ nitrified to NO_3^- transferred to river/estuary/open ocean	soil to ground water $\rightarrow$ river $\rightarrow$ ocean	eutrophication
ocean uptake in phyto/zooplankton	shelf seas to open ocean	eutrophication
denitrification in ocean sediments $NO_3^- \rightarrow$ N2	returns to atmosphere as N_2 and N_2O	climate change

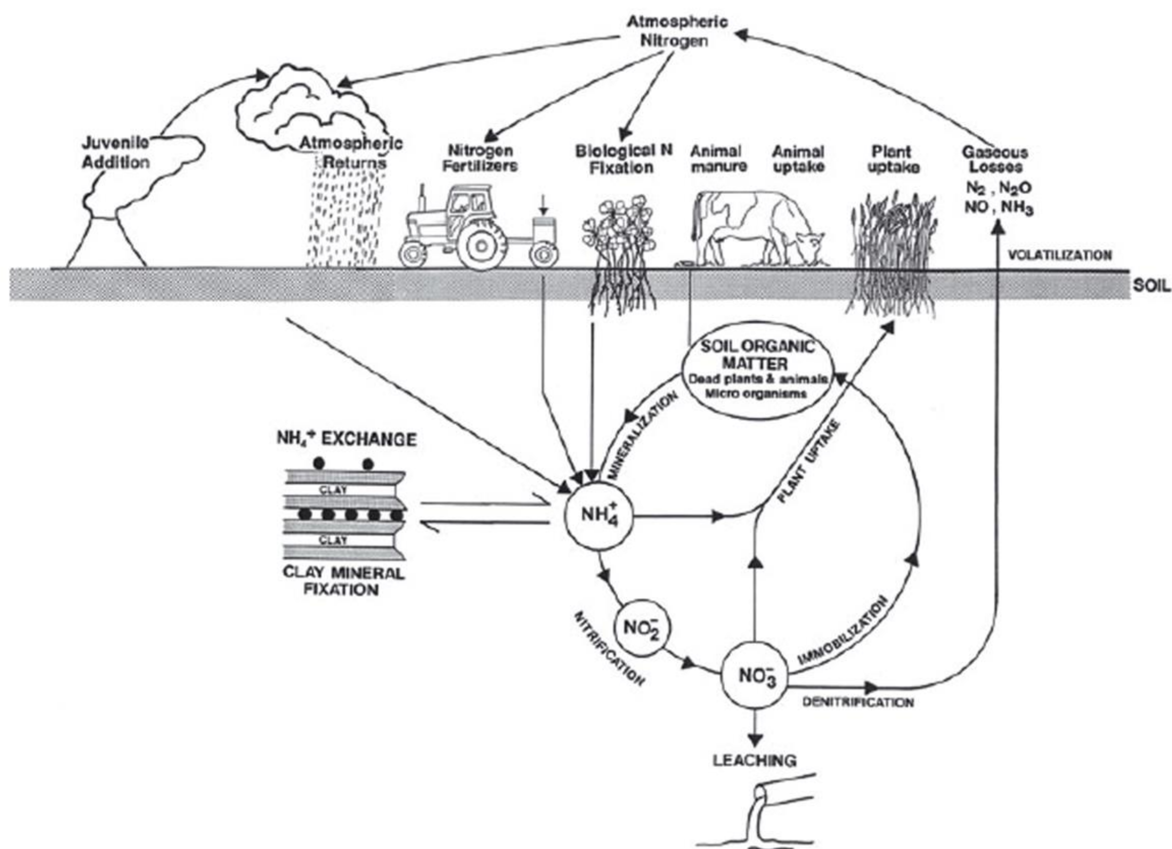


Figure 2. The soil/plant nitrogen cycle, from Cameron *et al*., 2012.

2. Nitrogen use efficiency (NUE)

Nitrogen use efficiency lacks exact definition and can be defined in various ways depending on the different methods used to measure it, starting from the first classical definition of Moll *et al*. (1982), in which NUE is defined as the yield of grain per unit of available N in the soil. As a function of multiple interacting genetic and environmental factors, NUE is inherently complex. The definition of NUE itself is also complex, and the term can mean different things in different contexts, including N use efficiency (NUE), N uptake efficiency (NUpE), N utilization (assimilation) efficiency (NUtE), apparent N recovery rate (ANR), agronomy efficiency of fertilizer N (AE), N physiological use efficiency (NpUE), N transport efficiency (NTE), and N remobilization efficiency (NRE) (definitions and formulae used here to describe nutrient use efficiency in plants are reviewed in Good *et al*., 2004 and Xu *et al*., 2012). Consequently, the different ways to measure and to define NUE depend on

the crop, on its harvest product and on the physiological aspects considered. In addition, in order to improve the nitrogen use efficiency, it is necessary to consider that NUE also depends on a large number of external factors, such as soil type and management, interactions with microorganisms, the nature of N source and the climate (Hirel *et al*., 2011). In this respect, agronomic approaches in improving NUE are related to the (i) organic farming (such as using green manure or cover crops for N fertilization, as well as to the application of appropriate livestock manure) and (ii) precision agriculture principles (for instance, the development of strategies to synchronize fertilization with the larger crop N demand periods), (iii) no or minimum tillage techniques in order to reduce leaching and volatilization of N and increase in turn, the N soil availability for the crop (Tilman *et al*., 2002; Good and Beatty, 2011; Hirel *et al*., 2011). The use of combined approaches including molecular biology and physiology for the breeding of crops with enhanced NUE is a further vital step in order to reduce N losses to the environment while maintaining yield and protein quality. In particular, in the last few years, the understanding of the genetic basis of plant nutrition has advanced rapidly and has allowed the identification of key elements involved in nutrient uptake, transport and assimilation, as well as in root physiology and morphology (López *et al*., 2013). However, the whole regulation and signaling systems of NUE are still far from a full comprehensive understanding (Hirel *et al*., 2007; Kant *et al*., 2011; McAllister *et al*., 2012) and new studies are required to better decipher the nitrogen use efficiency in crops both at molecular and morphological level.

Before discussing the attempts at modifying NUE in plants, a review of the key steps in primary N metabolism as well as the nitrate signaling pathways is given.

2.1 Nitrogen acquisition by plants

Regulation of plant N-metabolism is complex and influenced by several physiological and metabolic processes, such as circadian rhythms, sugars synthesis and transport, key N metabolite levels and NO_3^- itself (McAllister *et al*., 2012). Nitrate, beside being an essential nutrient for plants, also plays a signaling role in regulating important physiological and developmental processes, such as seed dormancy, flowering time, root development and the expression of a large number of nitrate-responsive genes (Bouguyon *et al*., 2012). In addition, plants are also capable to reprogramme their growth by modulating the activity of

N transport system, in order to modify root system architecture in response to different nitrogen availability in the soils (Dechorgnat *et al*., 2011).

Nitrogen (N) in fact, is present in the soil in several different N-containing compounds including inorganic forms, such as nitrate (NO_3^-) and ammonium (NH_4^+), and to a lesser extent, organic forms such as amino acids, peptides (di- and tri-peptides) and proteins (Miller *et al*., 2007). Under natural conditions, the content of inorganic and organic N forms in the soil depends on a very large number of factors, such as the physical and chemical properties of soils, pH, temperature and the presence and activity of microorganisms; however, under aerobic soil conditions (typical of most cultivated soils), soluble NO_3^- is the major N source taken up by crops (Krouk *et al*., 2010a). In the well-aerated agricultural soils however, high NO_3^- concentration are not maintained for a long time (because of losses mainly due to NO_3^- leaching and microbial denitrification) and the presence of the anion is extremely variable in both space and time, considering that it can vary by two to four orders of magnitude (Miller *et al*., 2007). Thus, plant roots have developed multiple strategies to cope with this extreme variability in nitrate soil concentration, evolving a very sophisticated and fine-tuned nitrate uptake and transport system, in order to optimize and regulate the acquisition and assimilation of NO_3^-.

2.1.1 Nitrate transporters and channels

Plants have developed four different families of nitrate transporters (Fig. 3):

- NRT1/PTR (nitrate transporter 1/peptide transporter);
- NRT2;
- CLC (chloride channels, with affinity also for nitrate);
- SLAC/SLAH (slow anion channels).

Among these, only some nitrate transporters belonging to the NRT1/PTR and the NRT2 family are directly involved in the acquisition of NO_3^- from soil; the *CLC* family in fact, which encodes two tonoplast H^+/NO_3^- antiporters and the *SLAC1/SLAH* family, which encodes two guard cell anion channels, are responsible, together with the remaining NRT1/PTR and NRT2 transporters, for the efflux, transport and allocation of nitrate within the plant (Kant *et al*., 2011; Wang Y. *et al*., 2012).

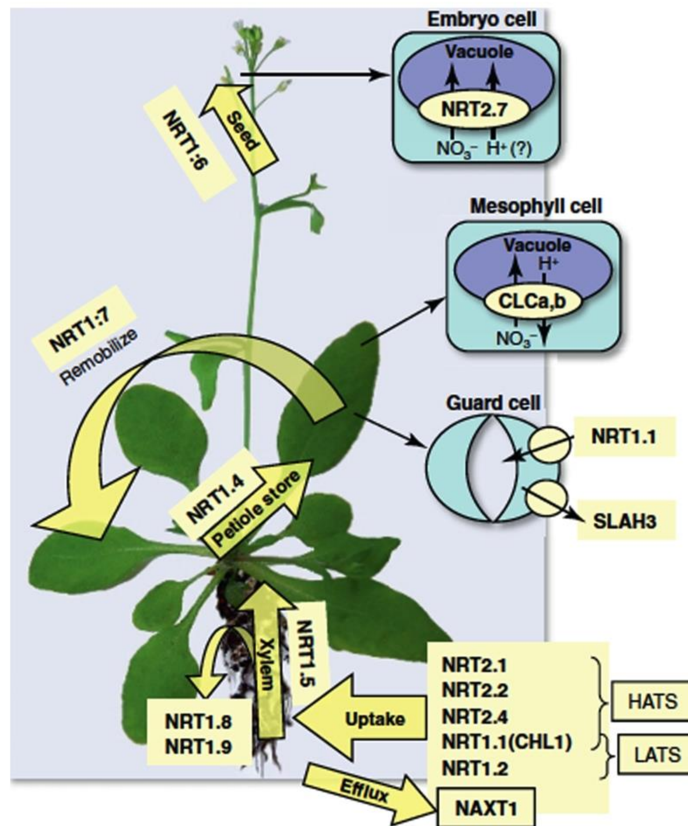


Figure 3. Physiological functions of *Arabidopsis* nitrate transporters, from Wang Y. *et al*., 2012.

2.1.1.1 Nitrate uptake from soil

Regarding the NO_3^- uptake system, plants have developed two kinetically different NO_3^- transport systems: the low affinity transport system (LATS), which operates and becomes significant at external NO_3^- concentration above 1 mM, and the high affinity transport system (HATS) that predominates in the micromolar range (Miller *et al*., 2007; Kraiser *et al*., 2011; Xu *et al*., 2012).

In *Arabidopsis*, there are 53 NRT1/PTR transporters although, so far, a role in nitrate uptake has been proposed for only two *NRT1* genes, *NRT1.1* and *NRT1.2* (Andrews *et al*., 2013). The gene family was named *NRT1/PTR* because in animals, fungi and bacteria they transport dipeptides (Wang Y. *et al*., 2012). In plants, some NRT1/PTR transport NO_3^- and some transport dipeptides. With the exception of AtNRT1.1, also known as CHL1 (Wang R. *et al*., 1998; Liu *et al*., 1999), and MtNRT1.3 (Morére-Le Paven *et al*., 2011), which are dual-affinity NO_3^- transporters, most of the NRT1 members are low-affinity NO_3^- transporters (LATS). *AtNRT1.1* was the first gene identified for its putative role in LATS, using chlorate

selection on the NO_3^- uptake mutant *chl1* (Tsay *et al*., 1993). Further investigations on *nrt1.1* mutants defective in low-affinity NO_3^- uptake demonstrated surprisingly a role for *NRT1.1/CHL1* also in high-affinity nitrate transport system (HATS), suggesting a dual role in taking up nitrate of this gene (Wang R. *et al*., 1998; Liu *et al*., 1999). A phosphorylation switch of threonine 101 by calcineurin B-like-interacting protein kinase 23 (CIPK23), in response to variations in external NO_3^- concentration, is responsible for the change of AtNRT1.1 mode of action, which functions as a low-affinity transporter in the dephosphorylated configuration and exhibits, on the contrary, high-affinity NO_3^- uptake activity in the phosphorylated isoform (Liu and Tsay, 2003). Very interestingly, increasing evidence suggest that AtNRT1.1 may also function as a major nitrate sensor, being involved in regulation of several plant physiological responses, such as the relief of seed dormancy and the stimulation of lateral root proliferation, similarly to other membrane proteins communally called "transceptors", that fulfil a dual transport/signaling function in yeast and animals, (Gojon *et al*., 2011). Thus, NRT1.1 can sense a wide range of NO_3^- concentration in the soil, switching between its transporting and signaling activities. In addition to NRT1.1/CHL1, in NRT1/PTR family also NRT1.2 participates in low-affinity nitrate uptake (Huang N. *et al*., 1999). In contrast with *NRT1.1*, which is induced by NO_3^-, *NRT1.2* is constitutively expressed (Huang N. *et al*., 1999), and does not show neither the dual-affinity mode of action nor, so far, a signaling function, and it is expressed only in root epidermal cells, whereas NRT1.1 has been found in epidermis, cortex and endodermis (Tsay *et al*., 2007). Finally, the role of *AtNRT1.3* in taking up NO_3^- remains unclear, considering that its expression in root is repressed by exposure to NO_3^- and conversely induced by NO_3^- deprivation, suggesting an insignificant contribution to LATS (Okamoto *et al*., 2003; Plett *et al*., 2010).

The NRT2 family of *Arabidopsis thaliana* includes seven genes. NRT2.1 is the main component of inducible HATS in *Arabidopsis*, as demonstrated in *nrt2.1* mutants that lack up to 75% of the high-affinity nitrate uptake activity (López *et al*., 2013). To be active, NRT2.1 forms a functional unit with the membrane protein NITRATE ACCESSORY PROTEIN2.1 (NAR2.1, also known as AtNRT3.1), which plays a key role in regulating both inducible and constitutive HATS (Laugier *et al*., 2012). In the *Arabidopsis nar2.1* mutant, the disappearance of NRT2.1 protein from the plasma membrane suggested that NAR2.1 is required for the plasma membrane targeting and/or for the NRT2.1 stability (Wirth *et al*., 2007). Thus, in

Arabidopsis three nitrate transporters are involved in HATS: NRT2.1, NRT2.2 and the dual-affinity NO_3^- transporter NRT1.1. The relative contribution of these transporters to high-affinity NO_3^- uptake is dependent on the developmental stage and to the N status of the plant (Tsay *et al*., 2007). Both *NRT2.1* and *NRT2.2* are inducible by provision of NO_3^-, in contrast with NRT2.4, which is similarly expressed in root, exhibiting a very high-affinity range at low NO_3^- concentration, but its gene expression appears to decrease following exposure to nitrate (Kiba *et al*., 2012).

To conclude, it is predictable that other candidate genes for both LATS and HATS will be identified in the future, especially in plant species different than the model species *Arabidopsis thaliana.* In fact, the determination of function of the *NRT2* genes in cereals simply based on sequence homology to functionally characterized *Arabidopsis NRT2* genes may not be possible (Xu *et al*., 2012). The phylogenetic analysis of the *NRT* gene families in *Arabidopsis* and monocot species in fact, has revealed some striking differences in gene family structure, thus providing a framework for future investigations on grass NO_3^- transporters and potentially future strategies for improving NUE in cereals through genetic manipulation of the *NRT* genes (Plett *et al*., 2010). However, regarding the three maize nitrate transporters analysed in this work, such as *ZmNRT1.1* and *ZmNRT2.1* and *ZmNRT2.2*, they all show gene expression and regulation patterns similar to those of *A. thaliana* (Quaggiotti *et al*., 2003; Santi *et al*., 2003; Trevisan *et al*., 2008; Yu P. *et al*., 2014).

2.1.1.2 Nitrate efflux, translocation and allocation in vegetative tissues and seeds, and nitrate-induced stomatal movement

Nitrate is taken up from soil through the root system by the activity of a few numbers of transporters belonging to the NRT1/PRT and NRT2, as mentioned above (NRT2.1, NRT2.2 and NRT2.4 acting as HATS, NRT1.2 as LATS and the dual-affinity transporter NRT1.1/CHL1). The following steps of the NO_3^- fate within the plant are mediated by the concerted action of the remaining NRT1/PRT and NRT2 family transporters, together with the vacuolar chloride channels CLCa and CLCb, and the anion-efflux channels SLAC1 and SLAH3.

The physiological role of NO_3^- efflux in root epidermal cells remains unclear so far, although it has been proposed the involvement of AtNAXT1, a member of the NRT1/PTR family (Segonzac *et al*., 2007) and of AtNRT1.5 (Lin *et al*., 2008) in this process. The

transporter NRT1.5 has a role also in the loading of nitrate into the xylem, in concert with NRT1.8 and NRT1.9 in modulating the translocation of NO_3^- from root to shoot. The cooperation of these transporters in the root-to-shoot NO_3^- transport suggests a fine-tuned distribution of the anion within plant, by different regulatoty pathways (Wang Y. *et al.*, 2012). In *Arabidopsis*, *NRT1.5* is expressed in the perycicle cells adjacent to the protoxylem and is responsible for exporting NO_3^- out the perycicle cells for xylem loading and is positive induced by the presence of the anion (Lin *et al.*, 2008). Otherwise, NRT1.8 is responsible for retrieving nitrate from the xylem parenchima both in root and shoot (Li J. *et al.*, 2010), thus working synergistically with NRT1.5 to control long-distance NO_3^- transport. Finally, NRT1.9 mediates the downward phloem transport of nitrate in root (Wang Y. and Tsay, 2011), suggesting that NRT1.9 together with NRT1.8 are negative effectors of root-to-shoot NO_3^- transport but through different mechanisms.

After transportation to shoot, nitrate can be assimilated or stored in vacuoles. The low- affinity NO_3^- transporter NRT1.4 shows a very specific pattern of expression in the leaf petiole (a NO_3^- storage site) indicating that NRT1.4 regulates NO_3^- distribution in leaves and that it plays, thus, an important role in regulating leaf nitrate homeostasis and leaf development (Chiu *et al.*, 2004). In addition to NRT1.4, also NRT1.7 has been identified as playing a role in the allocation of NO_3^- within leaves, remobilizing nitrate from the older to younger leaves through facilitating phloem loading (Fan *et al.*, 2009). Finally, NRT2.4 also participates in phloem NO_3^- transport in shoots and its expression in phloem parenchyma leaves is induced by N starvation (Kiba *et al.*, 2012). These last findings that suggest a contribution of phloem nitrate transporters in controlling NO_3^- distribution within plants, shed new light on the general opinion that nitrate can be only transported via the xylem, suggesting the phloem nitrate transport as a secondary route to modulate local NO_3^- distribution (Wang Y. *et al.*, 2012). Regarding nitrate membrane transporters through the tonoplast, the genes responsible for exporting NO_3^- out of vacuoles have not yet been identified, as opposed as those that are responsible for transporting NO_3^- into the vacuoles, which are well document: the proton-nitrate tonoplast exchangers CLCa and CLCb mediate, in fact, nitrate import into the vacuole (De Angeli *et al.*, 2006; von der Fecht-Bartenbach *et al.*, 2010).

Lastly, a brief summary about the nitrate transporters and channels associated with the stomatal activity and the NO_3^- movement in reproductive tissues is given. Very

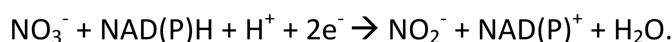
interestingly, *NRT1.1/CHL1* is expressed not only in roots but also in the guard cells where, in presence of NO_3^-, promotes stomatal opening in *Arabidopsis* (Guo *et al*., 2003). The anion-efflux channels SLAC1, which exhibits similar permeability for nitrate and chloride, and SLAH3, which shows a strong preference for nitrate over chloride, drive, on the opposite, the stomatal closure (Negi *et al*., 2008; Vahisalu *et al*., 2008; Geiger *et al*., 2011). In conclusion, the low-affinity NO_3^- transporters NRT1.6 (a plasma-membrane localized transporters, expressed only in the vascular bundles of the siliques and the funiculi) and the tonoplast-membrane transporter NRT2.7 are responsible in delivering nitrate to developing seeds (Alboresi *et al*., 2005 and Chopin *et al*., 2007, respectively).

After having discussed about nitrate uptake and efflux, translocation and allocation in vegetative tissues and seeds of NO_3^-, as well as the nitrate effects on stomatal movement, and the several transporters involved in all these physiological processes, in the next paragraph a review on nitrate assimilation within the root symplast is given.

2.2 Nitrate assimilation through the NR-NiR-GS-GOGAT pathway

Nitrate, after being taken up by root epidermal cells, can be stored in the vacuoles or be transported via the xylem (and, to a lesser extent, via the phloem) to vegetative tissues and seeds, where again it can be stored or assimilated, as discussed before. Otherwise, nitrate can be assimilated into organic forms directly in root cells, through the sequential reduction to nitrite (NO_2^-) and ammonium (NH_4^+), as showed in Fig. 4.

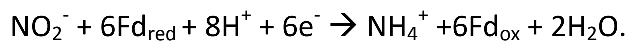
The first step of NO_3^- assimilation is the cytosolic reduction to NO_2^- that is carried out by nitrate reductase (NR; EC 1.6.6.1) through the subsequent reaction:



$$NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O.$$

Nitrate reductase is a multiple-subunit enzyme, in which each monomer contains three prosthetic groups (FAD, molybdenum cofactor and cytochrome 557), and can use, as electron donor, NADH or NADPH or both (Tischner, 2000). The most widespread NR is the NADH-dependent form, which is mainly expressed in photosynthetic tissues, whereas the major form occurring in root can use, as electron donor, both NADH and NADPH (Masclaux-Daubresse *et al*., 2010). *NR* transcription is induced within a few minutes by both NO_3^- and

light, but a post-translational mechanism of regulation, by reversible phosphorylation and the interaction with 14-3-3 proteins was also demonstrated (Heidari *et al*., 2011; Lambeck *et al*., 2012). High concentration of reduced N-forms, such as NH_4^+ or amino acids glutamine (Gln) and asparagine (Asn), and the darkness are the most potent inhibitors of NR activity (Lillo *et al*., 2004). Interestingly, high C/N ratio also induces *NR* expression, suggesting a complex interaction between the C and N signaling pathways and predictable matrix effects (Coruzzi and Zhou, 2001). Post-translational mechanisms for the regulation of NR activity are vital to have a faster control of the enzyme (minutes against hours).
Nitrite (NO_2^-), is fairly toxic to plant cells and is immediately converted into NH_4^+ by the plastidial or chloroplastal enzyme nitrite reductase (NiR; EC 1.7.7.1), which catalyses the transfer of six electrons to NO_2^-, using reduced ferrodoxin (Fd) as electron donor (Sakakibara *et al*., 2012). The reaction is the following:



$$NO_2^- + 6Fd_{red} + 8H^+ + 6e^- \rightarrow NH_4^+ + 6Fd_{ox} + 2H_2O.$$

Synthesis of NiR is regulated by a nitrate responsive *cis*-element, but there is no evidence of post-translation modifications (Konishi and Yanagisawa, 2011). Similarly to NR, also NiR is a substrate and light induced enzyme, whereas its gene expression is inhibited by Gln and Asn (Lillo, 2008).

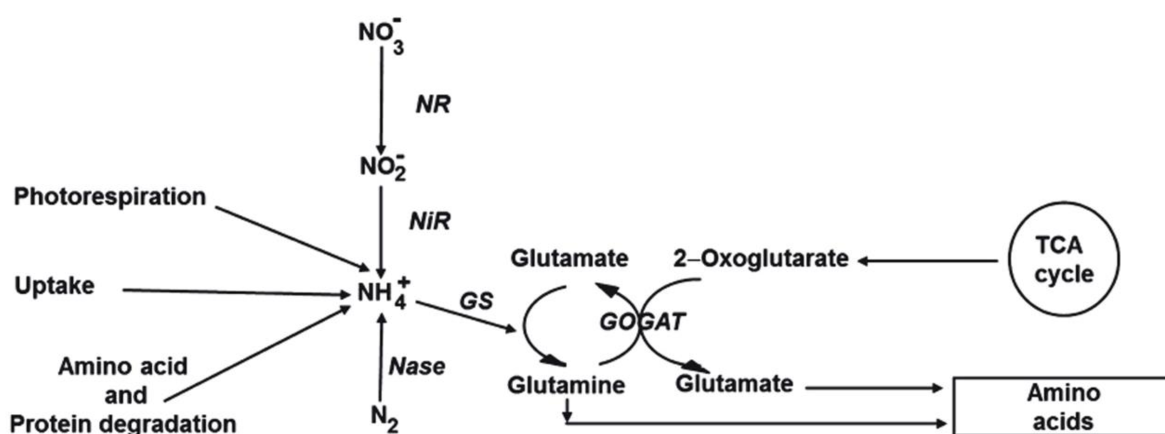
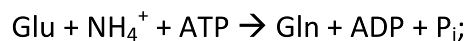


Figure 4. The assimilation of nitrogen (N) in higher plants. The main enzymes involved are indicated in italics: NR=nitrate reductase; NiR=nitrite reductase; Nase=nitrogenase; GS=glutamine synthetase; GOGAT =glutamate synthase. The ultimate source of inorganic N available to the plant is ammonium, which is incorporated into organic molecules in the form of glutamine and glutamate through the combined action of the two enzymes GS and GOGAT in the plastid or chloroplast, from Andrews *et al*., 2013.

Ammonium (NH_4^+) is rapidly converted, because of its toxicity, into the amino acid glutamate (Glu) by the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT), also known as glutamine:2-oxoglutarate aminotransferase (Masclaux-Daubresse *et al*., 2010). Ammonium, both directly taken up from soil and generated by the reduction of NO_2^- (as seen above) or from the secondary metabolism (photorespiration), is at first combined with glutamate to form glutamine, through an ATP-dependent reaction catalysed by GS (EC 6.3.1.2). The amide amino group of the newly formed glutamine is consequentially transferred to 2-oxoglutarate to yield two molecules of glutamate through a reaction catalysed by GOGAT, which uses, as electron donor, either reduced ferrodoxin (Fd-GOGAT; EC 1.4.7.1) or NADH (EC 1.4.1.13). Thus, the GS-GOGAT cycle can be represent in the following way:



$$\text{Glu} + NH_4^+ + \text{ATP} \rightarrow \text{Gln} + \text{ADP} + P_i;$$

$$\text{Gln} + \text{2-oxoglutarate} + Fd_{red}\ (\text{NADH} + H^+) \rightarrow \text{2Glu} + Fd_{ox}\ (NAD^+).$$

Two major isoforms of GS exist; GS1, which occurs in the cytosol of roots, phloem and leaf cells, and GS2, which occurs in plastids of roots and other non-photosynthetic tissues and in the chloroplasts of photosynthetic tissues (Suzuki and Knaff, 2005). As far as GOGAT is concerned, both Fd-GOGAT and NADH-GOGAT isoforms appear to be solely located in the plastids and chloroplasts. The NADH-dependent GOGAT is located predominantly in non-photosynthesizing tissues, where reductant power is initially supplied by the pentose phosphate pathway (Bowsher *et al*., 2007). The Fd-GOGAT activity is much greater than NADH-GOGAT in chloroplasts, where light energy is used directly for the synthesis of reduced Fd (Masclaux-Daubresse *et al*., 2010). Both GS isoforms and Fd-GOGAT are induced by high level of light and increasing level in C/N ratio, showing, once again, a complex convergence of signals between C and N metabolism (Vidal *et al*., 2010; Castaings *et al*., 2011). In addition, GS isoforms, as also observed for NR, show a post-translational regulation mechanism mediated by 14-3-3 proteins, suggesting a fine coordinated regulation of enzymatic activity in response to external and internal factors (Diaz *et al*., 2011).

Glutamate Dehydrogenase (GDH; EC 1.4.1.2) can also catalyse the incorporation of $NH4^+$ into amino acids; nevertheless, the conversion of 2-oxoglutarate to Glu is a very

improbable reaction, and GDH seems more likely to play a role in the reverse reaction in the remobilization of N during senescence ad grain filling (Tabuchi *et al*., 2007). The enzymatic reaction is showed below:

$$2\text{-oxoglutarate} + NH_4^+ + NAD(P)H \leftarrow Glu + NAD(P)^+ + H_2O.$$

Finally, once N has been assimilated in organic forms, it is transported throughout the plant predominantly as glutamine and glutamate (as seen above), but also as asparagine (Asn) and aspartate (Asp), to utilization and storage. The conversion of glutamine to asparagine and glutamate to aspartate requires two amino-trasferase enzymes, asparagine synthetase (AS; EC 6.3.5.4) and aspartate aminotransferase (AspAT; EC 2.6.1.1.) respectively (Hodges, 2000 and Masclaux-Daubresse *et al*. 2010). Transported via the xylem, these amino acids are distributed to mesophyll cells where they are either stored or utilized for C assimilation (Tegeder and Rentsch, 2010). All the above-mentioned enzymes and associated pathways are controlled by several factors, such as soil N availability, plant N status, external and internal C status and changes in plant hormones (Vidal *et al*., 2010; Castaings *et al*., 2011).

After having talked about nitrate uptake, transport and assimilation, it is now necessary to focus on nitrogen and nitrate signaling in plants, keeping always in mind the perspective of improvement on nitrogen use efficiency of crops.

2.3 Nitrate sensing and signaling in plants

Nitrate is not only a major nutrient for plants but also acts as a signal, regulating gene expression and several physiological and developmental processes. Strong evidences suggest that NO_3^- acts as a signal molecule *per se*, considering that on one hand, the anion induces the expression of many genes involved in its own assimilation pathway, such as *NR*, *NiR* and many nitrate transporters (Crawford and Glass, 1998), and on other hand, by observing that this induction is maintained also in *NR*-deficient mutants unable to reduce nitrate to nitrite (Wang R. *et al*., 2004). In order to better decipher the specific effects of nitrate as signal molecule, microarray analyses, performed especially in *Arabidospis thaliana* in the last decade, were very useful to identify a large number of genes responding specifically to NO_3^- (in particular by using *NR*-null mutants) and which do not required NO_3^- reduction. These

findings surprisingly have been revealed that almost 10% (*i.e*. >2000 genes) of the detectable transcriptome of *A*. *thaliana* responds to NO_3^-, involving many genes belonging to important metabolic pathways and many other regulatory components of plant signaling and development (Scheible *et al*., 2004; Gutiérrez *et al*., 2007; Krouk *et al*., 2010a). Since the gene regulation in response to NO_3^- occurs very rapidly (within few minutes) and does not require protein synthesis, it is referred as the "Primary Nitrate Response" (PNR; Krouk *et al*., 2010b). PNR affects a wide range of gene functional categories, such as ion transport, primary and secondary metabolism, biosynthesis of nucleic acids, transcription and RNA processing, hormone homeostasis (Bouguyon *et al*., 2012). The aim of this paragraph is focused at describing the most recent findings of NO_3^- sensing in plants, starting from nitrate sensors.

2.3.1 Nitrate sensors

Regarding nitrate sensing, the identification of NO_3^- sensor/receptors has remained elusive for long. In plants in facts, no external NO_3^- sensing systems similar to that of bacteria have been identified so far (Bouguyon *et al*., 2012). However, more recent investigations on *A*. *thaliana* actually suggest that nitrate early sensing in plant seems to be mediated directly by nitrate transporters, namely the dual-affinity NO_3^- transporter NRT1.1/CHL1 and the high-affinity NO_3^- transporter NRT2.1, similar to that observed in yeast, acting as the so-called "transceptors" (Gojon *et al*., 2011). The role of NRT1.1/CHL1 in early NO_3^- sensing was well documented by observing the strong altered regulation of many important plant activities, such as (i) gene expression of other NO_3^- transporters, including NRT2.1 (Muños *et al*., 2006; Krouk *et al*., 2006), (ii) seedling germination (Alboresi *et al*., 2005), (iii) root and shoot growth/development (respectively, Remans *et* al., 2006a and Krouk *et al*., 2010b; Hachiya *et al*., 2010) and (iv) cytokinins (CK) synthesis in roots (Kiba *et al*., 2011). The NRT1.1-dependet regulation of *NRT2.1* is very complex, having opposite actions depending on the timing of NO_3^- supply on one hand, and on NO_3^- concentration on the other hand. Short term NO_3^- supply increases NRT1.1-dependent *NRT2.1* gene expression, which is part of the PNR (Ho *et al*., 2009); by contrast, high NO_3^-concentration for several days down-regulates *NRT2.1* expression (Muños *et al*., 2006). Similarly to NRT1.1 transport activity, which is a dual-affinity NO_3^- transporter exhibiting both HATS and LATS phases, also NRT1.1-dependent *NRT2.1*

expression displays a two-affinity pattern (Ho *et al*., 2009), being slightly induced at low NO_3^- concentrations (HATS phase) and, on the opposite, full induced at high NO_3^- concentrations (LATS phase). As for the transport activity (already mentioned above), the switch from the two phases (HATS or LATS) of NRT2.1 by NRT1.1 activity is due to the phosphorylation of the T101 residue (Ho *et al*., 2009). To date, the nitrate signaling pathway elicited by NRT2.1 has only been reported for the regulation of the initiation of Lateral Root Primordia (LRP; Little *et al*., 2005; Remans *et al*., 2006b), as soon will be discussed, as well as the role of NRT1.1 in concert with the phytohormone auxin (IAA) in regulating lateral root growth in response to nitrate (Krouk *et al*., 2010b). Very interestingly, specific mutation able to uncouple the NRT1.1 activity to that of signaling, demonstrated there is no direct relationship between the NO_3^- signaling and the transport of the anion (Walch-Liu and Forde, 2008). In this regard, *nrt1.1* mutants do not shows decreased NO_3^- uptake as compared to wild-type controls (Muños *et al*., 2006). On the contrary, the T101A mutation, which prevents the phosphorylation of threonine 101 in NRT1.1, is able to suppress the NRT1.1-dependent root development response, but not NO_3^- transport activity (Walch-Liu and Forde, 2008), thus providing further strong parallel between NRT1.1 (and possibly NRT2.1) and the so-called "transceptors" (transporter/receptor) identified in yeast. Furthermore, a functional role for nitrate transporters in signaling has been recently reported in other plant species in addition to the model *A. thaliana*, such as *Medicago truncatula* (Yendrek *et al*., 2010; Morere-Le Pavene *et al*., 2011), suggesting that the concept of "transceptors" may be of general occurrence in eukaryotes, and also plant may have been developed this kind of sensing mechanism to cope with the extreme range of variability in nutrient concentration in soil (Gojon *et al*., 2011).

Despite the concerted role of the nitrate transporters NRT1.1/CHL1.1 and NRT2.1 in regulating a very large numbers of molecular and physiological responses to NO_3^-, specific NO_3^- signalling pathways are still active in *chl1* mutants (Wang R. *et al*., 2009), suggesting that different nitrate sensing systems have yet to be identified. Thus, further investigations to better understand the overall plant NO_3^- signaling are needed, especially considering the strong effects of NO_3^- in regulating root development and growth, in view of improving nitrogen use efficiency in crops.

2.3.2 Nitrate signal transduction

The overall network of signaling cascade triggered by nitrate in plants is largely unknown at molecular level to date, as mentioned before, although, in the last few years some advances in identifying key genes or transcription factors (TF) involved in NO_3^- signaling have been obtained, especially in *A. thaliana*. Regarding the regulation network involved in the uptake of NO_3^- and in its own assimilation pathway, two kinesis (*CIPK8* and *CIPK23*) and many TFs (*NLP7, LBD 37/38/39,* and *SPL9*) have been recently characterised.

As far as CIPK8 and CIPK23 are concerned, both of them are Ser/Thr kinases belonging to the CBL-interacting protein kinase (CIPK), a class of plant kinases that has shown an emerging role as calcium (Ca_2^+)-mediated signaling components in response to stresses, which are activated by specific Ca_2^+ sensors, collectively named CBL (Calcineurin B-like protein) (Chen *et al*., 2011). In *A. thaliana*, gene expression of both *CIPK8* and *CIPK23* is up regulated in response to NO_3^- but, on the contrary, strongly down-regulated in *chl1* mutants, suggesting their involvement in the NRT1.1-dependent signaling pathway (Ho *et al*., 2009; Hu H. *et al*., 2009). Interestingly, CIPK23 participates in the signaling pathway evoked by NO_3^- through the activity of the transceptor NRT1.1, but at the same time, the protein kinase is in turn a regulator of NRT1.1 by phosphorylating the T101 residue, showing a complex mechanism of retro-control loop for NRT1.1-dependent gene response to nitrate (Ho *et al*., 2009). The function of CIPK8 is, on the contrary, partially unknown, although it has been demonstrated that acts as a stimulator of the PNR in the low- but not in the high-affinity phase (Hu H. *et al*., 2009).

The transcription factor NLP7 (Nodule Inception-like protein 7) is a member of the NIN family of TFs. In the unicellular algae *Chlamydomonas reinhardtii* it was firstly reported a role in up-regulating the activity of NR (*NIA*) gene by the member of this family NIT2 (Camargo *et al*., 2007). In *Arabidopsis*, NLP7 is thought to be involved in the transduction of the NO_3^- signal, considering that *nlp7* knockout mutants display the characteristic phenotype of N-starved wild type seedlings, irrespective of N supply, and transcriptome analysis further confirmed the hypothesis of a N-starvation phenotype (Castaings *et al*., 2009). These Authors show that *nlp7* mutants are impaired in transduction of the NO_3^- signal, as they fail to induce NO_3^--responsive genes after a short nitrogen starvation followed by NO_3^- re-supply. More recently, a genome-wide analysis reported that NLP7 binds and modulates, via a nuclear retention mechanism evocated by NO_3^-, several hundred of known nitrate signaling

and assimilation genes within few minutes (the PNR), indicating that plants, similarly to fungi and mammals, depend on a nuclear retention mechanism to instantaneously respond to changes in nutrients availability (Marchive *et al.*, 2013). Thus, it has been suggested that NO_3^-, directly or indirectly, inhibits, through an as yet unknown mechanism, the export of NLP7 from the nucleus, leading to its rapid nuclear accumulation in response to the anion.

SPL9 (Squamosa Promoter-binding-like Protein 9) is another regulatory TF involved in the early PNR, emerging as a potential hub of a gene regulatory network in response to nitrate. By performing a high-resolution time course analysis of transcriptome modifications after NO_3^- supply, it has been shown to respond to NO_3^- very fast (3-6 minutes), (Krouk *et al.*, 2010a). Moreover, *spl9* mutants, characterized by over-expression of the TF, significantly modified the time-course of *NiR* gene expression, strongly increasing its transcript accumulation levels within the first few minutes as compared to the wt, but interestingly, reducing NiR expression after that period. Thus, *SPL9* gene provides a further example, similarly to the signaling activity of *NRT1.1* and *NRT2.1*, of genes able to adjust either the up- or down-regulation of nitrate-responsive genes, depending on the fluctuations in the NO_3^- availability.

Regarding the three TFs *LBD37/38/39*, they are zinc-finger DNA-binding protein belonging to the LATERAL ORGAN BOUNDARY DOMAIN family. Interestingly, all of them show a strong and very fast induction in response to NO_3^- but, at the same time, no differences in transcript accumulation when seedling were supply with other N sources (Rubin *et al*., 2009). LBDs play a role in down-regulating many NO_3^- responsive genes, among which *NRT2* and *NIA,* in response to nitrate supply; however NO_3^- induction of *NRT2* and *NIA* is not dependent by the activity of LBDs, thus, excluding an involvement of these TFs in the PNR but rather suggesting a link with other NO_3^- signaling pathways, responsible for feedback down-regulation of NO_3^- responsive genes by long-term high NO_3^- provision (Bouguyon *et al*., 2012). To conclude based on these last findings, the regulation of NO_3^- transporters and NO_3^- assimilation genes in the response to the anion depends on at least three major different signaling pathways, as illustrated in Fig. 5. The PNR can be consequently subdivided into the kinase CIPK8-dependent (low-affinity: high NO_3^- concentration) and CIPK8-independent (high-affinity: low NO_3^- concentration) pathways (Ho *et al*., 2009), while a third pathway involving LBD 37/38/39 ensures the longer-term feedback down-regulation by NO_3^-.

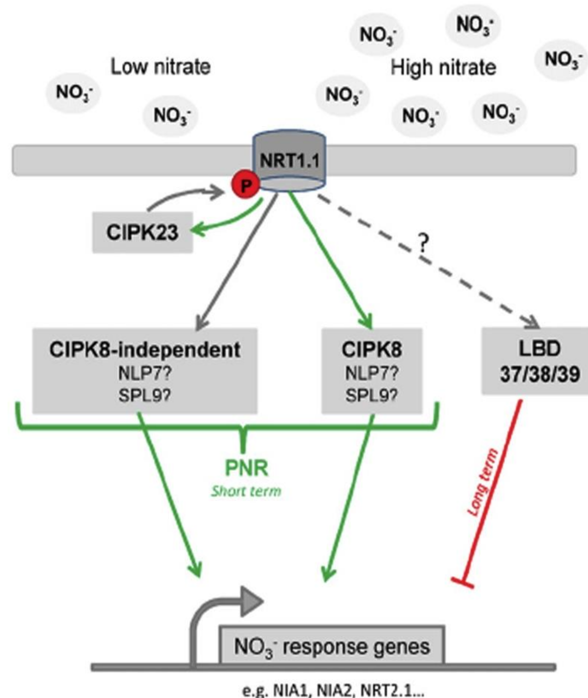


Figure 5. Molecular players in the nitrate regulation of nitrate transport and assimilation genes, from Bouguyon *et al*., 2012.

As far as the changes in root morphology are concerned, a specific paragraph is dedicated to better understand the phenotypic plasticity of the root system in response to NO_3^- and the signaling events underlying this process, especially in the view of improving NUE in plants.

2.4 Regulation of root growth and development in response to nitrate

The effects of nitrate on the root system are complex and depend on several factors, such as NO_3^- concentration in the soil, the N endogenous status of the plant and the sensibility of the different species. Investigations on root system architecture (RSA) and morphology are vital, considering that RSA determines the dramatic plasticity of plants to explore the soil for searching water and nutrients, including nitrate. Thus, breeding crop varieties that are more efficient at capturing soil NO_3^- is a compelling need to decrease NO_3^- leaching and denitrification losses and consequently to increase NUE. Despite the large numbers of reports published on NO_3^- effects on RSA, especially focused on lateral roots (LR) development, many aspects are still unclear and require further investigations, in particular at molecular level.

The effect of nitrate on primary root growth is controversial and strongly variable depending on NO_3^- concentration, on the exposure time and on the species (Andrews *et al*., 2013). For example, in a number of *Arabidopsis thaliana* accessions a stimulatory effect of nitrate on primary root growth was observed after nine days of NO_3^- exposure at concentration ranging from 0.05 mM to 5 mM (Walch-Liu and Forde, 2008), similarly to Gifford *et al*. (2013) after twelve days of exposure (NO_3^- concentration was ranging from 0 to 20mM). In contrast, Zhang and Forde (1998) did not observe any changes in primary root length in a range of concentration from 0.01 mM to 100 mM (fourteen days of exposure), as well as no effects were measured also by Signora *et al*. (2001) after seven days of exposure to NO_3^- concentration ranging from 0.1 mM to 10 mM. The same Authors reported conversely, an inhibitory effect at higher concentrations (> 50 mM). Finally, long-term exposure (17/18 days) to NO_3^- at low concentration (0.01/1 mM) showed additionally an inhibition of primary root elongation (Linkohr *et al*., 2002), making very ardours to clearly decipher the regulation of the growth of primary roots in response to nitrate in the model species *Arabidopsis thaliana.* In maize, a consistent inhibitory effect on primary root length was observed by Tian and co-authors (2005) after twelve days of growth at a nitrate concentration of 20 mM. A few years later, a more detailed study was published by the same Authors who demonstrated that nitrate concentrations lower than 0.5 mM had no effect on elongation of primary, seminal, and crown roots, while concentrations above 5 mM affected more significantly the root elongation after twelve days of treatment (Tian *et al*., 2008). A similar inhibitory effect was also observed in maize, after having grown up the seedlings in varying NO_3^- concentration (0.1/10 mM) for seven days and then exposed, respectively, to 0.1 mM and 1 mM NO_3^- for 48 hours (Zhao *et al*., 2007). A model for an interaction between nitrate and L-glutamate (Glu) signalling pathway mediated by the “transceptors” CHL1/NRT1.1 in regulating meristematic activity at the root tip has been proposed (Walch-Liu and Forde, 2008). According to these Authors, Glu, after being sensed at the primary root tip by an unknown receptor, would trigger a reduction in primary root growth and stimulate branching behind the root tip. Conversley, nitrate sensed by NRT1.1 would antagonises the Glu signalling pathway and alleviates the effect of Glu on root architecture. In fact, this action of NO_3^- is lost in *chl1* mutants, indicating the key role of NRT1.1 in mediating this effect (Forde and Walch-Liu, 2009).

Unlike to the nitrate-regulation of the primary root growth, which is for many aspects still unclear, more is known on the molecular and morphological mechanisms governing lateral roots (LR) formation and development in response to nitrate. LR elongation by localized supply of high NO_3^- patches is a classical example of the stimulatory effects of external NO_3^- availability, starting from the early studies by Drew *et al*. (1973) and Drew (1975) in barley. The proliferation of LRs within a localized nitrate-rich zone is a response that occurs in many plant species and can be considered a common adaptation phenomenon (Hodge, 2004). *ANR1* (*ARABIDOPSIS* NITRATE-REGULATED1), a MADS box transcription factor, and the NO_3^- "transceptor" CHL1/NRT1.1, were identified as key components governing this response in *Arabidopsis* (Zhang and Forde, 1998; Remans *et al*., 2006a). ANR1 is specifically expressed in root, especially in LR primordia and at the tip of LRs; the transgenic plants, in which *ANR1* expression is down-regulated or suppressed, are less responsive to the localized NO_3^- signal and consequently displays a strongly altered ability to preferentially colonize nitrate-rich patches (Remans *et al*., 2006a). Interestingly, tissue localizations of *ANR1* and *NRT1.1* mRNAs overlap. Furthermore, considering also that *chl1* mutants have strongly reduced ANR1 mRNA levels (Rahayu *et al*., 2005) it has been suggested that ANR1 acts downstream of the NRT1.1 in the signaling pathway stimulating LR development in response to nitrate (Remans *et al*., 2006a). In addition, the high-affinity uptake complex NRT2.1/NAR2.1 also participates in regulating LR development. Under N-limiting conditions, both of them are positive regulators of LR initiation, even in nitrate-free conditions, suggesting their involvement in the regulation of LR growth is independent from their uptake function (Remans *et al*., 2006b; Orsel *et al*., 2007). However, in conditions of high C/N ratio, they function as repressors, indicating that the NRT2.1/NAR2.1 complex has a dual effect on LR development (Little *et al*., 2005).

As far as the role of phytohormones in regulating RSA in response to nitrate is concerned, the IAA long-distance transport from shoot to root was proposed to be involved in the inhibition of early LR development by high rates of NO_3^- in *Arabidopsis* (Forde, 2002; Walch-Liu *et al*., 2006). A role of auxin (IAA) was also evidenced in maize, in which the inhibition of root growth by high NO_3^- supply was correlated with reduced IAA concentration in the roots (Tian *et al*., 2008). More recently, the possible involvement of IAA in the stimulation of LR growth by NO_3^- was reconsidered, when it was found that the nitrate "transceptor" NRT1.1 was able to transport IAA as well as NO_3^- (Krouk *et al*., 2010b).

According to these Authors, IAA moves from the primary root vascular tissues to the tip of the lateral root through the pro-vascular tissue, and it is redirected back to the primary root through a basipetal transport route in the epidermis. NRT1.1 is expressed in the epidermis, and under low NO_3^- is able to transport IAA. Thus, NRT1.1 seems to be involved in the repression of LR growth at low NO_3^- concentrations by promoting lateral basipetal transport of IAA out of the lateral root. Conversely, high NO_3^- levels seem to inhibit NRT1.1-dependent basipetal IAA transport, thus inducing IAA accumulation in the LR tip thus stimulating growth of these roots.

In addition, both miR167 and its target the AUXIN RESPONSE FACTOR 8 (ARF8) and miR393 and the auxin receptor AFB3 (AUXIN SIGNALING F-BOX PROTEIN 3) were also proposed as crucial components of regulating the ratio between initiating and emerging LRs (Gifford *et al*., 2008) and in modulating both LR and primary root growth in response to NO_3^- (Vidal *et al*., 2010) in *Arabidopsis* roots respectively. . AFB3 belongs to a group of F-box receptors for auxin and was found to be the unique IAA receptor transcriptionally induced by NO_3^-, suggesting that besides modulating IAA gradients in roots, through the NRT1.1 dual NO_3^-/IAA transport activity (Krouk *et al*., 2010b), NO_3^- also increases root IAA sensitivity by affecting *AFB3* expression (Bouguyon *et al*., 2012). In fact, analysis of both *afb3* insertional and miR393 overexpressors mutants demonstrated that NO_3^- is able to transcriptionally induce the expression of AFB3 in both primary and lateral roots, while metabolites of NO_3^- assimilation pathway were able to down-regulate AFB3 due to the induction of miR393 (Vidal *et al*., 2010). Thus, according to these Authors, stimulation of AFB3 by NO_3^- is only transient because the AFB3 transcript is rapidly targeted by miR393 for degradation as soon as NO_3^- enter the assimilation pathway, showing an interesting NO_3^--responsive feed-forward mechanism in controlling RSA in *Arabidopsis*, as summarized in Fig. 6.

There are also evidences that abscisic acid (ABA) could play a central role in mediating the regulatory effects of high NO_3^- levels on root branching in *A. thaliana* (Signora *et al*., 2001), as well as brassinosteroids (Kiba *et al*., 2011). A crosstalk between IAA and cytokinins (CK) signaling in coordinating the requirement and acquisition of NO_3^- and their effects on root branching in *Arabidopsis* has been also suggested (Kiba *et al*., 2011). Finally, a novel role for nitric oxide (NO) in regulating plant root growth is emerging in the last few years, despite the current knowledge about its signaling pathway, especially in regulating root system architecture, is still fragmentary. Thus, considering the large number of key

developmental processes signalled by this gaseous molecule, in the next section the state of the art on NO biology is reviewed.

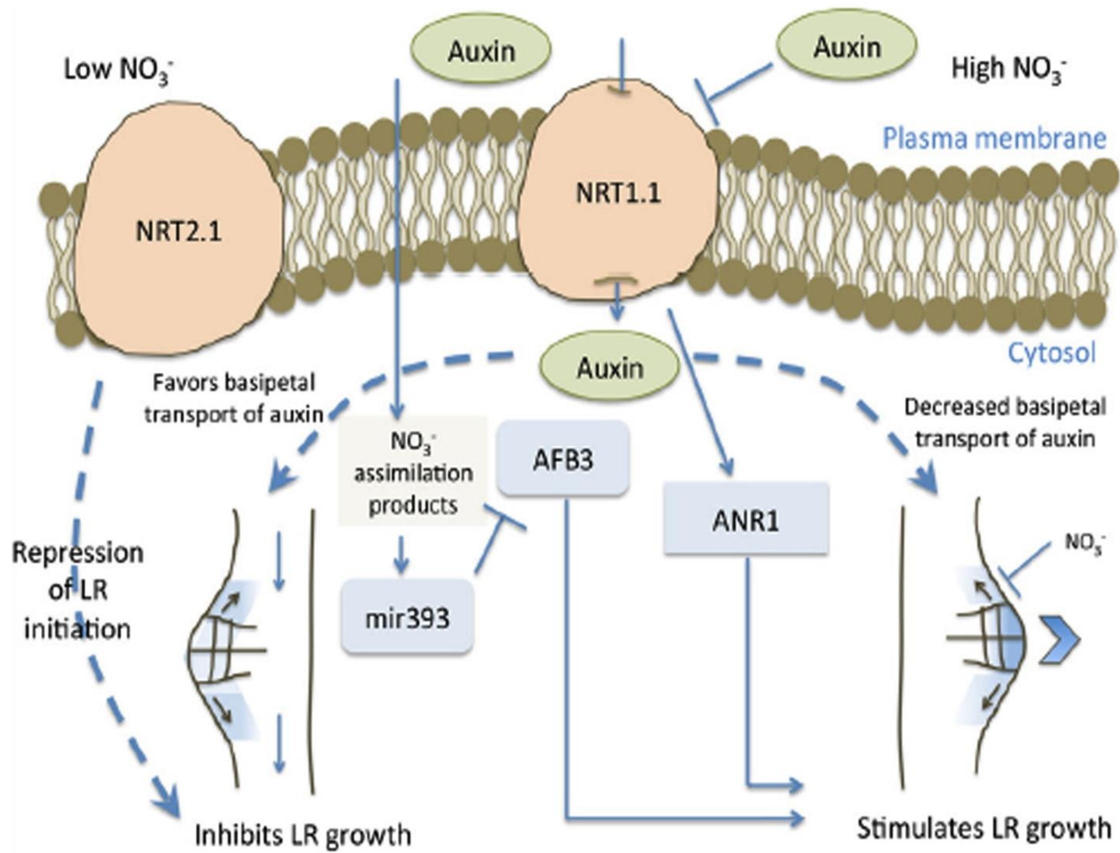


Figure 6. Schematic representation of the signaling pathways specifically involved in changes in root architecture in response to nitrate, from López-Arredondo *et al*., 2012.

2.5 Nitric oxide as a new regulator of root growth in response to nitrate

The participation of nitric oxide (NO) in root response to NO_3^- has been recently postulated in maize (Trevisan *et al*., 2011). In this work, through a cDNA-AFLP approach, the transcript profiling of maize seedlings roots grown with different NO_3^- availabilities was exploited and the expression of a number of selected genes was analysed in depth by both quantitative real-time polymerase chain reaction (qPCR) and *in situ* hybridisation (ISH). A new model for nitrate-induced signaling in maize roots involving the coordinate spatio-temporal expression of nitrate reductase and non-symbiotic hemoglobins for NO production and scavenging, respectively, was thus postulated. Considering that this gaseous free radical is involved in a variety of plant growth and developmental processes. These preliminary findings opened a very interesting scenario in studying plant root responses to nitrate.

Plant hemoglobins (Hbs) are hemeproteins that reversibly bind molecular oxygen (O_2) and were first identified in the 1930s as symbiotic hemoglobins (also known as leghemoblobins) in legume root nodules, in which it is universally accepted they function to facilitate the diffusion of O_2 to bacteroids, whereas the discovery of non-symbiotic hemoglobins (nsHbs) is a more recent uncovering (Garrocho-Villegas *et al*., 2007). The kinetic constants and localisation of nsHbs in metabolically active tissues as well as the over-expression of *nshb* genes in stressed plants suggest that nsHbs have functions other than or additionally to O_2 transport, such as to bind other gaseous ligands, including NO (Garrocho-Villegas *et al*. 2007; Dordas, 2009). A key role of nsHbs in NO detoxification under hypoxic conditions was first demonstrated in *Arabidopsis* (Dordas *et al*., 2003; Perazzolli *et al*., 2004), and now the participation of this class of Hbs in NO scavenging is widely recognized (Crawford and Guo, 2005; Hill, 2012). In addition, in the last few years nsHbs have been found to function in many crucial plant processes, such as seed development and germination, flowering, root development and differentiation, abiotic stress responses, pathogen invasion and symbiotic bacterial association (reviewed in Hill, 2012) as well as, very recently, plant nsHbs have been also identified as master regulators during *Arabidopsis* (Elhiti *et al*., 2013) and *in vitro* maize embryogenesis (Huang S. *et al*., 2014a,b). Non-symbiotic hemoglobins have been shown to be induced by nitrate (Wang R. *et al*., 2000; Ohwaki *et al*., 2005; Shimoda *et al*., 2005). The conversion of nitrite (NO_2^-) to NO by the activity of nitrate reductase (NR) was documented since the 1980s in *Leguminosae* family

(Dean and Harper, 1988) and has been successively demonstrated in detail in other plant species (Wildt *et al*., 1997), including maize (Yamasaki et al., 1999; Yamasaki and Sakihama, 2000), implying a putative responsibility for NR as NO signal emitter. Hence, in addition to its physiological role in nitrogen assimilation, NR is also required for the control of the production of active nitrogen species (Yamasaki and Sakihama, 2000) and, in this scenario, nsHbs could thus play a protective function against NR-derived NO, thus contributing to the modulation of NO-mediated signaling (Hebelstrup *et al*., 2007). The increasing evidence of the role of NO in hormone responses and the known involvement of nsHbs in scavenging NO provide a new and fascinating field of research, as summarized in Fig. 7.

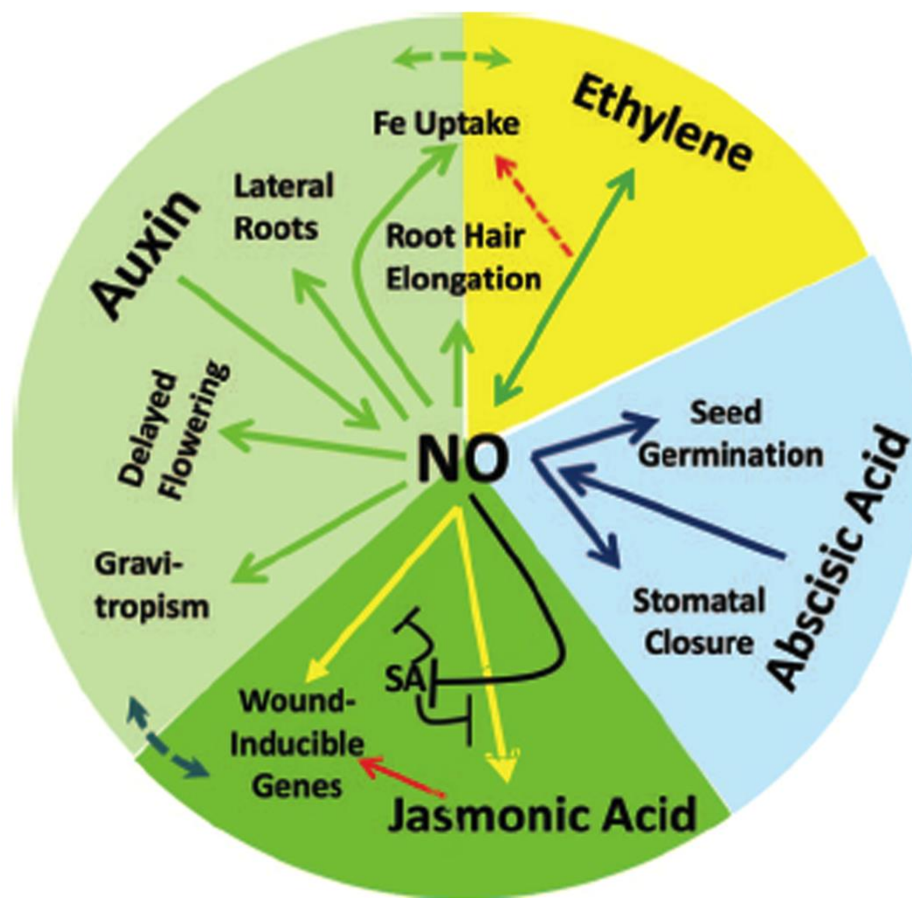


Figure 7. The relationship between NO, hormones and biological function, from Hill *et al*., 2012.

Nitric oxide, in fact, is gradually becoming established as a central regulator of many physiological and developmental processes, such as growth, cell differentiation, immunity and environmental interactions (Siddiqui *et al*., 2011). In plants, seven sources have been proposed as putative routes for NO generation, which depend upon either reductive or oxidative chemistry (reviewed in Gupta *et al*., 2011; Mur *et al*., 2013), including NR. As bioactive molecules in plants, NO takes part to the control of a variety of key cellular

processes. For example, NO acts in plant immunity and in hypersensitive cell death, as well as, a number of independent papers have suggested a role of NO in stress responses, including drought, salt, heat and cold stress (reviewed in Yu M. *et al*., 2014). In comparison to what observed in the other kingdoms, findings obtained in the last decade revealed a striking NO role in regulating plant development programmes, such as germination (Beligni and Lamattina, 2000), flower development (Lee *et al*., 2008; Kwon *et al*., 2012), flowering time (He *et al*., 2004; Kwon *et al*., 2012), and apical dominance (Lee *et al*., 2008; Kwon *et al*., 2012) processes. However, it is the influence of NO upon root growth and development that has gathered the most attention in plant biology research, including the connection existing between NO and the phytohormones auxin (IAA) in regulating root system architecture.

Root growth and development are complex processes involving and integrating several exogenous and endogenous signals, including NO. A physiological role for NO in regulating negatively primary root (PR) growth and promoting lateral root (LR) development has been described in tomato (Correa-Aragunde *et al*., 2004) and *Arabidopsis* (Méndez-Bravo *et al*., 2010); however, it has been suggested that NO probably acts in a dose-dependent manner in PR regulation (Fernández-Marcos *et al*., 2011). Furthermore, NO is also able to induce adventious root (AR) development in a variety of plant species, including monot, dicot and gymnosperm (Lanteri *et al*., 2008). In this content, a number of second messengers implicated in AR development are involved in signaling cascades regulated by NO, and two parallel and independent pathways have been unravelled so far. The first of these is thought to utilize cGMP through an NO-mediated activation of the enzyme guanylate ciclase (Pagnussat *et al*., 2003). A second pathway that involved a MAPK cascade has been also reported (Pagnussat *et al*., 2004). As far as LR formation is concerned, this process is associated with IAA action and is generally linked to the inhibition of PR elongation; NO is thought to be a downstream messenger in IAA signalling, promoting LR formation (Correa-Aragunde *et al*., 2004). Concerning primary root growth, the participation of NO in modulating PR development is still controversial. It has been previously mentioned that NO acts differentially in a dose-dependent manner: exogenous application of high levels of NO donors inhibits PR growth, whereas applications of lower NO donor concentrations promotes it (Fernández-Marcos *et al*., 2011). Interestingly, an investigation of plant gravitropic responses, which involved various endogenous NO levels, seems to confirm the hypothesis that NO promotes root elongation at low concentration, with opposite inhibitory

effects on PR growth at higher NO levels (Hu X. *et al*., 2005). In *Arabidopsis*, it has been proposed that elevated (> 100 µM) NO concentrations reduce auxin transport and responses via a PIN1-dependent mechanism and polar auxin transport is impacted negatively by over-accumulation of NO, considering that PIN1 protein levels appear to be reduced dramatically after application of exogenous NO (Fernández-Marcos *et al*., 2011). In this work, by using microscopic analysis, it has been also shown that the organization of primary root meristem is very sensitive to changes in NO levels and that increasing NO concentrations decrease PR growth by reducing the number of dividing cells in the meristem. In addition, they demonstrated that during early root development endogenous NO accumulates mainly in a zone situated between the apical meristem and the elongation zone (*i.e.* the "root transition zone"). Cytoskeleton proteins seem to be good candidates for these NO-related structural modifications and interestingly, in this scenario, a study published by Kasprowicz *et al*. (2009) reported that actin cytoskeleton acts as a downstream effector of NO signal transduction in root cells and that the extent of such modifications is cell-type and developmental stage-specific. Additionally, fluctuating NO concentrations can also modulate cellulose synthase activity and cell wall biosynthesis contributing to modification of cell growth (Yu M. *et al*., 2014). Taken together, these data showed that NO could act as a master regulator of primary root growth, by both modifying polar auxin transport, being PIN1 a target of NO signaling, and by affecting the functioning of the actin cytoskeleton and actin-dependent mechanisms.

To summarize, in order to link the need to study the molecular and physiological responses to nitrate in roots with a parallel analysis of the effects of NO upon root growth is clearly evident after this short review explaining the role of this gaseous molecule as emerging regulator of these processes. This is also vital in the view of better understanding the mechanisms underlying the nitrogen use efficiency in plants, considering that the assimilation of nitrate is strictly connect via nitrate reductase to the generation of NO. It therefore cannot be excluded that NO could operate at the interface between NO_3^- perception and transduction, taking part in the overall physiological and developmental plant adaptation to nitrate. In fact, as previously mentioned, the detection of NR and HB transcripts in tissues devoted to nutrient uptake and their spatial distribution in epidermal cells, the first layer of living cells in the root in contact with the external environment, strongly suggests that they could play an important role during the early perception and

signaling of nitrate in the rhizosphere (Trevisan *et al*., 2011). Moreover the co-localization of mRNA for NR and HB observed in the root apex matches with the major sites of NO accumulation, suggesting that these two genes may represent the pivotal elements of a fine-tuning system for NO homeostasis and signaling. Thus, deciphering how NO biosynthesis, turnover and downstream signaling pathway is interconnected with nitrate assimilation in roots, is a field of research that is worthy of much attention.

To conclude, this brief review on nitrate signal transduction and nitrate-dependent regulation of RSA showed the high complexity of the NO_3^- signaling cascades, which we should consider as parts of networks integrating multisignal responses, including striking connections with auxin and NO. Although many efforts to better understand the genetic basis of NUE in crops by identifying individual genes or gene clusters that are responsible for the variability of this complex trait have been made, attempts to improve crop NUE by directly regulating the activity of one or a few number of NO_3^- transport/assimilation gene, or by modulating phytohormones balance to coordinate root architecture is still likely too challenging. In the next paragraph, several approaches that are being taken to meet this goal are discussed.

3. Approaches to improve nitrogen use efficiency

Recently, systems biology has been demonstrated to be a promising tool to dissect the plant response to nitrate in order to improve NUE. With the aim of improving NUE, many genetic and molecular approaches based on either targeted approaches or quantitative genetics have been used during the past decade. A critical overview on these attempts is provided below.

Several experiments have allowed the identification of specific genes involved in the regulation of NO_3^- uptake, translocation and assimilation, as well as, amino acid biosynthesis and C/N homeostasis, as candidate genes to improve NUE. Transgenic approaches based on either overexpressing or knockout mutants for improving NUE, mainly by making use of CaMV35S promoter, have been carried out in both *Arabidopsis* and main crops in the last few years. However, in many cases, overexpression of these genes did not result in a direct effect on these traits (Hirel *et al*., 2011). Regarding nitrate transporters, despite they are the first components of the NO_3^- assimilation pathway in the roots, only few studies have been

carried out to check the effect of their overexpression on NUE to date. In *Arabidopsis*, overexpression of *AtNRT1.1* resulted in increasing NO_3^- uptake, but no improvement on net NUE was observed (Liu *et al*., 1999). Similar results were obtained when overexpressing the high affinity NRT2.1 transporter in tobacco (Fraisier *et al*., 2000). Similarly in rice, increased expression of *NRT2.1* slightly improved seedling growth, but did not have any effects on NUE (Katayama *et al*., 2009).

Nitrate and nitrite reductase are key regulatory checkpoints for improving NUE. However, overexpression of the tobacco NR-encoding gene *NIA1* and *NIA2* in different plants, such as potato (Dejannane *et al*., 2002), lettuce (Curtis *et al*., 1999) and *Nicotiana plumbaginifolia* (Lillo *et al*., 2003) showed no NUE-phenotype associated under N-limiting conditions. Regarding nitrite reductase, transgenic plants by overexpressing *NiR* in Arabidopsis and tobacco did not show any phenotypic differences (Crété *et al*., 1997). The lack of positive effects by overexpressing genes encoding NR and NiR has been associated with the known tight regulation of these two enzymes at the translational and post-translational levels.

As far as nitrate assimilation genes are concerned, changes in the expression of glutamine synthetase (either GS1 or GS2 isoforms) could have an effect on nitrate and N metabolism in plants, potentially affecting NUE (Fei *et al*., 2003; Brauer *et al*., 2011). In maize, GS single and double mutants show significant phenotypic effects on kernel size and yield, indicating that GS has a role in grain filling (Martin *et al*., 2006). However, in this investigation, NO_3^- uptake and assimilation were not analysed. Further studies carried out by overexpressing cytosolic GS1 in maize leaves (Hirel *et al*., 1997) and the leaf-specific GS2 in tobacco (Migge *et al*., 2000), showed changes in the amino acids concentration but no significant changes were observed on growth and morphology of plants. In rice, by comparing two varieties with different levels of GS2 activities, Kumagay and colleagues (2011) found that the variety with high GS2 activity has only a better ability to recycle and re-assimilated NH_3, indicating that the overexpression of GS genes may play a role in the general N economy within the plant by improving N recycling but not necessarily primary N assimilation. In addition, most of these experiments have not directly measured NUE, as well as, many of them have not grown plant on varying NO_3^- levels, making it difficult to provide an undoubtedly role of GS in modifying NUE by these transgenic approaches (Hirel *et al*., 2011).

The GOGAT isoenzymes conversely, (both Fd-GOGAT and NADH-GOGAT), seem to be a major candidate for improving NUE in crops. For example in rice, over-expression of NADH-GOGAT has been linked with enhanced grain filling (Yamaya *et al*., 2002; Tabuchi *et al*., 2007), while knockout mutations of NADH-GOGAT1 result in decreasing yield, overall biomass and panicle production (Tamura *et al*., 2010). Likewise, by suppressing both Fd-GOGAT and NADH-GOGAT in rice, the resulting phenotypes show that tilled number, total shoot dry weight and yield are decreased significantly respect to the control plants, and these results were also confirmed in plants grown in field conditions (Lu *et al*., 2011). However, given these observed phenotypes and those observed for GS enzymes, the interactions between isozymes of GOGAT with the GS isozymes and how this affects NUE, as well as post-transcriptional regulation of these enzymes, needs to be further investigated (McAllister *et al*., 2012).

Arabidopsis plant overexpressing asparagine synthetase (AS) were reported to have enhanced NUE, showing higher tolerance to N-limiting conditions (Lam *et al*., 2003), however these results, similarly to that obtained by the other transgenic approaches overexpressing enzymes involved in nitrate uptake and N assimilation pathway, need other investigations to assess whether AS could be used effectively to enhance NUE in crops. Finally, regarding the two aminotransferase enzymes AspAT and AlaAT, Cañas and collaborators (2010) suggest that both can serve as markers of NUE in maize. Conversely, no effects on phenotypes, biomass or yield were detected when a soybean AspAT was constitutively overexpressed in *Arabidopsis* as well as by overexpressing AspAT in *Brassica napus* (Murooka *et al*., 2002 and Wolasky *et al*., 2005, respectively).

Genetic modifications involving transcription factors and other regulatory elements are a further promising approach to modify plant metabolism for NUE improvement. For example, overexpression of the DNA-binding with One Finger1 (Dof1) TF appears to improve N uptake and assimilation, considering that maize *Dof1*-overexpression in *Arabidopsis* revealed increased growth, amino acids and total N contents under low N-conditions (Yanagisawa *et al*., 2004). More recently, *ZmDof1*-overexpressing experiments in rice showed enhanced N and C accumulation and photosynthesis rates in transgenic rice plants under N-limiting conditions, especially in roots that had also a higher biomass than the control plants (Kurai *et al*., 2011). In rice, overexpression of the endogenous *EARLY NODULIN93-1* (*ENOD93-1*) gene, which is potentially involved in amino acids transport,

resulted in increased shoot dry biomass and higher concentrations of total amino acids and total N in roots, especially under N stress (Bi *et al*., 2009). Finally, ANR1 overexpression induces a stronger LR initiation and elongation in *Arabidopsis* (Gan *et al*., 2012), potentially enhancing the exploratory capacity of the root system, which is one of the main critical steps limiting the efficient use of N fertilizers in modern crops.

Over the last decade, quantitative genetics through the detection of quantitative trait loci (QTL) has also became an important approach for identifying key regulatory or structural genes involved in crops NUE (Hirel *et al*., 2007). When QTLs for NUE phenotypic traits are located on a genetic map, it is possible to look for their genetic significance by establishing the co-localization of QTLs for physiological or biochemical traits with genes putatively involved in the control of the traits of interest (candidate genes). Validation of candidate genes can be carried out by transgenic technologies (forward genetics) and mutagenesis (reverse genetics), as discussed before and also reviewed more in detail in Kant *et al*. (2001) and references therein, or by studying the relationship between allelic polymorphism and the traits of interest (association genetics). One of the first QTL study in order to analysing NUE in crop plant was performed by Obara and colleagues (2001) in rice. They looked at QTLs associated with NUE and determined whether cosegregated with GS1 and NADH-GOGAT in rice. The analysis identified seven loci that cosegregated with GS1 and six with NADH-GOGAT. In a more detailed investigation by Bertin and Gallais (2001) using maize recombinant inbred lines, coincidences between QTLs for yield and two genes encoding cytosolic GS were detected, and more recently, an extensive QTL analysis in wheat has shown that the genome regions containing *GS* and *GOGAT* are linked to NUE traits (Quraishi *et al*., 2011). However, the role of the GS enzyme and other N-related physiological traits in the control of NUE still remains to be clearly established, considering that overexpressing these genes for improving NUE did not give unequivocal results (Hirel *et al*., 2011), as discussed before. Thus, it is probably necessary to identify other nitrate-responsive genes by transcriptomic data set (Hawkesford and Howarth, 2011) or using systems biology approaches (Simons *et al*., 2014).

In conclusion, many important crop traits like NUE are considered to be very complex, with no single gene contributing more than a small percentage to the phenotypes. Thus, it is questionable whether a single transgene approach could have a significant enough effect on NUE, considering that these targeted approaches does not adequately take in

account the variation in complex traits such as those controlling NUE. In addition, the components of NUE interact in multiple and complex ways with other signaling and metabolic pathways and therefore, in order to understand the complexity of the control of NUE of model crop species such as maize, this requires a holistic insight of nitrate and nitrogen flow within plant and the associated regulation at cellular, organ and whole-plant levels.

4. Aim of work

The involvement of NO in the nitrate signaling pathway has been recently proposed to be implicated in plant adaptation to environment, but its exact role in the response of plants to nutritional stress is still under evaluation. Aim of this work is to evaluate the contribution of NO in the nitrate-regulated pathway that directs maize root system architecture, unravelling the role of NO as a nitrate-related signal. To do this, firstly, the study has been focused on both the characterization of the expression profiles of selected genes putatively involved in NO homeostasis and on the determination of NO *in vivo* production by roots in response to nitrate. Secondly, in order to deepen the spatial regulation of NO homeostasis balance, the expression of few selected genes, those involved in NO production and scavenging, was analysed in four different root zones (meristem, transition zone, elongation zone, maturation zone). Additionally, this work has been also focused on deepening the knowledge of the effect of nitrate on root growth and especially on root elongation. In order to characterize the adaptive morphological strategies, the different root phenotypes in response to changing nitrogen availability and to unravel whether the nitrate-induced root length increase is dependent on a NO signalling pathway, a series of root morphological analyses has been performed. In conclusion, since it has known from the literature that NO signaling alters cell polarity and cytoskeleton-mediated vesicle trafficking processes, as well as, that NO and auxin act synergically to control diverse aspects of root biology, the final part of this work has been dedicated to analyse cytoskeleton-mediated cell wall xyloglucans recycling and PIN1-mediated auxin transport in the transition zone.

5. References

5.1 Articles and books

Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN. 2005. Nitrate, a signal relieving seed dormancy in *Arabidopsis*. *Plant Cell and Environment* **28,** 500-512.

Andrews M, Raven JA, Lea PJ. 2013. Do plants need nitrate? The mechanisms by which nitrogen form affects plants. *Annals of Applied Biology* **163,** 174-199.

Baligar VC, Fageria NK, He ZL. 2001. Nutrient use efficiency in plants. *Communications in Soil Science and Plant Analysis* **32,** 921-950.

Beligni MV, Lamattina L. 2000. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta* **210,** 215-221.

Bertin P, Gallais A. 2001 Physiological and genetic basis of nitrogen use efficiency in maize. II. QTL detection and coincidences. *Maydica* **46,** 53-68.

Bi YM, Kant S, Clarke J, Gidda S, Ming F, Xu J, Rochon A, Shelp BJ, Hao L, Zhao R, Mullen RT, Zhu T, Rothstein SJ. 2009. Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling. *Plant, Cell and Environment* **32,** 1749-1760.

Bowsher CG, Lacey AE, Hanke GT, Clarkson DT, Saker LR, Stulen I, Emes MJ. 2007. The effect of Glc6P uptake and its subsequent oxidation within pea root plastids on nitrite reduction and glutamate synthesis. *Journal of Experimental Botany* **58,** 1109-1118.

Bouguyon E, Gojon A, Nacry P. 2012. Nitrate sensing and signaling in plants. *Seminars in Cell and Developmental Biology* **23,** 648-654.

Brauer EK, Rochon A, Bi YM, Bozzo GG, Rothstein SJ, Shelp BJ. 2011. Reappraisal of nitrogen use efficiency in rice overexpressing *glutamine synthetase1*. *Physiologia Plantarum* **141,** 361-372.

Camargo A, Llamas A, Schnell RA, Higuera JJ, González-Ballester D, Lefebvre PA, Fernández E, Galván A. 2007. Nitrate signaling by the regulatory gene *NIT2* in *Chlamydomonas*. *The Plant cell* **19,** 3491-3503.

Cameron KC, Di HJ, Moir JL. 2013. Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology* **162,** 145-173.

Castaings L, Camargo A, Pocholle D, Gaudon V, Texier Y, Boutet-Mercey S, Taconnat L, Renou JP, Daniel-Vedele F, Fernandez E, Meyer C, Krapp A. 2009. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in *Arabidopsis*. *The Plant Journal* **57,** 426-435.

Castaings L, Marchive C, Meyer C, Krapp A. 2011. Nitrogen signalling in Arabidopsis: how to obtain insights into a complex signalling network. *Journal of Experimental Botany* **62,** 1391-1397.

Cerezo M, Tillard P, Filleur S, Muños S, Daniel-Vedele F, Gojon A. 2001. Major alterations of the regulation of root NO_3^- uptake are associated with the mutation of *Nrt2.1* and *Nrt2.2* genes in *Arabidopsis*. *Plant Physiology* **127,** 262-271.

Chen X, Gu Z, Xin D, Hao L, Liu C, Huang J, Ma B, Zhang H. 2011. Identification and characterization of putative *CIPK* genes in maize. *Journal of Genetics and Genomics* **38,** 77-87.

Chiu CC, Lin CS, Hsia AP, Su RC, Lin HL, Tsay YF. 2004. Mutation of a nitrate transporter, AtNRT1:4, results in a reduced petiole nitrate content and altered leaf development. *Plant and Cell Physiology* **45,** 1139-1148.

Chopin F, Orsel M, Dorbe MF, Chardon F, Truong HN, Miller AJ, Krapp A, Daniel-Vedele F. 2007. The *Arabidopsis* ATNRT2.7 nitrate transporter controls nitrate content in seeds. *The Plant Cell* **19,** 1590-1612.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* **218,** 900-905.

Coruzzi GM, Zhou L. 2001. Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. *Current Opinion in Plant Biology* **4,** 247-253.

Crawford NM, Glass ADM. 1998. Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Science* **3,** 389-395.

Crawford NM, Guo FQ. 2005. New insights into nitric oxide metabolism and regulatory functions. *Trends in Plant Sciences* **10,** 195-200.

Crété P, Caboche M, Meyer C. 1997. Nitrite reductase expression is regulated at the post-transcriptional level by the nitrogen source in *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. *The Plant Journal* **11,** 625-634.

Curtis IS, Power JB, de Laat AMM, Caboche M, Davey MR. 1999. Expression of a chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Reports **18,** 889-896.

Dean JV, Harper JE. 1988. The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean. *Plant Physiology* **88,** 389-395.

De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, Barbier-Brygoo H. 2006. The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. *Nature* **442,** 939-942.

Dechorgnat J, Nguyen CT, Armengaud P, Jossier M, Diatloff E, Filleur S, Daniel-Vedele F. 2011. From the soil to the seeds: the long journey of nitrate in plants. *Journal of Experimental Botany* **62,** 1349-1359.

Diaz C, Kusano M, Sulpice R, Araki M, Redestig H, Saito K, Stitt M, Shin R. 2011. Determining novel functions of *Arabidopsis* 14-3-3 proteins in central metabolic processes. *BMC Systems Biology* **5,** 192.

Djennane S, Chauvin JE, Quilleré I, Meyer C, Chupeau Y. 2002. Introduction and expression of a deregulated tobacco nitrate reductase gene in potato lead to highly reduced nitrate levels in transgenic tubers. *Transgenic Research* **11,** 175-184.

Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J, Hill RD. 2003. Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *The Plant Journal* **35,** 763-770.

Dordas C. 2009. Nonsymbiotic hemoglobins and stress tolerance in plants. *Plant Science* **176,** 433-440.

Drew MC, Saker LR, Ashley TW. 1973. Nutrient supply and the growth of the seminal root system in barley. The effect of nitrate concentration on the growth of axes and laterals. *Journal of Experimental Botany* **24,** 1189-1202.

Drew MC. 1975. Comparison of the effects of a localized supply of phosphate, nitrate, ammonium, and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytologist* **75,** 479-490.

Elhiti M, Hebelstrup KH, Wang A, Li C, Cui Y, Hill RD, Stasolla C. 2013. Function of type-2 *Arabidopsis* hemoglobin in the auxin-mediated formation of embryogenic cells during morphogenesis. *The Plant Journal* **74,** 946-958.

Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W. 2008. How a century of ammonia synthesis changed the world. *Nature Geoscience* **1,** 636-639.

Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AMR, Leach A, de Vries W. 2013. Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368,** 20130116.

Fan SC, Lin CS, Hsu PK, Lin SH, Tsay YF. 2009. The *Arabidopsis* nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. *The Plant Cell* **21,** 2750-2761.

Fei H, Chaillou S, Hirel B, Mahon JD, Vessey JK. 2003. Overexpression of a soybean cytosolic glutamine synthetase gene linked to organ-specific promoters in pea plants grown in different concentrations of nitrate. *Planta* **216,** 467-474.

Fernández-Marcos M, Sanz L, Lewis DR, Muday GK, Lorenzo O. 2011. Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. *Proceedings of the National Academy of Sciences, USA* **108,** 18506-18511.

Forde BG. 2002. Local and long-range signaling pathways regulating plant responses to nitrate. *Annual Review of Plant Biology* **53,** 203-224.

Forde BG, Walch-Liu P. 2009. Nitrate and glutamate as environmental cues for behavioural responses in plant roots. *Plant, Cell and Environment* **32,** 682-693.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey DW, Haywood J, Lean J, Lowe DC, Myhre G, Nganga J, Prinn R, Raga G, Schulz M, Van Dorland R. 2007. Changes in atmospheric constituents and in radiative forcing. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds.), Cambridge University Press, Cambridge, UK and New York, NY, USA.

Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN, Vitousek P, Leach A, Bouwman AF, Butterbach-Bahl K, Dentener F, Stevenson D, Amann M, Voss M. 2013. The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368,** 20130164.

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F. 2000. Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. *The Plant Journal* **23,** 489-496.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vörösmarty CJ. 2004. Nitrogen cycles: past, present and future. *Biogeochemistry* **70,** 153-226.

Galloway JN, Leach AM, Bleeker A, Erisman JW. 2013. A chronology of human understanding of the nitrogen cycle. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368,** 20130120.

Garnett T, Conn V, Kaiser BN. 2009. Root based approaches to improving nitrogen use efficiency in plants. *Plant, Cell and Environment* **32,** 1272-1283.

Garrocho-Villegas V, Gopalasubramaniam SK, Arredondo-Peter R. 2007. Plant hemoglobins: what we know six decades after their discovery. *Gene* **398,** 78-85.

Geiger D, Maierhofer T, Al-Rasheid KA, Scherzer S, Mumm P, Liese A, Ache P, Wellmann C, Marten I, Grill E, Romeis T, Hedrich R. 2011. Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. *Science Signaling* **4,** ra32.

Gifford ML, Dean A, Gutiérrez RA, Coruzzi GM, Birnbaum KD. 2008. Cell-specific nitrogen responses mediate developmental plasticity. *Proceedings of the National Academy of Science, USA* **105,** 803-808.

Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C. 2010. Food security: the challenge of feeding 9 billion people. *Science* **327,** 812-818.

Gojon A, Krouk G, Perrine-Walker F, Laugier E. 2011. Nitrate transceptor(s) in plants. *Journal of Experimental Botany* **62,** 2299-2308.

Good AG, Shrawat AK, Muench DG. 2004. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends in Plant Science* **9,** 597-605.

Good AG, Beatty PH. 2011. Fertilizing nature: a tragedy of excess in the commons. *PLoS Biology* **9,** e1001124.

Grizzetti B, Bouraoui F, Aloe A. 2011. Changes of nitrogen and phosphorus loads to European seas. *Global Change Biology* **18,** 769-782.

Guo FQ, Young J, Crawford NM. 2003. The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in *Arabidopsis*. *The Plant Cell* **15,** 107-117.

Gupta KJ, Fernie AR, Kaiser WM, van Dongen JT. 2011. On the origins of nitric oxide. *Trends in Plant Science* **16,** 160-168.

Gutiérrez RA, Gifford ML, Poultney C, Wang R, Shasha DE, Coruzzi GM, Crawford NM. 2007. Insights into the genomic nitrate response using genetics and the Sungear Software System. *Journal of Experimental Botany* **58,** 2359-2367.

Hachiya T, Watanabe CK, Boom C, Tholen D, Takahara K, Kawai-Yamada M, Uchimiya H, Uesono Y, Terashima I, Noguchi K. 2010. Ammonium-dependent respiratory increase is dependent on the cytochrome pathway in *Arabidopsis thaliana* shoots. *Plant, Cell and Environment* **33,** 1888-1897.

Hawkesford MJ, Howarth JR. 2011. Transcriptional profiling approaches for studying nitrogen use efficiency. In *Annual Plant Reviews, Nitrogen Metabolism in Plants in the Post-genomic Era*; Foyer CH, Zhang H, Eds.; Wiley-Blackwell: Chichester, UK, **42,** 41-62.

He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM, Pei ZM. 2004. Nitric oxide represses the *Arabidopsis* floral transition. *Science* **305,** 1968-1971.

Hebelstrup KH, Igamberdiev AU, Hill RD. 2007. Metabolic effects of hemoglobin gene expression in plants. *Gene* **398,** 86-93.

Heidari B, Matre P, Nemie-Feyissa D, Meyer C, Rognli OA, Møller SG, Lillo C. 2011. Protein phosphatase 2A B55 and A regulatory subunits interact with nitrate reductase and are essential for nitrate reductase activation. *Plant Physiology* **156,** 165-172.

Hill RD. 2012. Non-symbiotic haemoglobins—what's happening beyond nitric oxide scavenging? *AoB Plants*. **2012,** pls004.

Hirel B, Le Gouis J, Ney B, Gallais A. 2007. The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany* **58,** 2369-2387.

Hirel B, Tétu T, Lea PJ, Dubois F. 2011. Improving nitrogen use efficiency in crops for sustainable agriculture. *Sustainability* **3,** 1452-1485.

Ho CH, Lin SH, Hu HC, Tsay YF. 2009. CHL1 functions as a nitrate sensor in plants. *Cell* **138,** 1184-1194.

Hodge A. 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist* **162,** 9-24.

Hodges M. 2002. Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *Journal of Experimental Botany* **53,** 905-916.

Hu HC, Wang YY, Tsay YF. 2009. AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. *The Plant Journal* **57,** 264-278.

Hu X, Neill SJ, Tang Z, Cai W. 2005. Nitric oxide mediates gravitropic bending in soybean roots. *Plant Physiology* **137,** 663-70.

Huang NC, Liu KH, Lo HJ, Tsay YF. 1999. Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. *The Plant Cell* **11,** 1381-1392.

Huang S, Hill RD, Wally OS, Dionisio G, Ayele BT, Jami SK, Stasolla C. 2014a. Hemoglobin control of cell survival/death decision regulates in vitro plant embryogenesis. *Plant Physiology* **165,** 810-825.

Huang S, Hill RD, Stasolla C. 2014b. Plant hemoglobin participation in cell fate determination. *Plant Signaling and Behaviour* **9,** DOI:10.4161/psb.29485

Kant S, Bi YM, Rothstein SJ. 2011. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *Journal of Experimental Botany* **62,** 1499-1509.

Kasprowicz A, Szuba A, Volkmann D, Baluška F, Wojtaszek P. 2009. Nitric oxide modulates dynamic actin cytoskeleton and vesicle trafficking in a cell type-specific manner in root apices. *Journal of Experimental Botany* **60,** 1605-1617.

Katayama H, Mori M, Kawamura Y, Tanaka T, Mori M, Hasegawa H. 2009. Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (OsNRT2.1). *Breeding Science* **59,** 237-43.

Kiba T, Kudo T, Kojima M, Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *Journal of Experimental Botany* **62,** 1399-1409.

Kiba T, Feria-Bourrellier AB, Lafouge F, Lezhneva L, Boutet-Mercey S, Orsel M, Bréhaut V, Miller A, Daniel-Vedele F, Sakakibara H, Krapp A. 2012. The *Arabidopsis* nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. *The Plant Cell* **24,** 245-258.

Konishi M, Yanagisawa S. 2011. The regulatory region controlling the nitrate-responsive expression of a nitrate reductase gene, *NIA1*, in *Arabidopsis*. *Plant and Cell Physiology* **52,** 824-836.

Kraiser T, Gras DE, Gutiérrez AG, González B, Gutiérrez RA. 2011. A holistic view of nitrogen acquisition in plants. *Journal of Experimental Botany* **62,** 1455-1466.

Krouk G, Tillard P, Gojon A. 2006. Regulation of the high-affinity NO_3^- uptake system by NRT1.1-mediated NO_3^- demand signaling in *Arabidopsis*. *Plant Physiology* **142,** 1075-1086.

Krouk G, Crawford NM, Coruzzi GM, Tsay YF. 2010a. Nitrate signaling: adaptation to fluctuating environments. *Current Opinion in Plant Biology* **13,** 265-272.

Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K, Zazimalova E, Benkova E, Nacry P, Gojon A. 2010b. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Developmental Cell* **18,** 927-937.

Kumagai E, Araki T, Hamaoka N, Ueno O. 2011. Ammonia emission from rice leaves in relation to photorespiration and genotypic differences in glutamine synthetase activity. *Annals of Botany* **108,** 1381-1386.

Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R. 2011. Introduction of the ZmDof1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. *Plant Biotechnology Journal* **9,** 826-837.

Kwon E, Feechan A, Yun BW, Hwang BH, Pallas JA, Kang JG, Loake GJ. 2012. *AtGSNOR1* function is required for multiple developmental programs in *Arabidopsis*. *Planta* **236,** 887-900.

Lam HM, Wong P, Chan HK, Yam KM, Chen L, Chow CM, Coruzzi GM. 2003. Overexpression of the *ASN1* gene enhances nitrogen status in seeds of *Arabidopsis*. *Plant Physiology* **132,** 926-935.

Lambeck IC, Fischer-Schrader K, Niks D, Roeper J, Chi JC, Hille R, Schwarz G. 2012. Molecular mechanism of 14-3-3 protein-mediated inhibition of plant nitrate reductase. *The Journal of Biological Chemistry* **287,** 4562-4571.

Lanteri ML, Laxalt AM, Lamattina L. 2008. Nitric oxide triggers phosphatidic acid accumulation via phospholipase D during auxin-induced adventitious root formation in cucumber. *Plant Physiology* **147,** 188-198.

Laugier E, Bouguyon E, Mauriès A, Tillard P, Gojon A, Lejay L. 2012. Regulation of high-affinity nitrate uptake in roots of *Arabidopsis* depends predominantly on posttranscriptional control of the NRT2.1/NAR2.1 transport system. *Plant Physiology* **158,** 1067-1078.

Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E. 2008. Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. *The Plant Cell* **20,** 786-802.

Li JY, Fu YL, Pike SM, Bao J, Tian W, Zhang Y, Chen CZ, Zhang Y, Li HM, Huang J, Li LG, Schroeder JI, Gassmann W, Gong JM. 2010. The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. *The Plant Cell* **22,** 1633-1646.

Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY, Glass AD. 2007. Dissection of the *AtNRT2.1*:*AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. *Plant Physiology* **143,** 425-433.

Lillo C, Lea US, Leydecker MT, Meyer C. 2003. Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. *The Plant Journal* **35,** 566-573.

Lillo C, Meyer C, Lea US, Provan F, Oltedal S. 2004. Mechanism and importance of post-translational regulation of nitrate reductase. *Journal of Experimental Botany* **55,** 1275-1282.

Lillo C. 2008. Signalling cascades integrating light-enhanced nitrate metabolism. *Biochemical Journal* **415,** 11-19.

Lin SH, Kuo HF, Canivenc G, Lin CS, Lepetit M, Hsu PK, Tillard P, Lin HL, Wang YY, Tsai CB, Gojon A, Tsay YF. 2008. Mutation of the *Arabidopsis NRT1.5* nitrate transporter causes defective root-to-shoot nitrate transport. *The Plant Cell* **20,** 2514-2528.

Linkohr BI, Williamson LC, Fitter AH, Leyser HM. 2002. Nitrate and phosphate availability and distribution have different effects on root system architecture of *Arabidopsis*. *The Plant Journal* **29,** 751-760.

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE. 2005. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *Proceedings of the National Academy of Science, USA* **102,** 13693-13698.

Liu KH, Huang CY, Tsay YF. 1999. CHL1 is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. *The Plant Cell* **11,** 865-874.

Liu KH, Tsay YF. 2003. Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. *The EMBO Journal* **22,** 1005-1013.

Lobell DB, Schlenker W, Costa-Roberts J. 2011. Climate trends and global crop production since 1980. *Science* **333,** 616-620.

López-Arredondo DL, Leyva-González MA, Alatorre-Cobos F, Herrera-Estrella L. 2013. Biotechnology of nutrient uptake and assimilation in plants. *The International Journal of Developmental Biology* **57,** 595-610.

Lu Y, Luo F, Yang M, Li X, Lian X. 2011. Suppression of glutamate synthase genes significantly affects carbon and nitrogen metabolism in rice (*Oryza sativa* L.). *Science China Life Sciences* **54,** 651-663.

Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A. 2013. Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. *Nature Communications* **4,** 1713.

Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C, Dubois F, Balliau T, Valot B, Davanture M, Tercé-Laforgue T, Quilleré I, Coque M, Gallais A, Gonzalez-Moro MB, Bethencourt L, Habash DZ, Lea PJ, Charcosset A, Perez P, Murigneux A, Sakakibara H, Edwards KJ, Hirel B. 2006. Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *The Plant Cell* **18,** 3252-3274.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of Botany* **105,** 1141-1157.

McAllister CH, Beatty PH, Good AG. 2012. Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnology Journal* **10,** 1011-1025.

Méndez-Bravo A, Raya-González J, Herrera-Estrella L, López-Bucio J. 2010. Nitric oxide is involved in alkamide-induced lateral root development in *Arabidopsis*. *Plant and Cell Physiology* **51,** 1612-1626.

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM. 2007. Nitrate transport and signalling. *Journal of Experimental Botany* **58,** 2297-2306.

Miller AJ, Fan X, Shen Q, Smith SJ. 2008. Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *Journal of Experimental Botany* **59,** 111-119.

Moll RH, Kamprath EJ, Jackson WA. 1982. Analysis and interpretation of factors which contribute to efficiency to nitrogen utilization. *Agronomy Journal* **74,** 562-564.

Morère-Le Paven MC, Viau L, Hamon A, Vandecasteele C, Pellizzaro A, Bourdin C, Laffont C, Lapied B, Lepetit M, Frugier F, Legros C, Limami AM. 2011. Characterization of a dual-affinity nitrate transporter MtNRT1.3 in the model legume *Medicago truncatula*. *Journal of Experimental Botany* **62,** 5595-5605.

Mulvaney RL, Khan SA, Ellsworth TR. 2009. Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. *Journal of Environmental Quality* **38,** 2295-2314.

Muños S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A. 2006. Transcript profiling in the *chl1-5* mutant of *Arabidopsis* reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. *The Plant Cell* **16,** 2433-2447.

Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJ, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* **5,** pls052.

Murooka Y, Mori Y, Hayashi M. 2002. Variation of the amino acid content of *Arabidopsis* seeds by expressing soybean aspartate aminotransferase gene. *Journal of Bioscience and Bioengineering* **94,** 225-230.

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K. 2008. CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. **452,** 483-486.

Ohwaki Y, Kawagishi-Kobayashi M, Wakasa K, Fujihara S, Yoneyama T. 2005. Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. *Plant and Cell Physiology* **46,** 324-331.

Okamoto M, Vidmar JJ, Glass AD. 2003. Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. *Plant and Cell Physiology* **44,** 304-317.

Orsel M, Krapp A, Daniel-Vedele F. 2002. Analysis of the NRT2 nitrate transporter family in *Arabidopsis*. Structure and gene expression. *Plant Physiology* **129,** 886-896.

Orsel M, Chopin F, Leleu O, Smith SJ, Krapp A, Daniel-Vedele F, Miller AJ. 2007. Nitrate signaling and the two component high affinity uptake system in *Arabidopsis*. *Plant Signaling and Behavior* **2,** 260-262.

Pagnussat GC, Lanteri ML, Lamattina L. 2003. Nitric oxide and cyclic GMP are messengers in the IAA-induced adventitious rooting process. *Plant Physiology* **132,** 1241-1248.

Pagnussat GC, Lanteri ML, Lombardo MC, Lamattina L. 2004. Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. *Plant Physiology* **135,** 279-286.

Parry MA, Hawkesford MJ. 2010. Food security: increasing yield and improving resource use efficiency. *Proceedings of the Nutrition Society* **69,** 592-600.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C, Delledonne M. 2004. *Arabidopsis* nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *The Plant Cell* **16,** 2785-2794.

Plett D, Toubia J, Garnett T, Tester M, Kaiser BN, Baumann U. 2010. Dichotomy in the *NRT* gene families of dicots and grass species. *PLos One* **5,** e15289**.**

Quaggiotti S, Ruperti B, Borsa P, Destro T, Malagoli M. 2003. Expression of a putative high-affinity $NO3^-$ transporter and of an H^+-ATPase in relation to whole plant nitrate transport physiology in two maize genotypes differently responsive to low nitrogen availability. *Journal of Experimental Botany* **54,** 1023-1031.

Quraishi UM, Abrouk M, Murat F, Pont C, Foucrier S, Desmaizieres G, Confolent C, Rivière N, Charmet G, Paux E, Murigneux A, Guerreiro L, Lafarge S, Le Gouis J, Feuillet C, Salse J. 2011. Cross-genome map based dissection of a nitrogen use efficiency ortho-metaQTL in bread wheat unravels concerted cereal genome evolution. *The Plant Journal* **65,** 745-756.

Rahayu YS, Walch-Liu P, Neumann G, Romheld V, von Wiren N, Bangerth F. 2005. Root-derived cytokinins as long-distance signals for NO_3^--induced stimulation of leaf growth. *Journal of Experimental Botany* **56,** 1143-1152.

Raun WR, Johnson GV. 1999. Improving nitrogen use efficiency for cereal production. *Agronomy Journal* **91,** 357-363.

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A. 2006a. The *Arabidopsis* NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proceedings of the National Academy of Science, USA* **103,** 19206-19211.

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A. 2006b. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. *Plant Physiology* **140,** 909-921.

Rich SM, Watt M. 2013. Soil conditions and cereal root system architecture: review and considerations for linking Darwin and Weaver. *Journal of Experimental Botany* **64,** 1193-1208.

Robertson GP. 2004. Abatement of nitrous oxide, methane and other non-CO2 greenhouse gases: the need for a systems approach. In *The global carbon cycle. Integrating Humans, Climate, and the Natural World*, Field CB, Raupach MR (eds.), SCOPE 62, Island Press, Washington D.C., 493-506.

Robertson GP, Vitousek PM. 2009. Nitrogen in agriculture: balancing the cost of an essential resource. *Annual Review of Environment and Resources* **34,** 97-125.

Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR. 2009. Members of the *LBD* family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. *The Plant Cell* **21,** 3567-3584.

Sakakibara Y, Kimura H, Iwamura A, Saitoh T, Ikegami T, Kurisu G, Hase T. 2012. A new structural insight into differential interaction of cyanobacterial and plant ferredoxins with nitrite reductase as revealed by NMR and X-ray crystallographic studies. *The Journal of Biochemistry* **151,** 483-492.

Santi S, Locci G, Monte R, Pinton R, Varanini Z. 2003. Induction of nitrate uptake in maize roots: expression of a putative high-affinity nitrate transporter and plasma membrane H^+-ATPase isoforms. *Journal of Experimental Botany* **54,** 1851-1864.

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiology* **136,** 2483-2499.

Segonzac C, Boyer JC, Ipotesi E, Szponarski W, Tillard P, Touraine B, Sommerer N, Rossignol M, Gibrat R. 2007. Nitrate efflux at the root plasma membrane: identification of an *Arabidopsis* excretion transporter. *The Plant Cell* **19,** 3760-3777.

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S, Uchiumi T. 2005. Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant and Cell Physiology* **46,** 99-107.

Siddiqui MH, Al-Whaibi MH, Basalah MO. 2011. Role of nitric oxide in tolerance of plants to abiotic stress. *Protoplasma* **248,** 447-455.

Signora L, Smet ID, Foyer CH, Zhang H. 2001. ABA plays a central role in mediating the regulatory effects of nitrate on root branching in *Arabidopsis*. *The Plant Journal* **28,** 655-662.

Suzuki A, Knaff DB. 2005. Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. *Photosynthesis Research* **83,** 191-217.

Tabuchi M, Abiko T, Yamaya T. 2007. Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.). *Journal of Experimental Botany* **58,** 2319-2327.

Tamura W, Hidaka Y, Tabuchi M, Kojima S, Hayakawa T, Sato T, Obara M, Kojima M, Sakakibara H, Yamaya T. 2010. Reverse genetics approach to characterize a function of NADH-glutamate synthase1 in rice plants. *Amino Acids* **39,** 1003-1012.

Tegeder M, Rentsch D. 2010. Uptake and partitioning of amino acids and peptides. *Molecular Plant* **3,** 997-1011.

Tian QY, Chen FJ, Zhang FS, Mi GH. 2005. Possible involvement of cytokinin in nitrate-mediated root growth in maize. *Plant and Soil* **100,** 1-12.

Tian QY, Chen FJ, Liu J, Zhang FS, Mi GH. 2008. Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. *Journal of Plant Physiology* **165,** 942-951.

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. 2002. Agricultural sustainability and intensive production practices. *Nature* **418,** 671-677.

Tilman D, Balzer C, Hill J, Befort BL. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Science, USA* **108,** 20260-20264.

Tischner R. 2000. Nitrate uptake and reduction in higher and lower plants. *Plant, Cell and Environment* **23,** 1005-1024.

Trevisan S, Borsa P, Botton A, Varotto S, Malagoli M, Ruperti B, Quaggiotti S. 2008. Expression of two maize putative nitrate transporters in response to nitrate and sugar availability. *Plant Biology* **10,** 462-475.

Trevisan S, Manoli A, Begheldo M, Nonis A, Enna M, Vaccaro S, Caporale G, Ruperti B, Quaggiotti S. 2011. Transcriptome analysis reveals coordinated spatiotemporal regulation of hemoglobin and nitrate reductase in response to nitrate in maize roots. *New Phytologist* **192,** 338-352.

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM. 1993. The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. *Cell* **72,** 705-713.

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK. 2007. Nitrate transporters and peptide transporters. *FEBS Letters* **581,** 2290-2300.

Vahisalu T, Kollist H, Wang YF, Nishimura N, Chan WY, Valerio G, Lamminmäki A, Brosché M, Moldau H, Desikan R, Schroeder JI, Kangasjärvi J. 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452,** 487-491.

Vidal EA, Tamayo KP, Gutiérrez RA. 2010. Gene networks for nitrogen sensing, signaling, and response in *Arabidopsis thaliana*. *Wiley Interdisciplinary Reviews. Sistems Biology and Medicine* **2,** 683-693.

von der Fecht-Bartenbach J, Bogner M, Dynowski M, Ludewig U. 2010. CLC-b-mediated NO_3^-/H^+ exchange across the tonoplast of *Arabidopsis* vacuoles. *Plant and Cell Physiology* **51,** 960-968.

Walch-Liu P, Ivanov II, Filleur S, Gan Y, Remans T, Forde BG. 2006. Nitrogen regulation of root branching. *Annals of Botany* **97,** 875-881.

Walch-Liu P, Forde BG. 2008. Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *The Plant Journal* **54,** 820-828.

Wang R, Liu D, Crawford NM. 1998. The *Arabidopsis* CHL1 protein plays a major role in high-affinity nitrate uptake. *Proceedings of the National Academy of Science, USA* **95,** 15134-15139.

Wang R, Guegler K, LaBrie ST, Crawford NM. 2000. Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *The Plant Cell* **12,** 1491-1509.

Wang R, Tischner R, Gutiérrez RA, Hoffman M, Xing X, Chen M, Coruzzi G, Crawford NM. 2004. Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. *Plant Physiology* **136,** 2512-2522.

Wang R, Xing X, Wang Y, Tran A, Crawford NM. 2009. A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene *NRT1.1*. *Plant Physiology* **151,** 472-478.

Wang YY, Tsay YF. 2011. *Arabidopsis* nitrate transporter NRT1.9 is important in phloem nitrate transport. *The Plant Cell* **23,** 1945-1957.

Wang YY, Hsu PK, Tsay YF. 2012. Uptake, allocation and signaling of nitrate. *Trends in Plant Science* **17,** 458-467.

Wildt J, Kley D, Rockel A, Rockel P, Segschneider HJ. 1997. Emission of NO from several higher plant species. *Journal of Geophysical Research* **102,** 5919-5927.

Wirth J, Chopin F, Santoni V, Viennois G, Tillard P, Krapp A, Lejay L, Daniel-Vedele F, Gojon A. 2007. Regulation of root nitrate uptake at the NRT2.1 protein level in *Arabidopsis thaliana*. *The Journal of Biological Chemistry* **282,** 23541-23552.

Wolansky MA. 2005. *Genetic manipulation of aspartate aminotransferase levels in Brassica napus: effects on nitrogen use efficiency*. M.Sc. thesis: University of Alberta.

Xu G, Fan X, Miller AJ. 2012. Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology* **63,** 153-182.

Yamasaki H, Sakihama Y, Takahashi S. 1999. An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends in Plant Science* **4,** 128-129.

Yamasaki H, Sakihama Y. 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters* **468,** 89-92.

Yamaya T, Obara M, Nakajima H, Sasaki S, Hayakawa T, Sato T. 2002. Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. *Journal of Experimental Botany* **53,** 917-925.

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T. 2004. Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. *Proceedings of the National Academy of Science, USA* **101,** 7833-7838.

Yendrek CR, Lee YC, Morris V, Liang Y, Pislariu CI, Burkart G, Meckfessel MH, Salehin M, Kessler H, Wessler H, Lloyd M, Lutton H, Teillet A, Sherrier DJ, Journet EP, Harris JM, Dickstein R. 2010. A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. *The Plant Journal* **62,** 100-112.

Yu M, Lamattina L, Spoel SH, Loake GJ. 2014. Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytologist* **202,** 1142-1156.

Yu P, White PJ, Hochholdinger F, Li C. 2014. Phenotypic plasticity of the maize root system in response to heterogeneous nitrogen availability. *Planta* **240,** 667-678.

Zhang H, Forde BG. 1998. An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279,** 407-409.

Zhao DY, Tian QY, Li LH, Zhang WH. 2007. Nitric oxide is involved in nitrate-induced inhibition of root elongation in *Zea mays*. *Annals of Botany* **100,** 497-503.

5.2 Websites cited

http://www.census.gov/popclock/

http://faostat.fao.org/site/567/default.aspx#ancor

Chapter II - NO homeostasis is a key regulator of early nitrate perception and root elongation in maize

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This work is dedicated to our friend and master Angelo Ramina.

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1. Abstract

Crop plant development is strongly dependent on the nitrogen availability in the soil and on the efficiency of its utilization. However, knowledge about molecular responses to nitrogen availability derives mainly from the study of model species. The understanding of the molecular events underlying the root adaptation to nitrogen fluctuations is a primary goal to develop biotechnological tools for sustainable agriculture.

Nitric oxide (NO) has been recently proposed to be implicated in plant response to environmental stresses, but its exact role in the response of plants to nutritional stress is still under evaluation.

In this work the role of nitric oxide production by maize roots after nitrate perception was investigated by focusing on the regulation of transcription of genes involved in the NO homeostasis and by measuring the NO production in roots. Moreover, its involvement in the root growth response to nitrate was also investigated.

Our results provided evidence that NO is produced by nitrate reductase, as an early response to nitrate supply, and that the coordinated induction of ns-haemoglobins could finely regulate the NO steady-state. This seems to be implicated on the modulation of the root elongation in response to nitrate perception.

Moreover an improved agar-plate system for growing maize seedlings was developed. Thanks to this system, which allows to perform localized treatments on specific root portions, it has been possible to discriminate between localized and systemic effects of nitrate supply to roots.

Keywords: Maize, Nitrate (NO_3^-), Nitrate reductase (NR), Nitric oxide (NO), Ns-haemoglobin (Hb), Root, Transition zone (TZ).

Abbreviations: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO); L-NG-Nitroarginine methyl ester (L-NAME); non-symbiotic haemoglobin (nsHb); (±)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR); Sodium nitroprusside (SNP); Sodium tungstate dihydrate ($Na_2WO_4 \bullet 2H_2O$).

2. Introduction

Soil nutrients acquisition intensely affects global crop production (Forde and Clarkson, 1999; Robertson and Vitousek, 2009). In poor nations drought and low soil fertility cause low yields and food insecurity, while in rich nations intensive fertilization leads to soil leaching and/or greenhouse gas emission (Donner and Kucharik, 2008). The development of new crop cultivars with enhanced soil resource acquisition is therefore an important strategic goal for modern agriculture (Lynch, 1998; Vance *et al*., 2003; Lynch, 2007). Understanding nutrient responses at the organism level will be useful to modify plant metabolism, physiology, growth and developmental programs to improve nutrient use efficiency and productivity in crops.

The macronutrient nitrogen is essential for plant growth and development as it is a component of proteins, nucleic acids, many co-factors and secondary metabolites. In aerobic soils nitrate is the major source of nitrogen for most plant species (Ahmad *et al*., 2007; Nischal *et al*., 2012).

Plants have the potential for adaptation to dramatic fluctuations of nitrogen availability by modulating their capacity for nutrient acquisition and by alteration of whole-plant morphology and metabolism, such as increasing the root/shoot ratio (Rubio *et al*., 2009). Developmental adaptive mechanisms stimulate growth in organs that directly participate in nutrient acquisition, such as primary roots (Walch-Liu and Forde, 2008). A dual effect of external nitrate on root system architecture (RSA) development has been depicted in the model species *Arabidopsis thaliana*: (i) a systemic inhibition of lateral primordia by uniformly high nitrate concentrations at a post-emergence stage and (ii) a localized stimulation of elongation on N-starved roots at the site of contact with a nitrate rich supply, known as the foraging capacity (Zhang and Forde, 1998; Zhang *et al*., 1999; Linkohr *et al*., 2002; Zhang *et al*., 2007; Ruffel *et al*., 2011). Apart from a few known pathways that involve transcription factors, micro-RNAs, hormonal signals and, more recently, nitrate transporters with dual affinity for nitrate and auxin (Little *et al*., 2005; Remans *et al*., 2006; Miller *et al*., 2007; Chiou, 2007; Gifford *et al*., 2008; Krouk *et al*., 2010; 2011; Vidal *et al*., 2010; Castaings *et al*., 2011; Rubio-Somoza and Weigel, 2011; Ruffel *et al*., 2011; Trevisan *et al*., 2012; Xu *et al*., 2012), our understanding of sensing external nitrate conditions and of the signal transduction system that leads to an altered development of root is still poor.

To trigger adaptive responses and to induce fast switching from starvation metabolism to nutrient assimilation, the nitrate itself or its primary assimilation products serve as signalling molecules (Schulze *et al*., 1994; Crawford, 1995; Scheible *et al*., 1997; Stitt, 1999). Significant advances have been made during the recent period concerning the molecular mechanisms of NO_3^- sensing and signalling in *Arabidopsis*, and the striking action of NO_3^- as a signal in regulating genome expression has been unravelled (Bouguyon *et al*., 2012).

A prolonged nitrate starvation was demonstrated to largely affect gene transcription, producing effects on the early nitrate signalling mechanisms. Transcriptomic analyses evidenced co-regulated transcriptional patterns in maize root epidermal cells for genes putatively involved in nitric oxide synthesis/scavenging (Trevisan *et al*., 2012).

Nitric oxide is a free radical that is considered to be a general plant signal, since it regulates both normal developmental processes and biotic or abiotic stress responses involving cross-talk with phytohormones (for reviews, see: Durner and Klessig, 1999; Wojtaszek, 2000; Beligni and Lamattina, 2001; Lamattina *et al.,* 2003).

NO has been reported to be required for root organogenesis (Pagnussat *et al.,* 2002), formation of adventitious roots (Pagnussat *et al.,* 2003), lateral root (LR) development (Correa-Aragunde *et al.,* 2004) and root hair formation (Lombardo *et al*., 2006). Recently Correa-Aragunde *et al.,* (2004) suggested the possibility that auxin and NO might be on a linear signalling pathway in the process of LR formation in tomato. However, our knowledge of the molecular mechanisms by which NO regulates growth and development is still fragmentary.

NO is produced in plant tissues by two major pathways, one enzymatic and the other non enzymatic (Wendehenne *et al*., 2004). The NO-producing enzymes identified in plants are nitrate reductase (NR), and several nitric oxide synthase-like proteins, including one localized in peroxisomes that has been biochemically characterized (del Río *et al*., 2004). Interestingly, it was recently shown that non-symbiotic haemoglobin 1 enzyme could reduce NO_2^- to NO with a constant rate that was far in excess of that reported for haemoglobins (Hbs) (Sturms *et al*., 2011). Plant Hbs are able to regulate several NO effects, as recently reviewed by Hill (2012). Class II nsHbs contributes to NO removal when over-expressed (Hebelstrup *et al*., 2006; 2012). Moreover, several studies have demonstrated a role for

plant Hbs in catalysing the turnover of nitric oxide to nitrate (Dordas *et al*., 2003*a; b*, 2004; Perazzolli *et al*., 2004; Hebelstrup *et al*., 2006; 2012).

The nitrate-regulated expression and spatial distribution in epidermal cells of NR and Hb transcripts which have been recently evidenced in maize roots, strongly suggests that they could play an important role during the early perception and signalling of nitrate in the rhizosphere (Trevisan *et al*., 2011). Moreover the co-localization of mRNAs for NR and Hb observed in the root apex matches with the major sites of NO accumulation, as shown in *Arabidopsis* (Stöhr and Stremlau, 2006), suggesting that these two genes may represent the pivotal elements of a fine-tuning system for NO homeostasis and signalling.

The involvement of NO in the pathway of nitrate signalling opens a wide field of research. In this report we evaluated the contribution of NO in the nitrate-regulated pathway that directs RSA, unravelling the role of NO as a nitrate-related signal.

The present study is focused on both the characterization of the expression profiles of selected genes putatively involved in nitric oxide homeostasis and the determination of NO production by roots in response to different N treatments. In addition, since the genes therein selected have proved to be very good candidates for monitoring nitrate sensing in maize roots, we propose them as early physio-molecular markers for the response to this anion. Furthermore, the effect of nitrate on root growth and especially on root elongation was also deepened. Moreover, an improved agar-plate culture system for studying the *Zea mays* L. root response to nutrients has been developed. Thanks to this system it has been possible to discriminate between localized and systemic effects of nitrate supply to roots.

Overall, our data provided evidence that in maize roots NO is produced by nitrate reductase as an early response to nitrate supply. Moreover, the coordinated induction of nsHbs, finely regulate the steady-state level of this molecule, which in turns seems to be involved in the modulation of the root growth in response to nitrate perception.

3. Materials and Methods

3.1 Maize growth and experimental design

Seeds of maize inbred line B73 were sown and then transferred to nutrient solution as described by Quaggiotti *et al.* (2003). For a first set of expression analyses seedlings were grown in different nutrient solutions for five days and then treated few hours as described in Fig. 1. For the analysis of root parameters with WinRhizo, seedlings were grown in the

nutrient medium for eight days. Nitrate, ammonium or ammonium-nitrate were supplied at a concentration of 1 mM. In the nitrogen depleted nutrient solution KNO_3 was replaced by 1 mM KCl and NH_4SO_4 by $MgSO_4$, respectively.

For nitric oxide content measurement, for subsequent expression analyses and for the analysis of root elongation rate, seedlings were grown only 24 h in the nutrient solution, to allow the manipulation of younger roots. To deepen the role of NO in the maize root response to nitrate Tungstate (1 mM), cPTIO (1 mM), L-NAME (0.2 mM), SNP (0.01 mM) and NOR (1 mM) were supplied to the nutrient solution (either NO_3^- -supplied or NO_3^--deprived) depending on the treatment.

Seedlings of the same age were also utilized to evaluate the expression of selected genes in four different portions of roots, as indicated by Baluska *et al*. (2010), after nitrate supply. The four zones sampled were: the root meristem (M, 4 mm), the transition zone (TZ, 1 cm), the elongation zone (EZ, 1 cm) and the maturation zone (MZ, residual portion). Roots were harvested after two hours of nitrate provision and the four fragments were immediately cut and frozen, both for root treated and for the negative control ($-NO_3^-$).

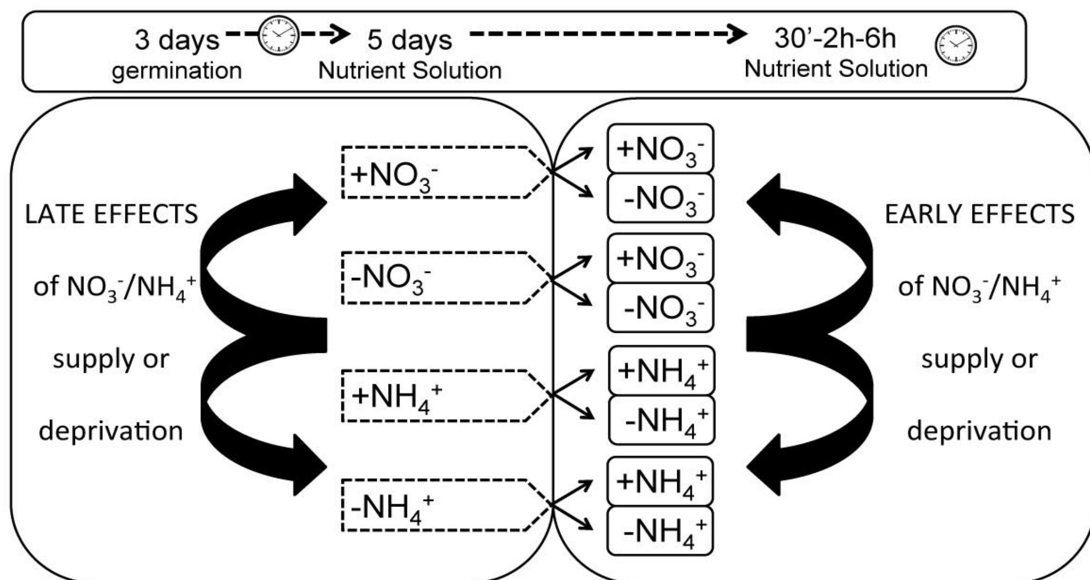

Figure 1. Workflow model of the experimental conditions. Seeds were sowed on filter paper, and three days after germination seedlings were divided into four groups and transferred for five days to four different hydroponic solutions: '+N' solution ($+NO_3^-$ and $+NH_4^+$, as reported in Materials and Methods section) and '–N' solution (NO3- and NH4+ - depleted nutrient solution, as reported in Material and Methods section). After five days, seedlings were transferred to eight different nutrient solutions, four '+N' solutions (two + NO_3^- and two + NH_4^+ groups) and four '–N' solutions (two - NO_3^- and two - NH_4^+ groups), and treated for different time (30 min, 2 h and 6 h). At the end of the treatments the eight groups of seedlings grown in different nitrogen availabilities were used to compare the effects of long/short term of nitrogen supply/depletion by means of a multifaceted transcriptomic approach.

3.2 Growth of maize seedlings in agar medium

A novel method was developed to grow maize seedlings on agar. To this aim specific plastic boxes (17.9 x 12.9 x 2.6 cm) modified with suitable holes on one side were utilized (Fig. S1). This system permits the insertion of young roots, which can grown vertically along the agar medium allowing the shoot to develop outside of the box, and enabled us to perform localized treatments to single portion of roots, as described in Fig. 2. The agar concentration utilized was 1%, after a preliminary test with concentrations ranging from 0.8 to 1.2%. The nutrients were supplied as indicated for hydroponics.

Roots of seedlings grown 24 h in a nitrate-depleted agar plate were transferred on an identical medium to which, in correspondence of specific root regions, round slices (about 1-1.5 cm in diameter) of agar were removed and substituted with new ones containing nitrate 1 mM. For the negative control the slices were substituted with new nitrate-depleted ones, to subject roots to a similar mechanical stress, thus avoiding false positives due to the perception of the discontinuance of agar and not to the nitrate presence.

3.3 Morphological analyses

For the analysis with WinRhizo, germinated seeds of maize inbred line B73 were transferred to 2-l-tanks containing five different aerated nutrient solutions (changed every two days) according to the treatments: a) + NO_3^-, b) – NO_3^-, c) + NH_4^+, d) – NH_4^+, e) +NH_4NO_3 and then placed in a growth chamber for eight days. The morphological analyses including total root length (cm), total surface area (cm^2), average root diameter (mm) and number of root tips were performed on thirty randomly chosen plants for each treatment (two biological replicates) by means of a STD-1600 EPSON scanner set and an image analysis software (WinRhizo Pro, Regent Instruments, QC, Canada). Statistical analyses were performed by using R software (version 2.14.2).

For the analysis of primary root elongation rate, seedlings were grown 24 h in a 500-ml beaker and subjected to six different treatments according to the growing medium, as follows: a) +NO_3^-, b) +NO_3^- +cPTIO, c) +NO_3^- +Tungstate, d) –NO_3^-, e) –NO_3^-+SNP, f) +NH_4^+. The measures of primary root length were made with a ruler on sixteen seedlings for each group, in four independent biological repetitions. To investigate possible effects of toxicity due to the use of chemicals, both total root weight and leaf weight were also measured. Statistical analyses were performed by using R software (version 2.14.2).

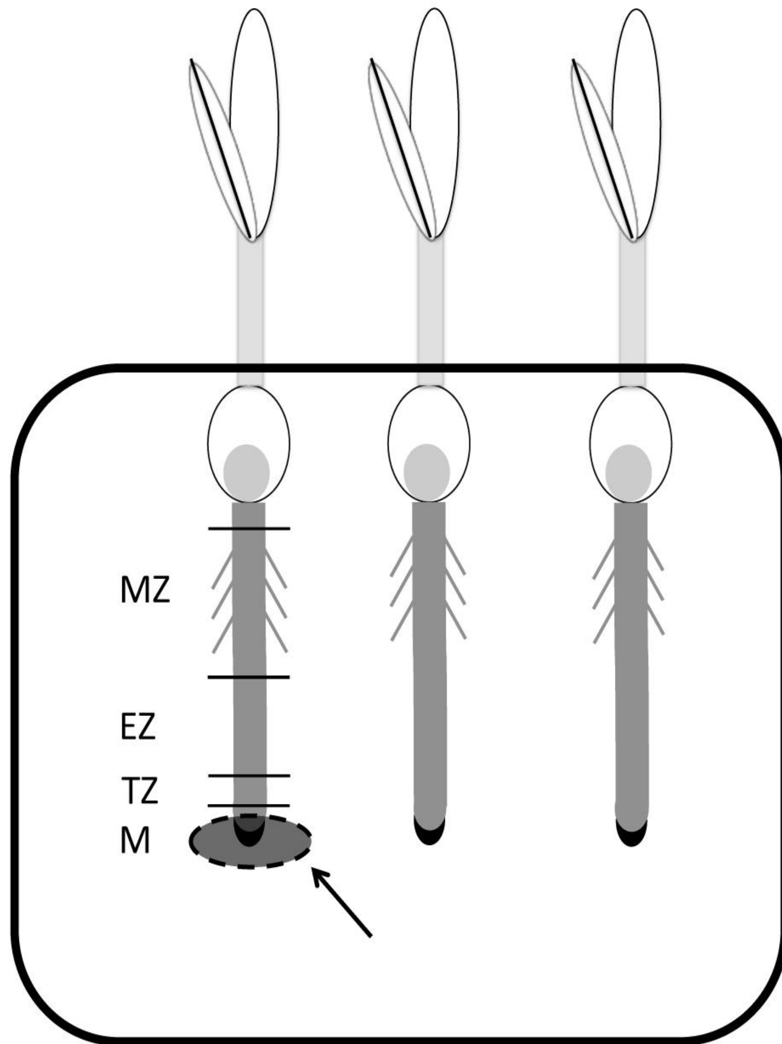

Figure 2. Design of the split-root system used to investigate the localized effects of nitrate on the intact root apex of maize seedlings. Seeds of maize inbred line B73 were sowed in paper and then seedlings were transferred to a vertical plate system. Plate prepared with N-depleted solution and 1% agar were either supplied with nitrate 1 mM (+N plants) or depleted (-N plants) by cutting and replacing a rounded portion of the agar, thus only apical portion of the root system could perceive the change of treatment. Seedlings continued to grow after the replacement of the rounded portion of agar, and at the end of the treatment they were removed from the system and harvested.

3.4 RNA extraction and cDNA synthesis

Tissues used for gene expression analyses were collected and immediately frozen in liquid nitrogen and kept at −80 °C for subsequent RNA extraction.

Total RNA was extracted as described by Trevisan *et al.* (2011) starting from 250 mg of frozen tissue and using the TRIzol method as described by the manufacturer (Invitrogen, San Giuliano Milanese, Italy). An aliquot of total RNA was treated with RQ1 RNAse-free DNAse (Promega, Milano, Italy) as described by Falchi *et al.* (2010). One µl of total RNA was quantified using a Nanodrop 1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE,

USA). cDNA was synthesized starting from 500 ng of total RNA mixed with 1 µl of Oligo dT 10 µM as described by Manoli *et al.* (2012).

3.5 Selection of genes to be evaluated, maize sequences identification and primers design

The list of genes analyzed is reported in Table S1, together with the primers utilized for RT-qPCR expression analysis. They were chosen according to previously published results (Trevisan *et al*., 2011; 2012). The *Hb* (NCBI: AF236080.1), the *NR1* (NCBI: AF153448.1) were then chosen for further more detailed analysis and the analysis was extended to the expression of *Hb2* (NCBI: NM_001112349.1), *NiR* (NCBI: ACG29734.1), *NOA1* (NCBI: NM_001174573) genes which were selected by screening the B73 genome database (http://www.maizesequence.org/index.html) and to *Nrt2.1* (NCBI: AY129953.1, Quaggiotti *et al*., 2003), that was used as a positive control for nitrate perception. The *NOA1* sequence was identified based on its similarity with the *AtNOA1* (At3g47450.1) gene of *Arabidopsis*.

Primers were designed with Primer3 web tool (ver. 0.4.0; http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky, 2000) and further verified with the PRATO web tool (Nonis *et al*., 2011; http://prato.daapv.unipd.it).

3.6 Real time qPCR

Relative quantification of transcripts by Real-Time PCR (RT-qPCR) was performed in a StepOne Real-Time PCR System (Applied Biosystems, Monza, Italy) as described by Nonis *et al. (*2007). Experiments were conducted using SYBR Green chemistry (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions. For each reaction 2.5 ng of retrotranscribed RNA were used as template. Three technical replicates were performed on six independent biological replicates using the conditions described by Trevisan *et al*. (2011). Melting curve analysis was performed to confirm the absence of multiple products or primer dimers formation. Data were exported and analyzed according to the Livak and Schmittgen (2001) method using *LUG* (leunig primers, forward 5'-TCCAGTGCTACAGGGAAGGT and reverse 5'- GTTAGTTCTTGAGCCCACGC) and *MEP* (Membrane protein PB1A10.07c, primers: forward 5'-TGTACTCGGCAATGCTCTTG and reverse 5'- TTTGATGCTCCAGGCTTACC) as reference genes according to Manoli *et al*. (2012). For each transcript, the ratio between the expression measured for a given treatment and that of its own control was used to estimate

up or down-regulation of genes. The ratios obtained were then expressed as base-2 logarithm to build the graphs.

3.7 NO detection

Germinated seeds were transferred to a nitrogen-depleted nutrient solution, and after 24 h root apices of 2 cm length ca. were excised and incubated for 30 min in 2 ml detection buffer (10 mM Tris-HCl, pH 7.4) added with 15 µM of DAF-2DA. Subsequently the apices were washed twice for 5 min with fresh detection buffer and placed on a microscope slide fixed with a Secure-Seal™ hybridization chamber gasket (Life Technologies, Carlsbad, CA, USA) (20-mm diameter, 0.8-mm deep) and analysed for NO production by stereo- and confocal microscopy. For each chamber one apex was incubated as described below.

For stereomicroscope analyses the chambers were immediately filled with nutrient solution containing 1 mM KNO_3 (+NO_3^-), or nitrogen-depleted nutrient solution containing 1 mM KCl (negative control, -NO_3^-), and examined by epi-fluorescence with a SteReo Lumar V.12 (Carl Zeiss, Oberkochen, Germany). Images were captured with an MRc5 Axiocam Zeiss color camera every five min for 50 min and processed with Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

Confocal NO measurements were carried out filling the chambers alternatively with: a) the +NO_3^- solution; b) -NO_3^-solution; c) +NO_3^- nutrient solution supplied with the NO scavenger cPTIO; d) -NO_3^- nutrient solution supplemented with the NO donor NOR-3; e) +NO_3^- solution with sodium tungstate. The incubation in DAF-2DA was carried out as previously described.

All apices were observed with a Leica TCS-SP2 confocal microscope (Leica Microsystems CMS, Mannheim, Germany) and images were acquired every five min for 45 min form the beginning of the incubation. Images were then analysed using the Leica Confocal Software application. Normalization of the data and ratios of average fluorescence intensities were calculated as described by Calcagno *et al*. (2012). Five root pieces were tested for each condition and five independent repeats were analyzed for each treatment.

4. Results

4.1 Nitrate exerts specific effects on genes involved in NO homeostatic control

The expression of a number of previously identified genes (Quaggiotti *et al*., 2003; Trevisan *et al*., 2011; 2012) together with that of some new ones (Table S1), was measured in roots and leaves of seedlings grown five days in a nutrient solution containing 1 mM nitrate (+NO_3^-) or 1 mM ammonium (+NH_4^+) or N-deprived (both -NO_3^- and -NH_4^+) (Fig. 3).

The transcriptional response of five of them (*NR1*, *Hb*, *Hb2*, *Nrt2.1*, *NiR*) evidenced a very strong nitrate responsiveness in roots. A similar behaviour was observed in leaves, even if to a lower extent. The rest of genes selected, on the contrary, did not evidence specific nitrate responsiveness.

The expression of the same set of genes was also assessed on root and leaf tissues of five-days old seedlings, but after only 30 min, 2 and 6 h of nitrate/ammonium provision or depletion. The time-course of the expression of the five nitrate specific targets in both roots and leaves after few hours of nitrate/ammonium supply/starvation is shown in Fig. 4 (the expression patterns of the other genes tested is reported in Fig. S2).

The nitrate supply induced a significant increase of transcript accumulation for all the five genes both in roots and in leaves (Fig. 4A and 4B, upper side), even if in roots it was much more noticeable (from 4-16 fold already after 30 min of NO_3^- supply, to 8->100 fold after six hours). Conversely, the transcription of the five genes did not show a similar increase when ammonium was supplied as unique nitrogen form, neither in roots nor in leaves (Fig. 4A and 4B, lower side), confirming the specificity of responsiveness to nitrate. Also in the case of N-deprivation all five genes displayed a more evident response (decrease of expression) to nitrate deprivation in comparison to that measured for ammonium removal (Fig. 4A and 4B, left column), both in leaves and in roots.

These five genes specifically nitrate inducible were thus selected for the subsequent and more detailed expression analyses.

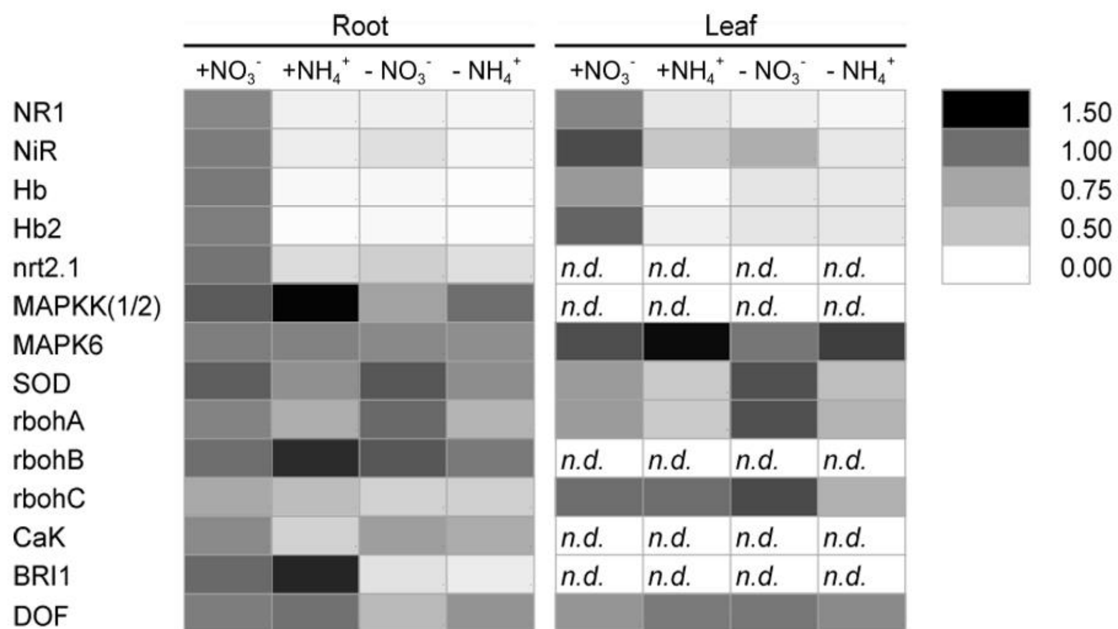

Figure 3. Heat map showing gene expression of 14 genes significantly regulated by long-term nitrate or ammonium supply and depletion in *Zea mays* L. roots and leaves. Seedlings were grown for five days in a nutrient solution containing 1 mM nitrate (+NO_3^-) or 1 mM ammonium (+NH_4^+) or N-deprived (both -NO_3^- and -NH_4^+). At the end of the treatment seedlings were harvest and roots were separated from leaves. The colour scale represents the level of a gene expression. Values are reported as arbitrary unit and are the means of 3 technical repetitions performed on six independent biological replicates.

4.2 Root growth responds specifically to nitrate availability

The effect of nitrate supply on root development was evaluated in comparison to that of both ammonium and NO_3NH_4 in plants grown in nutrient solution for eight days (Table 1). The analysis of root length, root surface area and number of tips evidenced a similar pattern, showing the strongest root growth stimulation in seedlings grown with nitrate 1 mM (treatment 1). Values measured for these three parameters in plants grown with ammonium (treatment 3) were significantly lower (50-60%) then those observed for nitrate-supplied roots and closest to rates observed for NO_3^--depleted roots (treatment 2, nitrate negative control). Furthermore, an inhibitory effect of ammonium supply was visible for both root length and tips number, which showed values even lower with respect to negative control (treatment 4). The supply of NO_3NH_4 (treatment 5) slightly stimulated these three parameters, even if to a significantly lower extent with respect to nitrate.

The average root diameter showed an opposite trend with the maximum rate observed for ammonium treated roots (treatment 3) and the lowest one for nitrate-supplied plants (treatment 1), which evidenced values even lower than those observed for nitrate-depleted roots (treatment 2). These observations, beside suggesting a compensatory

mechanism between the growth in length and in thickness in maize root, highlight the specificity of nitrate in affecting the root growth, which conversely did not showed any similar response when nitrogen was supplied as ammonium.

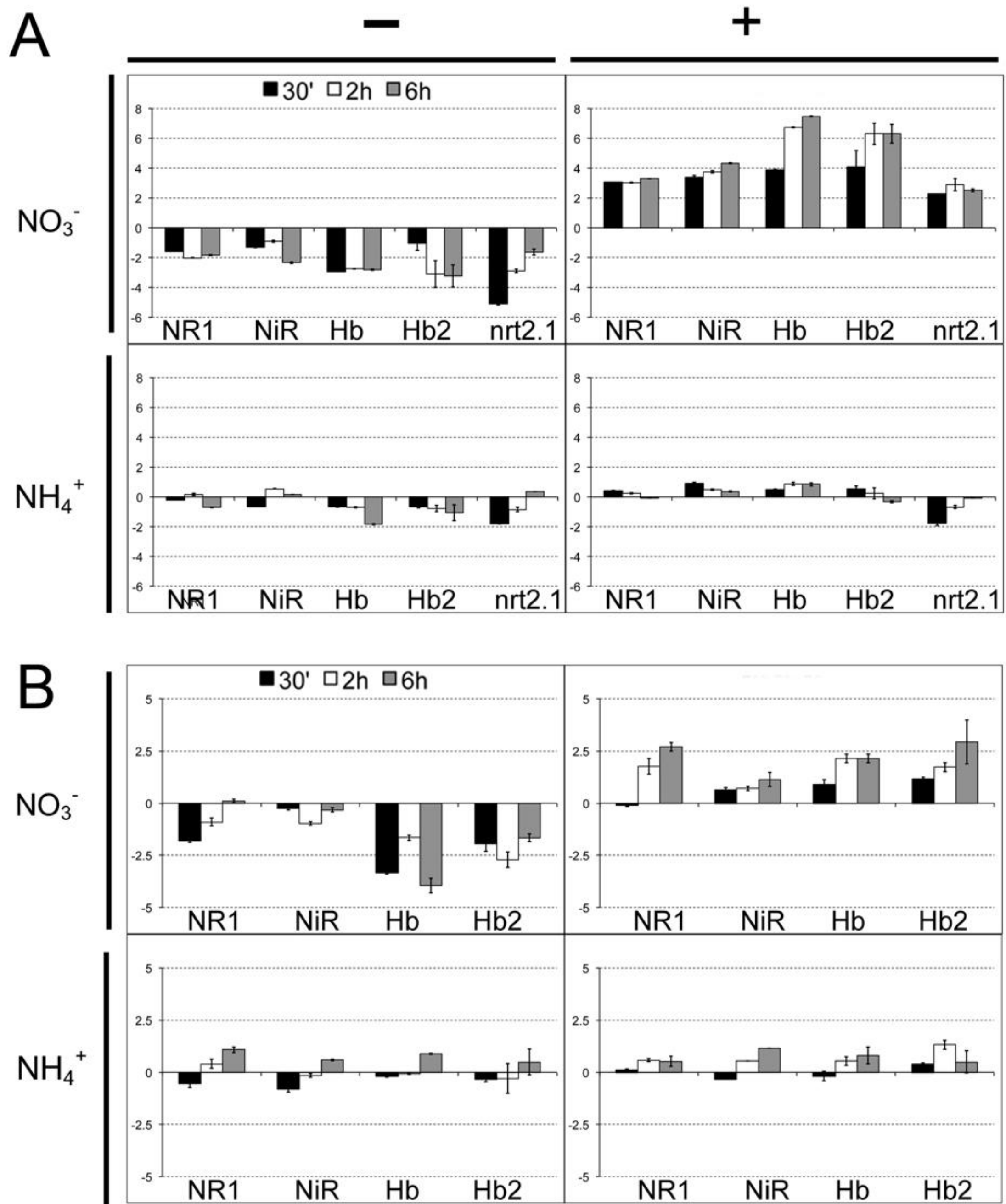

Figure 4. Time course of the expression of 5 genes significantly regulated by short-term nitrate/ammonium treatments in *Zea mays* L. roots (A) and leaves (B). The expression values were investigated at 30 min, 2 h and 6 h.

Data are expressed as base-2 logarithm of the ratio between the expression measured for a treatment and that of its own control. The same expression analyses were carried out in nutrient solution either supplied or depleted with nitrate or ammonium. In the left column the time course analyses of gene expressed in nitrogen-starved seedling are reported (-), while values correspondent to nitrogen supplied system (+) are reported in the right column.

Table 1. Effects of nitrate supply on root development. Root length, root surface area and number of tips were evaluated in plants grown in nutrient solution for eight days. The treatments investigated were 5: nitrate supplied roots (treatment 1); NO_3^--depleted roots (treatment 2); ammonium supplied roots (treatment 3); ammonium depleted roots (treatment 4); NO_3NH_4 supplied roots (treatment 5). Different letters indicate statistically significant differences among samples ($p<0.05$, ANOVA Test).

Treat.	Lenght (cm)	Surface Area (cm^2)	Av.diameter (mm)	Tips (n°)
1	79.48±3.98 [a]	12.58±0.55 [a]	0.50±0.01 [d]	91.20±5.07 [a]
2	51.61±2.96 [c]	8.60±0.46 [c]	0.55±0.01 [c]	67.40±4.57 [b]
3	36.59±1.39 [d]	8.18±0.28 [c]	0.70±0.01 [a]	55.43±3.34 [c]
4	51.34±2.15 [c]	9.03±0.43 [c]	0.52±0.01[d]	72.90±2.75 [b]

4.3 NR-dependent NO production after nitrate supply

To better understand the role of NO in nitrate signalling, its production was monitored by measuring the DAF-2T fluorescence in stereomicroscopy.

Seedlings grown for 24 h without nitrate were supplied with 1mM nitrate and the fluorescence produced was observed (Fig. 5A, panels I-P) in comparison to that measured in negative control (Fig. 5A, panels A-H). The nitrate supply caused a slight but consistent increase in DAF fluorescence since the first minutes after treatment (panels J and K). No fluorescence increase was induced by NO_3^--deprived control treatments, where by contrast a signal decrease was observed after ten minutes, probably due to the decay of the probe (panels A-H). Based on these observations the increment of fluorescence was mainly localized immediately above the meristematic apex and more precisely in the transition zone, as defined by Verbelen *et al*. (2006) and Mugnai *et al*. (2012).

In order to get a more detailed imaging and quantification of DAF fluorescence, we repeated the experiment in confocal microscopy and also evaluated the effects of a NO donor (NOR), a NO scavenger (cPTIO) and a NR inhibitor (tungstate).

Fig. 5B shows two pictures for both $–NO_3^-$ and $+NO_3^-$ treatments at T_0 and after 30 min of observation. Figure clearly evidences a difference between the two treatments, with a strong increase in the DAF fluorescence in response to nitrate provision (panel D), which was not observed in the case of negative control ($-NO_3^-$ roots) (panel C).

Moreover, higher magnification analyses (Fig. S3) revealed a few cytological details on the different cell types observed, which typically distinguish the transition zone (TZ). In the distal part of the portion of root examined nuclei are positioned in the centre of the cell, similarly to the meristem, whereas the more distal zone cells resembled those of the elongation zone with large central vacuoles and nuclei pushed to the side cell walls.

The same observations were performed in the presence of tungstate, NOR and cPTIO. Results obtained on five biological repetitions are reported in Fig. 5C. Data were expressed as relative fluorescence increase/decrease after 30 min of observation. Results showed a significant increase of fluorescence for nitrate supplied and for NOR-treated roots. On the contrary, when seedlings were supplied with a $–NO_3^-$-solution (negative control) or treated with both nitrate plus tungstate and nitrate plus cPTIO, the fluorescence did not increase throughout the experiment. These results globally suggest that a NO burst is produced immediately after nitrate supply to roots and that this is probably due to the nitrate reductase activity, which is activated in response to the anion, as also supported by the expression analysis data.

4.4 Genes putatively involved in the control of NO homeostasis are involved in the early response to nitrate

To allow the NO content determination via both stereomicroscope and confocal analyses roots were harvested from 24 h-old seedlings, in order to obtain root segments of size compatible with the mini-chamber utilized. For this reason for the following expression analyses we decided to shift the experimental plan to younger seedlings and to focus only on the early events after nitrate provision. Plants were, thus, grown 24 h in a $–NO_3^-$ solution and then transferred to a $+NO_3^-$ medium for two hours. The transcript accumulation of the previously selected genes (*NR1, Hb, Hb2, NiR*) together with a new one (*NOA1*) encoding a putative *AtNOA1* orthologous was examined after nitrate supply and in the presence of cPTIO, tungstate and L-NAME. The expression of *Nrt2.1* was also included among the analyses, as a positive control of the nitrate perception.

The nitrate addition induced strong increments of transcription for all the genes analyzed, except for *NOA1* (Fig. 6, first two columns of each gene). The expression of nitrate reductase gene reached rates six/nine fold higher in comparison to that measured in $–NO_3^-$ roots, whereas the two isoforms of ns-haemoglobin increased their transcription even by 27-72 fold. The *NiR* and the *Nrt2.*1 showed an induction of their expression of 21 and six fold respectively.

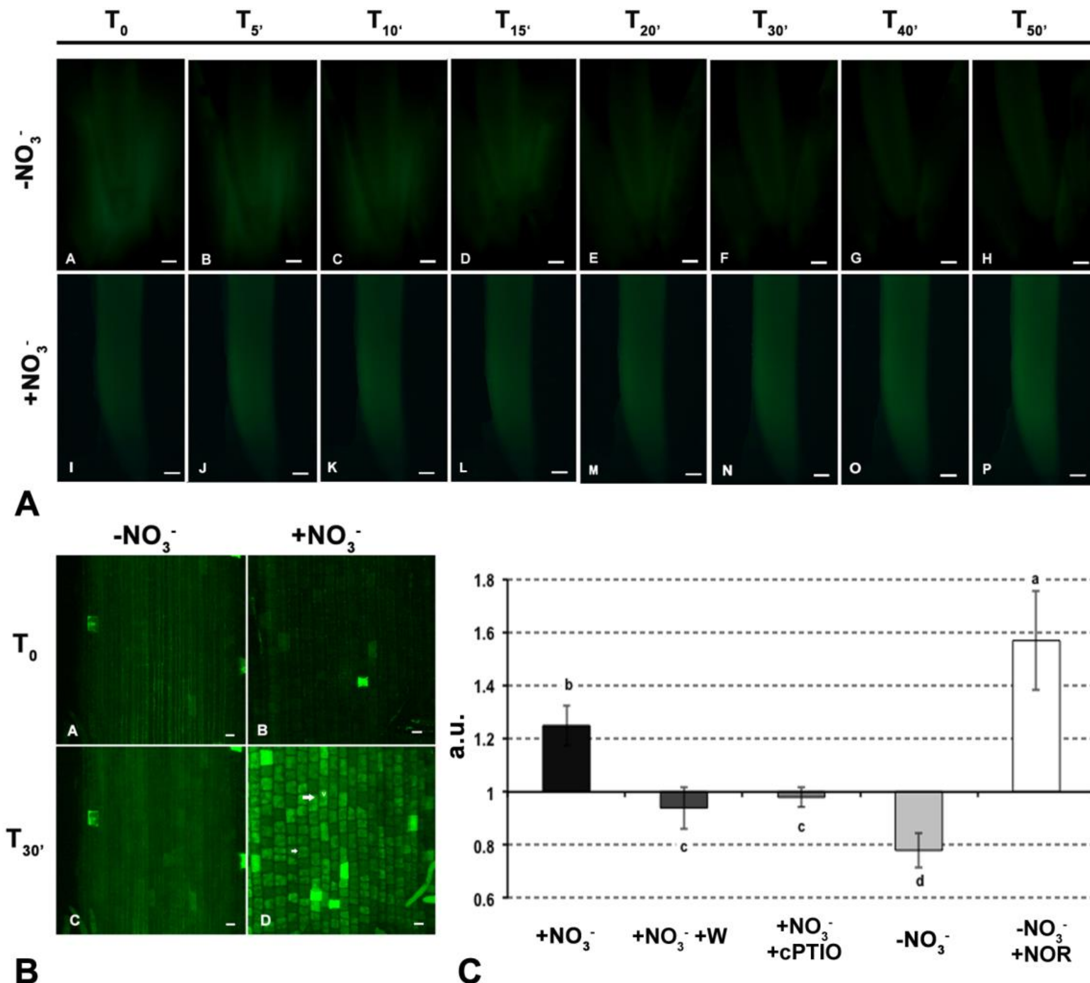

Figure 5. NO detection on 2 cm maize root apices excised from seedlings grown for 24 h in nitrogen-depleted nutrient solution. A) Stereomicroscope time course imaging of DAF-2T fluorescence (T0-T50') on apices treated for 30 min with 1 mM KCl (negative control, -NO3-) (A-H) and 1 mM KNO3 solution (I-P). Scale bar 500 µm. B) Confocal detection of DAF-2T in the transition zone of nitrate treated and untreated apices at T0 and T30'. Arrows indicate two different type of cells of this root zone: small square shape cell with central nucleus and elongated cell with a more developed vacuole (V) Scale bar 50 µm.
C) DAF-2T fluorescence intensity values at 30 min after treatment of root segments with NO3-; NO3- and tungstate (W); NO3- with the NO scavenger cPTIO; KCl (-NO3-); KCl (-NO3-) and NO donor NOR.
Average fluorescence values are reported as a ratio of the fluorescence intensity at 30 min to the fluorescence intensity at time 0 (a.u.). Different letters indicate statistically significant differences among samples ($p<0.05$, Kruskal–Wallis test)

When the cPTIO was given together with nitrate (third column), the expression of both *Hb* and *Hb2* was very strongly inhibited, whereas the other genes analyzed did not evidence significant differences of expression in comparison to the positive control ($+NO_3^-$). Similarly, the addition of tungstate (fourth column), led to an inhibition of the 75-90% of the transcription of all genes, with the exception of *NOA1*. Conversely, the provision of L-NAME, an inhibitor of the nitric oxide synthase, induced only slight and rarely significant decreases of the expression of these genes.

These results confirmed the role of the regulation of *NR1*, *Hb* and *NiR* genes in the early response to nitrate even in younger roots. Moreover the use of chemicals interfering with NO biosynthesis and scavenging provided further evidence of the involvement of NR-derived nitric oxide as a key signal in the nitrate signalling in roots of maize.

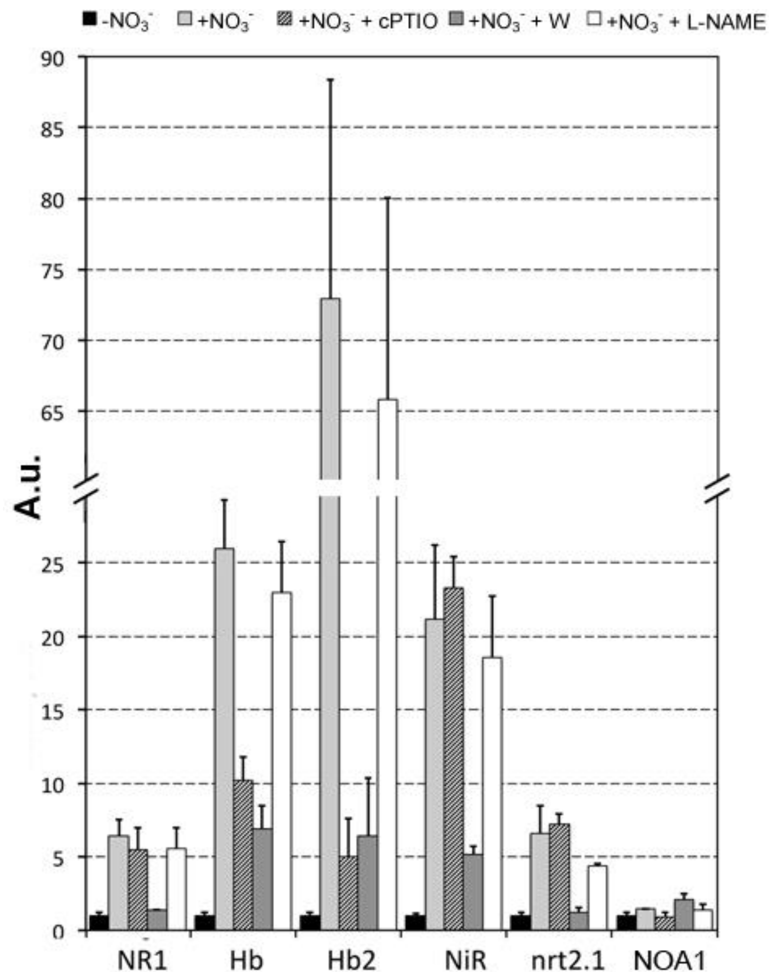

Figure 6. Effects of five different chemicals interfering with NO biosynthesis and scavenging on the expression profile of five genes differentially regulated by nitrate supply/depletion. Plants were grown 24 h in a $–NO_3^-$ solution and then transferred to a $+NO_3^-$ medium for two hours The transcript accumulation of six genes (*NR1, Hb, Hb2, NiR, Nrt2.1,* and *NOA1* was examined after nitrate supply and in the presence of cPTIO (1 mM), tungstate (W; 1 mM), and L-NAME (0.2 mM).

4.5 The transcription for genes involved in NO production and scavenging is maximally induced in the transition zone (TZ) of roots after nitrate induction

Results on NO measurements suggest that the production of this molecule after nitrate provision is preferably localized immediately above the meristematic apex, and more precisely at the level of the transition and elongation zones. The expression of the genes encoding nitrate reductases, haemoglobins, nitrite reductase and of *Nrt2.1* was, therefore,

studied in four different root portions (M: meristem, TZ: transition zone, EZ: elongation zone, MZ: maturation zone; as schematized in Fig. 7A), both in nitrate-depleted roots and after 2 h of nitrate provision.

All the five genes considered evidenced a significant change of localization when seedlings grown without nitrate were treated with the anion (Fig. 7B). In fact, in nitrate-starved root (left columns of Fig. 7B) the 70-80% of the mRNA was concentrated in the meristematic cells (M) for all five genes, with the remaining 20-30% of mRNAs prevalently localized in the elongation (EZ) and maturation zones (MZ). In these conditions the amount of transcript detected at the transition zone level (TZ) was extremely low or even negligible. On the contrary, in seedlings supplied with 1 mM nitrate for two hours (after being grown 24 h in a $-NO_3^-$ solution), the transcripts of all five genes were more equally distributed between the apical meristem (M) and the transition zone (TZ), with a significant increase of accumulation in the TZ which showed an amount of mRNA for each gene ranging from 20 to 40% of the total. Moreover, after nitrate supply the maturation zone also evidenced an increase in terms of gene expression if compared with nitrate-depleted roots.

Fig. 7C describes the increases of transcription for each gene in each of the four portions, independently from their relative abundance. All five genes evidenced an induction of their expression in all the four portions sampled, with the maximum in the TZ, that showed a transcription rate more then 30 times higher if compared with that measured in the same portion of nitrate-depleted roots (except the case of *Nrt2.1* that increased more then 10 times). In the MZ and EZ of nitrate-supplied roots the amount of mRNAs increased by around 8-20 and 4-8 times respectively. On the contrary, in the meristematic cells the increase of gene transcription measured was very low or insignificant.

In general, it would seem that the nitrate supply induces a redistribution of transcripts in zones of roots different from the meristem, which in turns appears to be the main site of their accumulation in conditions of nitrate starvation.

4.6 The nitrate induced root length increase is dependent on a NO signalling pathway

After germination seedlings were transferred to six different nutrient solutions ($+NO_3^-$, $-NO_3^-$, $+NH_4^+$, $+NO_3^-$ +tungstate, $-NO_3^-$ +SNP, $+NO_3^-$ +cPTIO, $+NO_3^-$ +L-NAME) and the growth of primary root was monitored for 24 h (Fig. 8). Nitrate-supplied seedlings and $-NO_3^-$

+SNP-seedlings showed the more elevated rate of root elongation, with values significantly higher in comparison to all the remaining treatments. The supply of ammonium did not produce any increase in the elongation rate, which was similar to that measured for negative control plants, as already observed also for older seedlings. The provision of tungstate together with nitrate inhibited even more significantly the root growth in comparison with nitrate-depleted roots. A similar decrease was also observed in roots supplied with nitrate plus cPTIO. On the contrary, the addition of SNP to nitrate depleted roots stimulated the root growth to levels comparable to those measured for positive control. Conversely, as also observed in the case of gene transcription, the supply of L-NAME, that inhibits the NOS activity, did not produce significant effects on root lengthening.

These results besides suggesting the involvement of NO in the regulation of nitrate induced root elongation, clearly confirm the key role of nitrate reductase for this signalling pathway.

The fresh weight of both roots and shoots were also determined to exclude toxicity effects of chemicals utilized (Table S2).

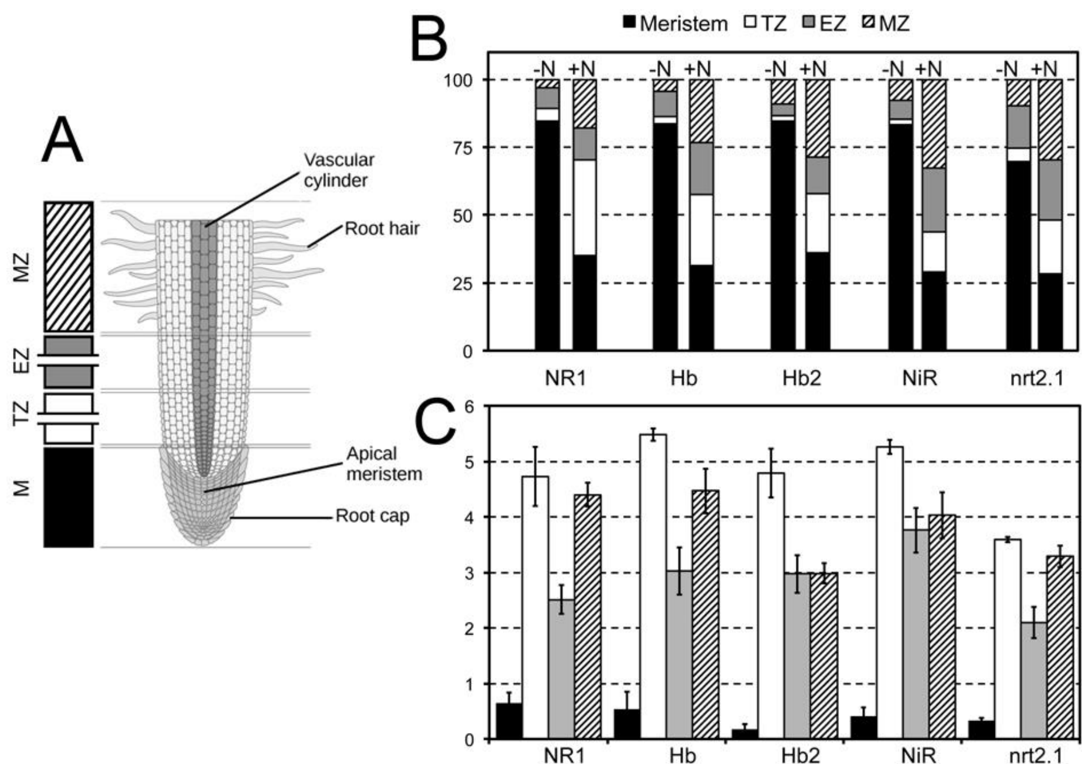

Figure 7. Spatial distribution of five genes differentially regulated by supply/depletion of nitrate. A) Graphical representation of the different part of primary root analyzed: M (Meristem), TZ (Transition Zone), EZ (Elongation Zone) and MZ (Maturation Zone). (B) Gene expression values in the different zones are reported as percentage in both nitrate starved and supplied roots. Increases of transcription for each gene in each of the four portions were reported in panel C. Data are reported as log_2 of the ratio +N/-N of the values recorded.

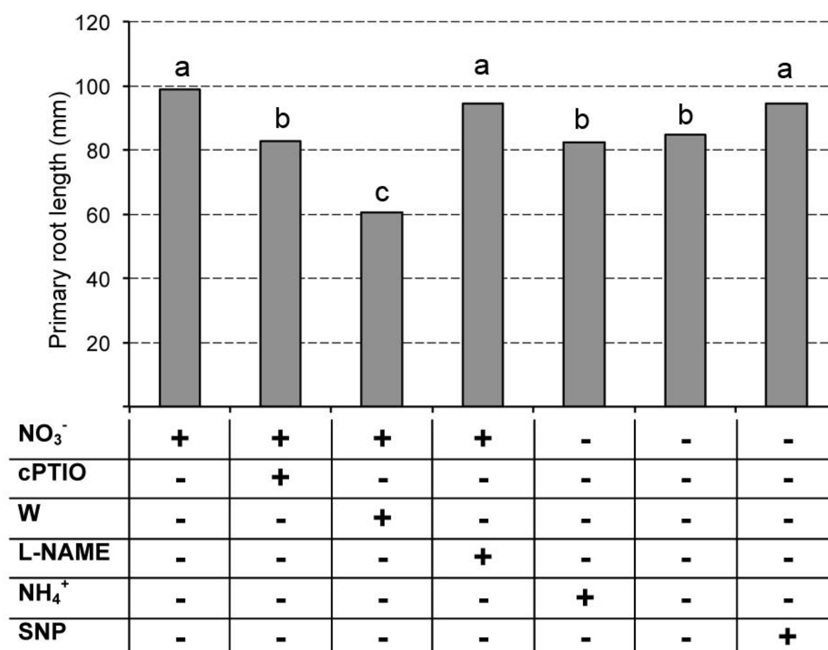

Figure 8. Effect of different nitrate treatments on primary root growth. After germination seedlings were transferred to six different nutrient solutions (+NO_3^-, -NO_3^-, +NH_4^+, +NO_3^- +tungstate, - NO_3^- +SNP, +NO_3^- +cPTIO, + NO_3^- + L-NAME) and the growth of primary root was measured for 24 h with a ruler on sixteen seedlings for each group. Each value represents the average ± S.E. of four independent biological repetitions.

4.7 The nitrate-induced NO signalling pathway is a localized effect

The setup of a method to grow maize seedlings on a semisolid agar medium allowed us to perform targeted treatments to single zone of root, as illustrated in Fig. 2 of the methodological section.

This system permits to treat only specific zones of root allowing thus to discriminate local from systemic effects on gene expression.

As a preliminary experiment, to test the validity of this method as an alternative to hydroponic, seedlings were grown in the agar plates which were nitrate-supplied or nitrate-deprived, by using the same timing and concentrations described for experiments in hydroponics and the expression of the previously selected genes was evaluated. RT-qPCR were carried out on both roots and shoots and for all the genes and the nutritional conditions described in the first paragraph of the Results (data not shown), but for simplicity in Fig. 9A we decided to show only those closely related to the induction of NO pathway in roots after nitrate supply. Results fully confirmed those obtained for seedlings grown in hydroponics. Furthermore, the root growth, analyzed by means of WinRhizo software, evidenced the same behaviour of plants grown in nutrient solution (data not shown), further confirming the validity of this method.

Seedlings were then submitted to a treatment with nitrate localized only to the meristematic apex (4 mm, for details see the M&M). The transcription of the five genes previously chosen was evaluated independently in the four different root zones (M, TZ, EZ, MZ) in both seedlings whose tip was treated with 1 mM nitrate and negative control (Fig. 9B). *NR1*, *Hb1*, *Hb2* strongly increased their expression in apex of nitrate supplied roots, whereas *NiR* transcription increased to a lesser extent. On the contrary, the mRNA abundance of *Nrt2.1* did not evidence any increase, indicating that this high affinity nitrate transporter is not involved in the influx of nitrate by root meristem. Furthermore, in all the other three root zones (TZ, EZ and MZ) no differences in terms of transcript accumulation were detected after local nitrate provision to apex, suggesting that the NO signalling activation by nitrate should represent a localized effect of nitrate.

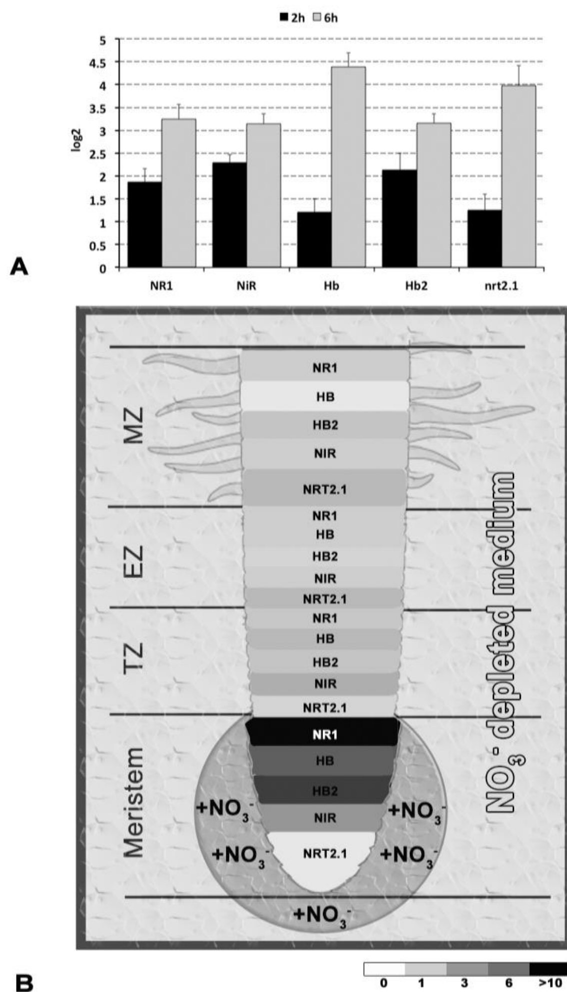

Figure 9. Expression analysis of NO and nitrate metabolism related genes *NR1*, *Hb*, *Hb2*, *NiR*, *Nrt2.1* on roots of 24 h old seedlings grown on nitrate-depleted agar medium and treated in a fresh medium added with nitrate in the whole plate or locally at the root tip. In panel A the RT-qPCR on roots at two (black bar) and six hours (grey bar) after treatment. Data are expressed as log2 ratios of the normalized expression levels measured in treated roots with respect to the control (no nitrate) grown at the same conditions. The results are averages ± SE of six independent biological replicates, each performed in three technical repetitions. In panel B the fold change (reference: untreated meristem) in expression of the genes along the root treated locally at the meristem zone with nitrate. The size of the different zones (Meristem; TZ transition zone; EZ elongation zone; MZ maturation zone) doesn't reflect the real values of length; please refer to the material and methods for the exact measures. Values of fold change are expressed by means of a grey scale. The authors arbitrarily choose the size of each block occupied by single gene investigated and it does not refer to any quantitative value.

5. Discussion

Nitrogen is a major element for plant life and crops strongly depend on intense fertilization programmes throughout the world. However N fertilization seriously affects environment quality and the identification of crop cultivars better adapted to low nitrogen input continues to be a real priority for plant scientists (Robertson and Vitousek, 2009; Xu G. *et al*., 2012).

Nitrate represents the principal nitrogen form in a standard agricultural soil and it is able to act also as a signal, triggering a number of molecular and physiological events leading to the overall plant's response to its availability.

The control of nitric oxide homeostasis through the spatio-temporal coordination of nitrate reductase and haemoglobin gene expression has been recently hypothesised to participate to nitrate sensing in maize roots (Trevisan *et al*., 2011). In the present work we tried to more deeply characterize the role of nitric oxide in the maize root response and adaptation to nitrate fluctuations.

To better discriminate nitrate specific effects from those more generally N-dependent, the expression of a list of previously selected genes (Quaggiotti *et al*., 2003; Trevisan *et al*., 2011, 2012) was evaluated in response to nitrate or ammonium supply and deprivation. This first screening allowed us to focus later in this work only on genes responding exclusively to nitrate (and not to ammonium), which coincided with those involved in the control of NO biosynthesis and scavenging. In particular, genes encoding the cytosolic nitrate reductase and two different ns-haemoglobins, together with a gene encoding nitrite reductase evidenced both in short-term and long-term experiments a clear and noticeable responsiveness to nitrate supply or starvation, but did not change their expression in response to ammonium. A gene encoding a high affinity root nitrate transporter was also used as internal control, in light of its putative role in the nitrate influx and of its transcriptional inducibility during the first phases of nitrate supply (Quaggiotti *et al.*, 2003). The expression profile recovered for this gene provided indirect evidence of the entry of nitrate into the root epidermal cells, hence enabling the activation of the signalling pathways in which nitrate is involved.

Besides being the first enzyme of nitrate assimilation, NR represents also one of the most important sources of NO in plants (Mur *et al.,* 2012). It is a cytosolic enzyme that could both reduce nitrate to nitrite and nitrite to nitric oxide, even if it shows a better affinity for

nitrate than for nitrite. However, NR seems to be switched to the latter reaction when high nitrite levels are produced (Gupta *et al*., 2011; Mur *et al*., 2012). This occurs, for example, as a consequence of an increased NO_3^- influx, as it might be happened in this case study. Once inside the root cells, nitrate is promptly converted to nitrite by NR leading to nitrite accumulation. The *NiR* transcript increase observed in roots already after 30 min of nitrate supply is an indirect evidence of nitrite formation. Besides serving as substrate for NiR, nitrite accumulation could also shift the NR equilibrium toward its second mode of action, promoting thus the biosynthesis of nitric oxide in response to nitrate. This scenario seems consistent with the main findings showed in this paper.

Nitrate reductase is involved in the NO production during bacteria induced defence (Modolo *et al*., 2005), disease development in certain pathogenic interactions (Shi and Li, 2008), drought (Freschi *et al*., 2010), cold (Zhao *et al*., 2009), osmotic stress response in roots of *Arabidopsis* (Kolbert *et al*., 2010), stomatal regulation (Srivastava *et al*., 2009) and many developmental processes as, for example, the initiation of flowering (Seligman *et al*., 2008).

The parallel strong increase of the expression of both the *nsHbs* genes observed already after 30 min of nitrate supply, is not surprising if considering the high reactivity of NO, which besides serving as a signal in regulating several physiological events, must also be kept at a steady state level to avoid damages due to its toxicity. Recently, several studies have indicated a role for haemoglobins in the detoxification from high intracellular NO concentrations (Dordas *et al*., 2003*a, b*; Perazzolli *et al*., 2004; Vieweg *et al*., 2005). The patterns of expression of non-symbiotic haemoglobins vary depending on tissues and in response to different types of stress (Hunt *et al*., 2001). Perazzolli *et al.* (2004) provided evidence that *Arabidopsis* non-symbiotic haemoglobin AtHb1 functions as a NO dioxygenase, metabolizing NO to nitrate. Moreover plant haemoglobins seem to be involved in the control of NO accumulation during rhizobial and mycorrhizal symbioses (Vieweg *et al*., 2005) and in the response to hypoxia in different tissues such as seeds, roots, and stem tissue (Dordas *et al*., 2003a, b). Plant Hbs can control developmental and physiological reactions by modulating cellular NO levels (Hill 2012) and should be considered to be as important as NO generation in regulating *in planta* NO signalling (Mur *et al*., 2012).

Our results, besides confirming our previous hypothesis of an involvement of nitric oxide control homeostasis in the maize root response to nitrate (Trevisan *et al*., 2011),

demonstrate also that this is a prerogative of NO_3^--signalling, since ammonium did not produce any similar effects on “NO genes” transcription. On the contrary, the expression of the other genes here analysed did not show similar specific nitrate responsiveness. In addition, data obtained by analysing root morphological parameters by the WinRhizo software highlighted the same specificity of nitrate, which significantly affected root growth when supplied to N-deprived roots, in contrast to what happens when the same concentration of ammonium is given to roots.

According to these results it would seem that nitric oxide might be produced by roots as an early signal of nitrate perception. To deepen this hypothesis an *in vivo* NO detection was carried out. Results obtained by using the DAF-2DA probe (Kojima *et al*., 1998) at both stereo- and confocal microscope evidenced a clear induction of fluorescence after nitrate provision. The main zone of NO production seems to be located immediately above the meristematic apex. A similar localization has been recently observed in this same species by Mugnai *et al.* (2012) as a response to hypoxic conditions.

The fluorescence detected after nitrate supply was not relieved in the presence of tungstate or cPTIO, giving support to the role of nitrate reductase in this process and confirming the specificity of NO detection by the probe utilized. This was also corroborated by the strong increase of fluorescence measured when the NOR was supplied to nitrate-depleted roots. Even if the DAF specificity has been recently questioned (Mur *et al*., 2011), well-established alternative methods to reveal tissue-specific patterns of high NO generation are not available yet. To give more strength to our results, we have tried to operate by following the steps indicated by Mur *et al*. (2012), being well conscious that it should be always preferable to employ parallel approaches for NO measurements.

The NR-dependent NO production observed after nitrate supply was then corroborated by the expression analyses performed on roots of one day olds seedlings. In particular, our results proved the strong induction of *NR1*, *NiR* and *nsHbs* transcription in the early phases of nitrate perception. As also observed in the case of NO production, the transcription of all genes was significantly inhibited after tungstate and cPTIO addition, confirming the cooperation between nitrate reductase and haemoglobin activities in the fine-tuning control of NO homeostasis. However, to avoid excluding the possible involvement of sources of NO other than NR, the study was extended also to the orthologous of the *Arabidopsis NOA1* (Guo *et al*., 2003), which encode the Nitric Oxide

Associated 1 protein (Zemojtel *et al*., 2006). NOA1 was previously named AtNOS1 and it has been described as a potential nitric-oxide synthase (NOS) in *Arabidopsis thaliana*, despite lack of sequence similarity to animal NOSs. It has been, successively, established to be a GTPase (Moreau *et al*., 2008) and not to possess NOS activity and for this reason it has been renamed AtNOA1. Previous studies have shown that NOA1-dependent NO synthesis is involved in hormonal signaling, stomatal movement, flowering, pathogen defence, and oxidative stress (Guo *et al*., 2003; He *et al*., 2004; Zeidler *et al*., 2004; Zhao *et al*., 2007). The transcription of the *AtNOA1* orthologous in maize did not evidence any alteration neither in response to nitrate nor to the other chemicals utilized.

Moreover, to exclude the involvement of a more generic nitric oxide synthase (NOS) activity, nitrate supplied seedlings were also treated with L-NAME, which is commonly used to inhibit NOS activity in mammalians and also in plants. No effects nor on transcription of nitrate-responsive genes (especially with regards to *nsHbs*), neither on the nitrate induced root lengthening were evidenced, giving more strength to the idea that the nitric oxide production after nitrate provision is predominantly dependent on the activity of nitrate reductase.

To deepen the spatial regulation of NO homeostasis balance, the expression of the five genes was analysed in four different root zones (M, TZ, EZ, MZ) both in nitrate-depleted and in nitrate-treated (1 mM) seedlings. In N-starved roots all five transcripts evidenced their maximum accumulation at the meristem level. This pattern radically changed when nitrate was furnished to roots with a very significant increase of transcript abundance in the transition zone (TZ), which is located between the meristem (M) and the region of fast cell elongation (EZ).

Cells of the TZ undergo a series of fundamental changes in their cytoarchitecture and physiology, and accomplish dramatic rearrangements of the actin cytoskeleton (Baluska *et al*., 1997; 2001). This is essential for the developmental switch into rapidly elongating root cells that expand strictly uniaxially (Baluska *et al*., 1997). The distal part of this zone in characterized by a prevalence of cells that optionally can re-enter the cell cycle, whereas the proximal part is equipped with cells competent to rapidly enter into the fast cell elongation zone. As this developmental passage of cells can be differentially regulated at the opposite root flanks, this unique zone provides the root apices with an effective mechanism to re-orientate growth in response to environmental stimuli (Verbelen *et al*., 2006). A number of

experimental proofs suggest that the TZ should be considered as a sort of sensory and information processing zone, enabling the growing root apex to monitor environmental parameters continuously and to trigger appropriate responses (Mugnai *et al*., 2012). If this is true and hypothesizing a role for NO homeostasis control through the combined action of NR and nsHB in the early perception of nitrate by roots, our results on transcript accumulation re-distribution along root apex are not surprising. Based on our finding it would seem that nitrate supply could activate its own sensing by stimulating the NO production by the TZ cells, thus initiating a signalling pathway contributing to the physiological adaptation (e.g. root growth) to nitrate fluctuations.

The most important example of the plasticity that plant express to fit with nutrients withdrawal in soil is, in fact, represented by the capability of rearranging root architecture to maximize their capture (López-Bucio *et al*., 2003; Hermans *et al*., 2006; Zhang *et al*., 2007; Zolla *et al*., 2010; Giehl *et al*., 2012; De Pessemier *et al*., 2013). Nitrate affects root development by finely regulating the growth of lateral roots depending on its external concentration and localization, as described above (Péret *et al*., 2009; Mounier *et al*., 2013; Yu *et al*., 2013) and as also showed by our findings obtained with the WinRhizo software.

Based on our preliminary results showing the preferential localization of NO production at the level of the transition zone, we decided to focus on nitrate effects on root elongation, which takes place in the zone immediately above and neighbouring the TZ. Our results evidenced a strong and specific induction of root elongation of young maize seedlings supplied with 1 mM nitrate and a drastic inhibition in the presence of ammonium, cPTIO and tungstate. No effects were recorded in the presence of L-NAME. On the contrary, when the negative control (-NO_3^-) was supplied with a NO donor (SNP) the root length increased significantly. These results strongly suggest that the NO generated through nitrate reductase should significantly contribute to the root lengthening noticed after nitrate provision.

The involvement of NO in root development has been observed in numerous studies, as for example those published by the Lamattina group (Pagnussat *et al*., 2002; Pagnussat *et al*., 2003; Corre-Aragunde *et al*., 2004; Lombardo *et al*., 2006), but it had already been hypothesised in 1997 by Gouêva *et al*., who found that NO was able to induce cell elongation in a way similar to auxin.

Besides this, NO is involved in the regulation of actin cytoskeleton, endocytosis, vesicle trafficking and the polarity of growing tip cells (Prado *et al*., 2004; Lombardo *et al*.,

2006; Salmi *et al*., 2007; Prado *et al*., 2008; Kasprowicz *et al*., 2009; Wang *et al*., 2009), which are all prerequisites to acquire competence for cell to elongate.

Considering also that NO is widely implicated in the plant response to environmental stresses (Beligni and Lamattina 2001; Dat *et al*., 2004), it seems to play crucial functions in at the cross-roads between developmental and abiotic stress tolerance. For this reason, it should also represent a very good molecular candidate to regulate root development in response to abiotic stresses, as for example nutrients or oxygen deprivation (Mugnai *et al*., 2012), but also an early player in symbiotic interactions establishment, which also need root architecture to be adapted to the environment.

In the present research, thanks to the set-up of a method allowing to grow maize seedlings in vertical plates with an agar medium, some major details on NO-mediated nitrate signalling have been attained. Our results suggest that the mechanism underling the root response to nitrate and involving NO signalling is directly activated on cells that enter in contact with external nitrate. Moreover, this alert system does not seem to be turned on by some nitrate derived compounds or by the nitrate that move up through the root. In fact, when only the meristematic apex was treated with nitrate, the induction of the transcription of *NR1* and *Hb* was exclusively restricted to the apex itself, whereas in the upper zone of the roots no differences were detected in comparison with the negative control. This is even more remarkably considering that, conversely, when the entire root gets in touch with nitrate, the apex is the portion that show the lower responsiveness to this anion in terms of induction of gene expression, being instead the transition zone the most receptive.

Moreover, these results indicate that nitrate transporters other than Nrt2.1 should be implicated in the nitrate perception at the root meristem, since the transcription of the gene encoding *Nrt2.1* is not activated at all by nitrate, in contrast to what observed in all the other three root zones when the whole root was supplied with nitrate. Basing on these data, it would seem that the NO mediated pathway here described represents an early alert system for external nitrate sensing by root cells, which seem to individually posses the competence to activate this pathway when external nitrate is perceived.

Since, root growth is modulated by the convergence of multiple environmental inputs which are integrated by specific signal pathways to decide how to explore the surrounding environment, additional experiments will be needed to better understand the functioning of

this NO-mediated pathways and to identify the downstream events linking the NO burst with the physiological re-direction of root growth.

Even if a high number of specific and comprehensive issues on the NO role in the complicated cross-point between root and nitrate (and more in general root and abiotic stress perception) need to be further deepened, our findings suggest that the triggering of a NO burst is a direct response to external nitrate and that it could mediate the root elongation observed after nitrate provision (Fig. 10).

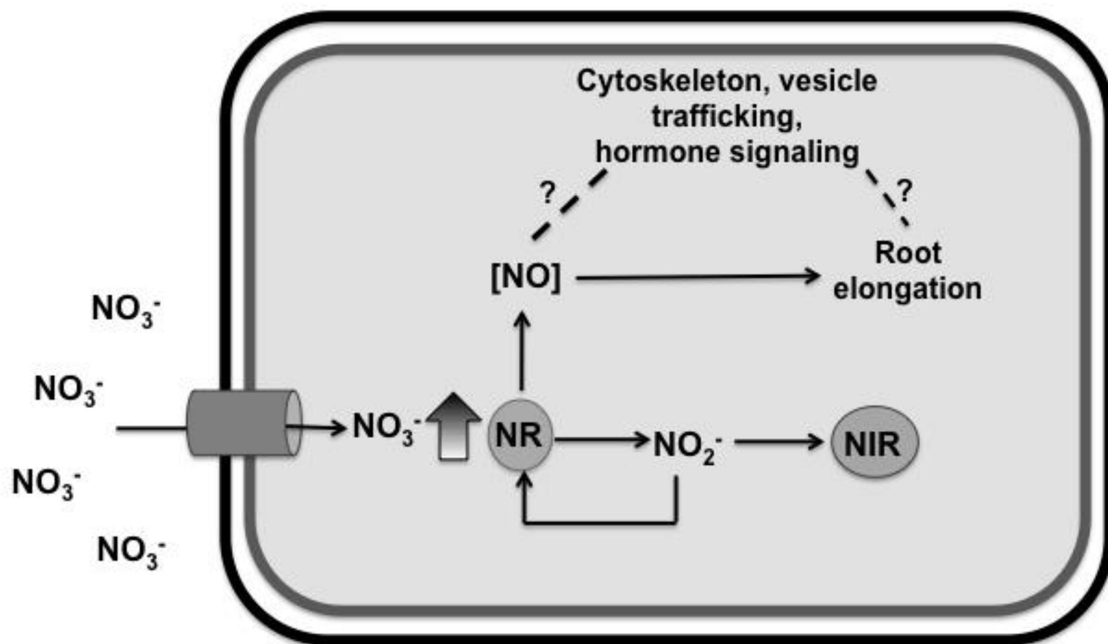

Figure 10. Model for the NO-mediated nitrate induction of root elongation. NO_3^- influx is performed by specific nitrate transporters (e.g. Nrt2.1 in the TZ, EZ and MZ). Once inside the root cells NO_3^- is able to act as a signal to induce its own sensing via the NR/Hb-dependent NO fine-tuning, which in turns seems to be involved in the root elongation stimulation. The cytological events and molecular targets linking the NO biosynthesis to root growth response could be involved in the rearrangements of the actin cytoskeleton (Baluska et al., 2001) and need to be further studied and characterized.

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7. References

Ahmad A, Jain V, Abrol YP. 2007. Physiological and biochemical aspects of nitrogen use efficiency in crop plants. In: Abrol YP, Raghuram N, Sachdev MS, eds. *Nitrogen in environment, industry and agriculture*. New Delhi: IK International, 115–128.

Baluška F, Mancuso S, Volkmann D, Barlow P. 2010. Root apex transition zone: a signalling-response nexus in the root. *Trends in Plant Science* **15,** 402–408.

Baluška F, Vitha S, Barlow PW, Volkmann D. 1997. Rearrangements of F-actin arrays in growing cells of intact maize root apex tissues: a major developmental switch occurs in the postmitotic transition region. *European Journal of Cell Biology* **72,** 113–121.

Baluška F, Volkmann D, Barlow PW. 2001. A polarity crossroad in the transition growth zone of maize root apices: cytoskeletal and developmental implications. *Journal of Plant Growth Regulation* **20,** 170–181.

Beligni MV, Lamattina L. 2001. Nitric oxide in plants: the history is just beginning. *Plant, Cell and Environment* **24,** 267–278.

Bouguyon E, Gojon A, Nacry P. 2012. Nitrate sensing and signaling in plants. *Seminars in Cell and Developmental Biology* **23,** 648–654.

Calcagno C, Novero M, Genre A, Bonfante P, Lanfranco L. 2012. The exudate from an arbuscular mycorrhizal fungus induces nitric oxide accumulation in *Medicago truncatula* roots. *Mycorrhiza* **22,** 259–269.**Castaings L, Marchive C, Meyer C, Krapp A.** 2011. Nitrogen signalling in *Arabidopsis*: how to obtain insights into a complex signalling network. *Journal of Experimental Botany* **62,** 1391**–**1397.

Chiou TJ. 2007. The role of microRNAs in sensing nutrient stress. *Plant, Cell and Environment* **30,** 323**–**332.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* **218,** 900–905.

Crawford NM. 1995. Nitrate: nutrient and signal for plant growth. *The Plant Cell* **7,** 859**–**868.

Dat JF, Capelli N, Folzer H, Bourgeade P, Badot PM. 2004. Sensing and signalling during plant flooding. *Plant Physiology and Biochemistry* **42,** 273**–**282.

del Río LA, Corpas FJ, Barroso JB. 2004. Nitric oxide and nitric oxide synthase activity in plants. *Phytochemistry* **65,** 783–792.

De Pessemier J, Chardon F, Juraniec M, Delaplace P, Hermans C. 2013. Natural variation of the root morphological response to nitrate supply in *Arabidopsis thaliana*. *Mechanisms of Development* **130,** 45–53.

Donner SD, Kucharik CJ. 2008. Corn-based ethanol production compromises goal of reducing nitrogen export by the Mississippi River. *Proceedings of the National Academy of Science, USA* **105,** 4513**–**4518.

Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J, Hill RD. 2003*a*. Expression of a stress-induced haemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *The Plant Journal* **35,** 763–770.

Dordas C, Hasinoff BB, Rivoal J, Hill RD. 2004. Class-1 haemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. *Planta* **219,** 66–72.

Dordas C, Rivoal J, Hill RD. 2003*b*. Plant haemoglobins, nitric oxide and hypoxic stress. *Annals of Botany* **91,** 173–178.

Durner J, Klessig DF. 1999. Nitric oxide as a signal in plants. *Current Opinion in Plant Biology* **2,** 369**–**374.

Falchi R, Cipriani G, Marrazzo T, Nonis A, Vizzotto G, Ruperti B. 2010. Identification and differential expression dynamics of peach small GTPases encoding genes during fruit development and ripening. *Journal of Experimental Botany* **61,** 2829–2842.

Forde BG, Clarkson DT. 1999. Nitrate and ammonium nutrition of plants: physiological and molecular perspective. *Advances in Botanical Research* **30,** 1**–**90.

Freschi L, Rodrigues MA, Domingues DS, Purgatto E, Van Sluys MA, Magalhaes JR, Kaiser WM, Mercier H. 2010. Nitric oxide mediates the hormonal control of crassulacean acid metabolism expression in young pineapple plants. *Plant Physiology* **152,** 1971**–**1985.

Giehl RF, Lima JE, von Wirén N. 2012. Localized iron supply triggers lateral root elongation in *Arabidopsis* by altering the AUX1-mediated auxin distribution. *The Plant Cell* **24,** 33**–**49.

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD. 2008. Cell-specific nitrogen responses mediate developmental plasticity. *Proceedings of the National Academy of Sciences, USA* **105,** 803**–**808.

Guo FQ, Okamoto M, Crawford NM. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100–103.

Gouvêa CMCP, Souza JF, Magalhaes CAN, Martins IS. 1997. NO-releasing substances that induce growth elongation in maize root segments. *Plant Growth Regulation* **21,** 183–187.

Gupta KJ, Fernie AR, Kaiser WM, van Dongen JT. 2011. On the origins of nitric oxide. *Trends in Plant Science* **16,** 160–168.

He Y, Tang RH, Hao Y *et al.* 2004. Nitric oxide represses the *Arabidopsis* floral transition. *Science* **305,** 1968–1971.

Hebelstrup KH, Hunt P, Dennis E, Jensen SB, Jensen EO. 2006. Haemoglobin is essential for normal growth of *Arabidopsis* organs. *Physiologia Plantarum* **127,** 157–166.

Hebelstrup KH, van Zanten M, Mandon J, Voesenek LACJ, Harren FJ, Cristescu SM, Moller IM, Mur LA. 2012. Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in *Arabidopsis thaliana*. *Journal of Experimental Botany* **63,** 5581–5591.

Hermans C, Hammond JP, White PJ, Verbruggen N. 2006. How do plants respond to nutrient shortage by biomass allocation? *Trends in Plant Science* **11,** 610–617.

Hill RD. 2012. Non-symbiotic haemoglobins-What's happening beyond nitric oxide scavenging? *AoB Plants*. **2012,** pls004.

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES, Peacock WJ. 2001. Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Molecular Biology* **47,** 677–692.

Kasprowicz A, Szuba A, Volkmann D, Baluška F, Wojtaszek P. 2009. Nitric oxide modulates dynamic actin cytoskeleton and vesicle trafficking in a cell type-specific manner in root apices. *Journal of Experimental Botany* **60,** 1605–1617.

Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T. 1998. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Analytical Chemistry* **70,** 2446–2453.

Kolbert Z, Ortega L, Erdei L. 2010. Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of *Arabidopsis thaliana* L. roots. *Journal of Plant Physiology* **167,** 77–80.

Krouk G, Lacombe B, Bielach A, *et al.* 2010. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Developmental Cell* **18,** 927–937.

Krouk G, Ruffel S, Gutiérrez RA, Gojon A, Crawford NM, Coruzzi GM, Lacombe B. 2011. A framework integrating plant growth with hormones and nutrients. *Trends in Plant Science* **16,** 178–182.

Lamattina L, García-Mata C, Graziano M, Pagnussat GC. 2003. Nitric oxide: the versatility of an extensive signal molecule. *Annual Review of Plant Biology* **54,** 109–139.

Linkohr BI, Williamson LC, Fitter AH, Leyser HMO. 2002. Nitrate and phosphate availability and distribution have differents effects on root system architecture of *Arabidopsis*. *The Plant Journal* **29,** 751–760.

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE. 2005. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *Proceedings of the National Academy of Sciences, USA* **102,** 13693–13698.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. *Methods* **25,** 402–408.

Lombardo MC, Graziano M, Polacco JC, Lamattina L. 2006. Nitric oxide functions as a positive regulator of root hair development. *Plant Signaling and Behavior* **1,** 28–33.

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L. 2003. The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology* **6,** 280–287.

Lynch JP. 1998. The role of nutrient efficient crops in modern agriculture. *Journal of Crop Production* **1,** 241**–**264.

Lynch JP. 2007. Roots of the second green revolution. *Australian Journal of Botany* **55**, 1–20.

Manoli A, Sturaro A, Trevisan S, Quaggiotti S, Nonis A. 2012. Evaluation of candidate reference genes for qPCR in maize. *Journal of Plant Physiology* **169,** 807–815.

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM. 2007. Nitrate transport and signalling. *Journal of Experimental Botany* **58,** 2297**–**2306.

Modolo LV, Augusto O, Almeida IMG, Magalhaes JR, Salgado I. 2005. Nitrite as the major source of nitric oxide production by *Arabidopsis thaliana* in response to *Pseudomonas syringae*. *FEBS Letters* **579,** 3814**–**3820.

Moreau M, Lee GI, Wang Y, Crane BR, Klessig DF. 2008. AtNOS/A1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric oxide synthase. *The Journal of Biological Chemistry* **283,** 32957–32967.

Mounier E, Pervent M, Ljung K, Gojon A, Nacry P. 2013. Auxin-mediated nitrate signalling by NRT1.1 participates in the adaptive response of *Arabidopsis* root architecture to the spatial heterogeneity of nitrate availability. *Plant, Cell and Environment* doi: 10.1111/pce.12143.

Mugnai S, Azzarello E, Baluška F, Mancuso S. 2012. Local root apex hypoxia induces NO-mediated hypoxic acclimation of the entire root. *Plant and Cell Physiology* **53,** 912–920.

Mur LAJ, Mandon J, Cristescu SM, Harren FJ, Prats E. 2011. Methods of nitric oxide detection in plants: a commentary. *Plant Science* **181,** 509–519.

Mur LAJ, Sivakumaran A, Mandon J, Cristescu SM, Harren FJ, Hebelstrup KH. 2012. Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. *Journal of Experimental Botany* **63,** 4375–4387.

Nischal L, Mohsin M, Khan I, Kardam H, Wadhwa A, Abrol YP, Iqbal M, Ahmad A. 2012. Identification and comparative analysis of microRNAs associated with low-N tolerance in rice genotypes. *PLoS One* **7,** e50261.

Nonis A, Ruperti B, Falchi R, Casatta E, Thamashebi SE, Vizzotto G. 2007. Differential expression and regulation of a neutral invertase encoding gene from peach (*Prunus persica*): evidence for a role in fruit development. *Physiologia Plantarum* **129,** 436–446.

Nonis A, Scortegagna M, Nonis A, Ruperti B. 2011. PRaTo: a web-tool to select optimal primer pairs for qPCR. *Biochemical and Biophysical Research Communications* **415,** 707–708.

Pagnussat GC, Lanteri ML, Lamattina L. 2003. Nitric oxide and cyclic GMP are messengers in the IAA-induced adventitious rooting process. *Plant Physiology* **132,** 1241–1248.

Pagnussat GC, Simontachi M, Puntarulo S, Lamattina L. 2002. Nitric oxide is required for root organogenesis. *Plant Physiology* **129,** 954–956.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C, Delledonne M. 2004. *Arabidopsis* nonsymbiotic haemoglobin AHb1 modulates nitric oxide bioactivity. *The Plant Cell* **16,** 2785–2794.

Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplaze L, Beeckman T, Bennett MJ. 2009. *Arabidopsis* lateral root development: an emerging story. *Trends in Plant Science* **14,** 399–408.

Prado AM, Colaço R, Moreno N, Silva AC, Feijó JA. 2008. Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signaling. *Molecular Plant* **1,** 703–714.

Prado AM, Porterfield DM, Feijó JA. 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* **131,** 2707–2714.

Quaggiotti S, Ruperti B, Borsa P, Destro T, Malagoli M. 2003. Expression of a putative high-affinity NO_3^- transporter and of an H^+-ATPase in relation to whole plant nitrate transport physiology in two maize genotypes differently responsive to low nitrogen availability. *Journal of Experimental Botany* **54,** 1023–1031.

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A. 2006. The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proceedings of the National Academy of Sciences, USA* **103**, 19206**–**19211.

Robertson GP, Vitousek PM. 2009. Nitrogen in agriculture: balancing the cost of an essential resource. *Annual Review of Environment and Resources* **34,** 97**–**125.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics methods and protocols: methods in molecular biology*. Totowa, NJ: Humana Press, 365–386.

Rubio V, Bustos R, Irigoyen ML, Cardona-López X, Rojas-Triana M, Paz-Ares J. 2009. Plant hormones and nutrient signaling. *Plant Molecular Biology* **69,** 361–373.

Rubio-Somoza I, Weigel D. 2011. MicroRNA networks and developmental plasticity in plants. *Trends in Plant Science* **16,** 258–264.

Ruffel S, Krouk G, Ristova D, Shasha D, Birnbaum KD, Coruzzi GM. 2011. Nitrogen economics of root foraging: transitive closure of the nitrate-cytokinin relay and distinct systemic signaling for N supply vs. demand. *Proceedings of the National Academy of Science, USA* **108,** 18524–18529.

Salmi ML, Morris KE, Roux SJ, Porterfield DM. 2007. Nitric oxide and cGMP signalling in calcium-dependent development of cell polarity in *Ceratopteris richardii*. *Plant Physiology* **144,** 94–104.

Scheible WR, Lauerer M, Schulze ED, Caboche M, Stitt M. 1997. Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. *The Plant Journal* **11,** 671–691.

Schulze W, Schulze ED, Stader J, Heilmeier H, Stitt M, Mooney HA. 1994. Growth and reproduction of *Arabidopsis thaliana* in relation to storage of starch and nitrate in the wild-type and in starch-deficient and nitrate-uptake-deficient mutants. *Plant, Cell and Environment* **17,** 795–809.

Seligman K, Saviani EE, Oliveira HC, Pinto-Maglio CA, Salgado I. 2008. Floral transition and nitric oxide emission during flower development in *Arabidopsis thaliana* is affected in nitrate reductase-deficient plants. *Plant and Cell Physiology* **49,** 1112–1121.

Shi FM, Li YZ. 2008. *Verticillium dahliae* toxins-induced nitric oxide production in *Arabidopsis* is major dependent on nitrate reductase. *Biochemistry and Molecular Biology Reports* **41,** 79–85.

Srivastava N, Gonugunta VK, Puli MR, Raghavendra AS. 2009. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*. *Planta* **229,** 757–765.

Stitt M. 1999. Nitrate regulation of metabolism and growth. *Current Opinion in Plant Biology* **2,** 178–186.

Stöhr C, Stremlau S. 2006. Formation and possible roles of nitric oxide in plant roots. *Journal of Experimental Botany* **57,** 463–470.

Sturms R, DiSpirito AA, Hargrove MS. 2011. Plant and cyanobacterial haemoglobins reduce nitrite to nitric oxide under anoxic conditions. *Biochemistry* **50,** 3873–3878.

Trevisan S, Manoli A, Begheldo M, Nonis A, Enna M, Vaccaro S, Caporale G, Ruperti B, Quaggiotti S. 2011. Transcriptome analysis reveals coordinated spatiotemporal regulation of haemoglobin and nitrate reductase in response to nitrate in maize roots. *New Phytologist* **192,** 338–352.

Trevisan S, Nonis A, Begheldo M, Manoli A, Palme K, Caporale G, Ruperti B, Quaggiotti S. 2012. Expression and tissue-specific localization of nitrate-responsive miRNAs in roots of maize seedlings. *Plant, Cell and Environment* **35,** 1137–1155.

Vance CP, Uhde-Stone C, Allan DL. 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytologist* **157,** 423–447.

Verbelen JP, De Cnodder T, Le J, Vissenberg K, Baluška F. 2006. The root apex of *Arabidopsis thaliana* consists of four distinct zones of growth activities: meristematic zone,

transition zone, fast elongation zone and growth terminating zone. *Plant Signaling and Behavior* **1,** 296–304.

Vidal EA, Tamayo KP, Gutiérrez RA. 2010. Gene networks for nitrogen sensing, signaling, and response in *Arabidopsis thaliana*. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* **2,** 683–693.

Vieweg MF, Hohnjec N, Küster H. 2005. Two genes encoding different truncated haemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. *Planta* **220,** 757–766.

Walch-Liu P, Forde BG. 2008. Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *The Plant Journal* **54,** 820–828.

Wang Y, Chen T, Zhang C, Hao H, Liu P, Zheng M, Baluška F, Šamaj J, Lin J. 2009. Nitric oxide modulates the influx of extracellular Ca_2^+ and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes. *New Phytologist* **182,** 851–860.

Wendehenne D, Durner J, Klessig DF. 2004. Nitric oxide: a new player in plant signalling and defence responses. *Current Opinion in Plant Biology* **7,** 449–455.

Wojtaszek P. 2000. Nitric oxide in plants: to NO or not to NO. *Phytochemistry* **54,** 1–4.

Xu G, Fan X, Miller AJ. 2012. Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology* **63,** 153–182.

Xu Z, Zhong S, Li X, Li W, Rothstein SJ, Zhang S, Bi Y, Xie C. 2011. Genome-wide identification of microRNAs in response to low nitrate availability in maize leaves and roots. *PLoS One* **6,** e28009.

Yu P, Li X, Yuan L, Li C. 2013. A novel morphological response of maize (*Zea mays*) adult roots to heterogeneous nitrate supply revealed by a split-root experiment. *Physiologia Plantarum* doi: 10.1111/ppl.12075.

Zeidler D, Zähringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. 2004. Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Science, USA* **101,** 15811–15816.

Zemojtel T, Fröhlich A, Palmieri MC *et al.* 2006. Plant nitric oxide synthase: a never-ending story? *Trends in Plant Science* **11,** 524–525.

Zhang H, Forde BG. 1998. An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279,** 407–409.

Zhang H, Jennings A, Barlow PW, Forde BG. 1999. Dual pathways for regulation of root branching by nitrate. *Proceedings of the National Academy of Sciences, USA* **96,** 6529–6535.

Zhang H, Rong H, Pilbeam D. 2007. Signalling mechanisms underlying the morphological responses of the root system to nitrogen in *Arabidopsis thaliana*. *Journal of Experimental Botany* **58,** 2329–2338.

Zhao MG, Chen L, Zhang LL, Zhang WH. 2009. Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. *Plant Physiology* **151,** 755–767.

Zhao MG, Zhao X, Wu YX, Zhang LX. 2007. Enhanced sensitivity to oxidative stress in *Arabidopsis* nitric oxide synthase mutant. *Journal of Plant Physiology* **164,** 737–745.

Zolla G, Heimer YM, Barak S. 2010. Mild salinity stimulates a stress-induced morphogenic response in *Arabidopsis thaliana* roots. *Journal of Experimental Botany* **61,** 211–224.

8. Supplementary data

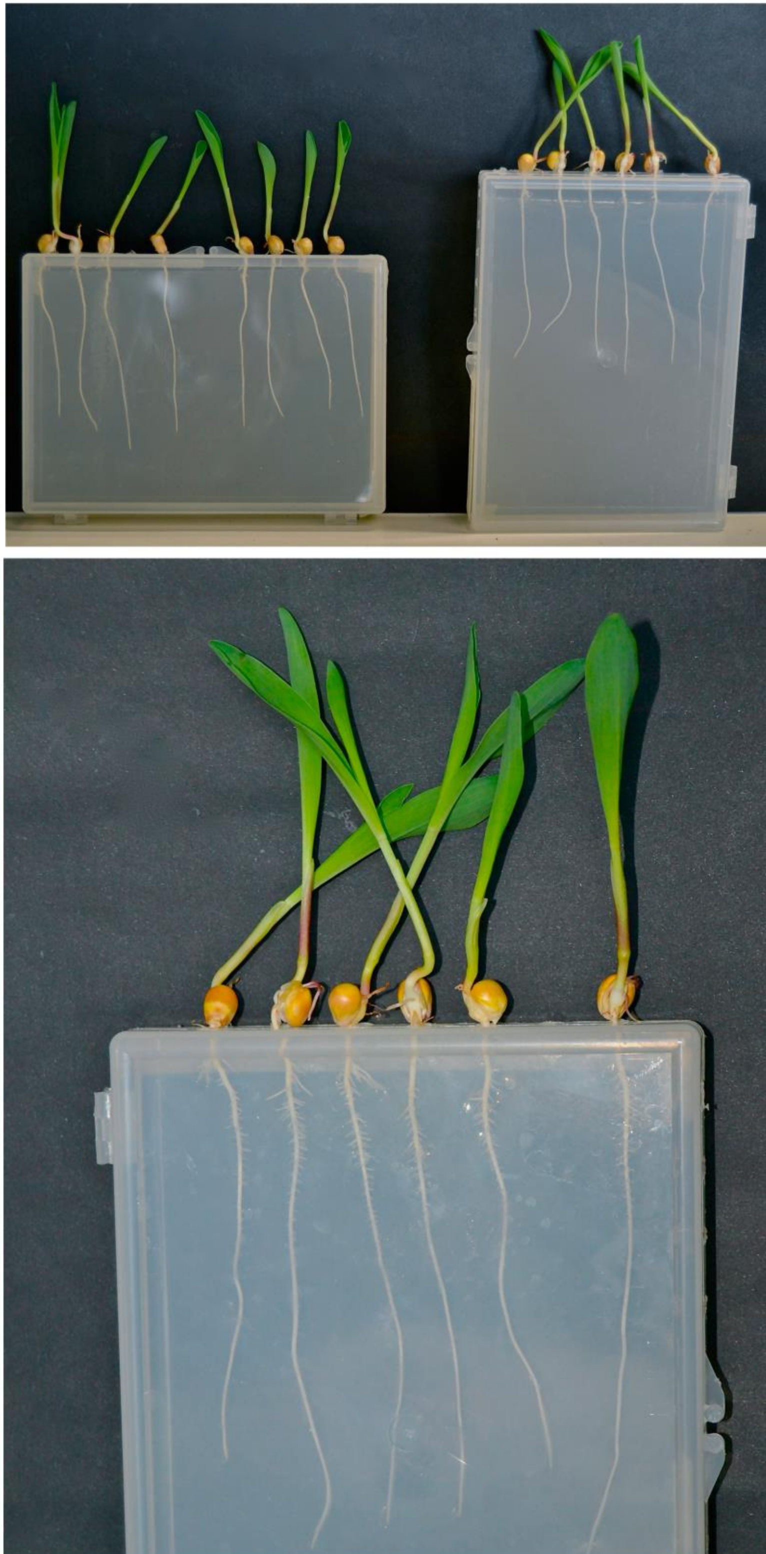


Figure S1. The new improved agar-plate culture system for studying the *Zea mays* L. root response to different nutrient availability.

Table S1. List of the genes analyzed by means of Real Time qPCR. Maize GDB and NCBI accessions are reported together with the gene functions and the primer sequences.

Maize GDB Accession ID	NCBI	Name	Gene Function	Fw	Rv
GRMZM2G067402 T02	AF236080.1	Hb	haemoglobin	GGAGCCTCGAGATGAAGAAA	ACAATACACGCTCCCTCCAG
GRMZM2G168898_T01	NM_001112349.1	Hb2	haemoglobin 2	GGCTGTTGATGCTTCCTAGC	ATGACGGGCCTTTTCTGAAT
GRMZM2G568636	AF153448.1	NR1	nitrate reductase	ATGATCCAGTTCGCCATCTC	GTCCGTGGTACGTCGTAGGT
GRMZM2G079381	EU957616.1	NiR	ferredoxin--nitrite reductase	CTTCATGGGCTGCCTCAC	CGCTTGACGAAGGTCCTACT
GRMZM2G010280 T01	AY129953.1	nrt2.1	putative high affinity nitrate transporter	GAGAAGAGCAAGGGACTCCA	CTCATGTCAACGGAGCACAC
GRMZM2G426953_T01	DQ855284.1	rbohA	respiratory burst oxidase protein A	CAGCGCACACAAGAACTCTC	CCCCGCATACATCAAAACTT
GRMZM2G138152_T03	EU807966.1	rbohB	respiratory burst oxidase protein B	TTGGGTTACACGTGAGCAAG	AATGGAGCAAAGGCAACTGT
GRMZM2G043435_T01	DQ897930.1	rbohC	respiratory burst oxidase protein C	GGCACAGGAACTAAGCAAGC	AAACTCATCGCCAAGAAAGC
GRMZM2G032852_T03	AY109304.1	CaK	Calcium-dependent protein kinase	AACCACTTCCCAAGGAGACC	CTGTGCGTCAGGAACTTGC
GRMZM2G015933_T01	EU954960.1	BRI1	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor	TCCTCTCCCTTGTCGTTGTT	AGCCTTGATCCAGGACTCTTC
GRMZM2G002100_T01	EU965114.1	MAPK6	mitogen-activated protein kinase 6	CACACCCTTACTTGGCATCA	ATCACCGGCTGAAATTGAAC
GRMZM2G106928_T01	EU959272.1	SOD	superoxide dismutase	CAGCGCACACAAGAACTCTC	CCCCGCATACATCAAAACTT
GRMZM2G089850_T01	NM_001156658.1	DOF	dof zinc finger protein MNB1A	CTCCTGCTTTGCTCTGCTCT	AATGGAGCAAAGGCAACTGT
GRMZM2G400470_T03	BT065734.1	MAPKK(1/2)	Mitogen-Activated Protein Kinase Kinase (1/2)	CAACGAGCTTGTGGAGAACA	TCTGACCGTCCTGGTAGTCC
GRMZM2G384293_T02	NM_001174573	NOA1	putative nitric oxide synthase	ATTCTACCTTCCGTGCGTGA	GACAACCCAGTCGCCTATACA

Table S2. Merged effects of different chemicals interfering with NO biosynthesis/scavenging and nitrate supply/depletion on root and leaf fresh weight.

Treatment	Total Root Weight (g)	Leaf Weight (g)
+NO_3^-	0.07 *a*	0.131 *a*
+ NO_3^- +cPTIO	0.057 *bc*	0.124 *a*
+ NO_3^- +W	0.049 *c*	0.127 *a*
+ NO_3^- + L-NAME	0.069 *a*	0.131 *a*
+ NH_4^+	0.057 *bc*	0.128 *a*
- NO_3^-	0.051 *bc*	0.116 *a*
- NO_3^- +SNP	0.059 *b*	0.132 *a*
	P < 0.01	ns

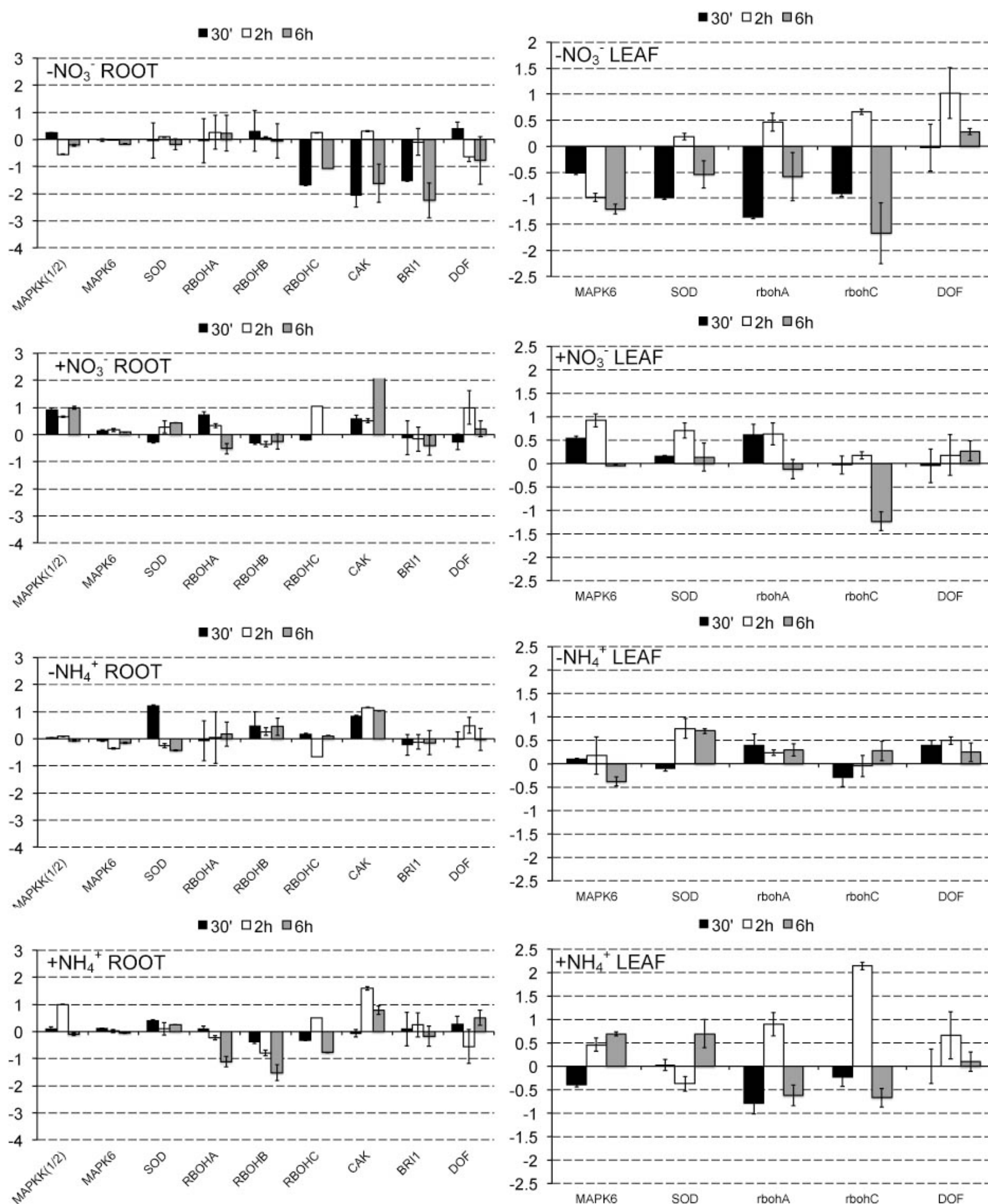


Figure S2. Time course of the expression of genes following short-term nitrate/ammonium treatments in maize roots (A) and leaves (B). The expression values were investigated at 30 min, 2 h and 6 h. Data are expressed as base-2 logarithm of the ratio between the expression measured for a treatment and that of its own control. The same expression analyses were carried out in nutrient solution either supplied or depleted with nitrate or ammonium.

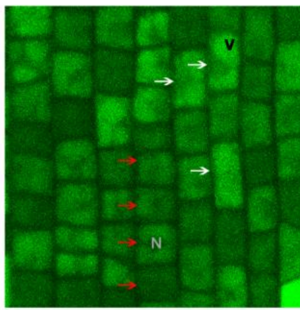
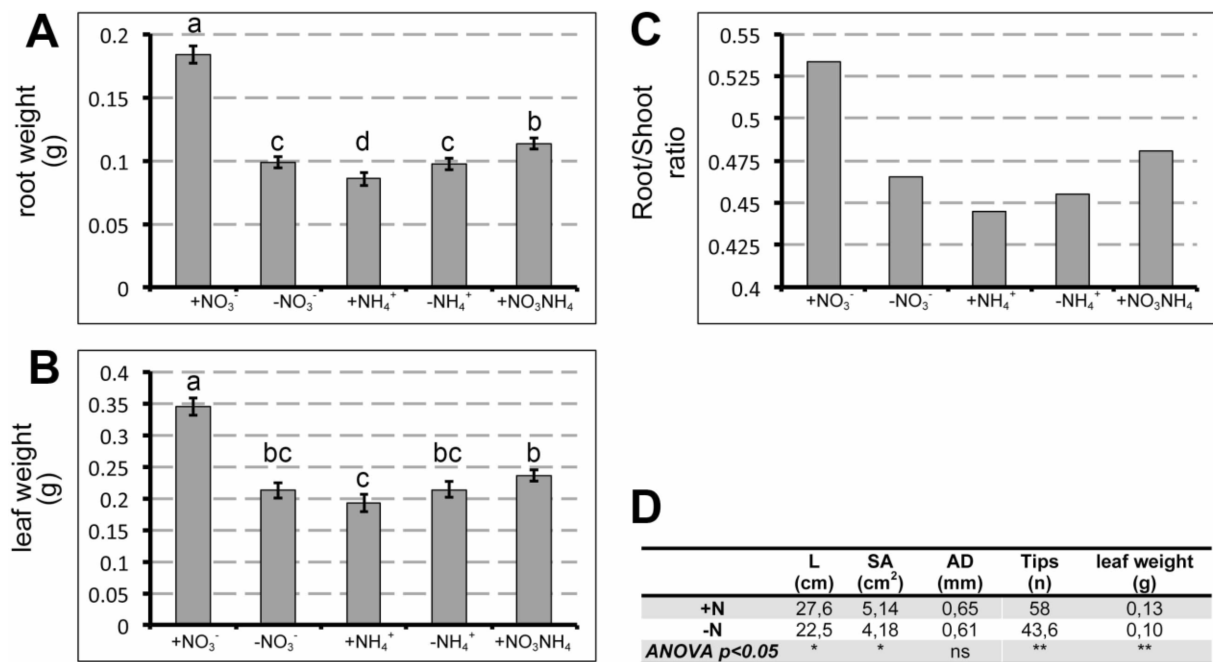


Figure S3. Confocal detection of DAF-2T in the transition zone of nitrate treated apices. Arrows indicate two different types of cells of this root zone: small square shape cells (red arrows) with central nucleus (N) and elongated cells (white arrows) with a more developed vacuole (V). Image was obtained by zooming the 40x lens.



	L (cm)	SA (cm^2)	AD (mm)	Tips (n)	leaf weight (g)
+N	27,6	5,14	0,65	58	0,13
-N	22,5	4,18	0,61	43,6	0,10
ANOVA p<0.05	*	*	ns	**	**

Figure S4. Root and leaf fresh weight and relative root/shoot ratio in seedlings grown in nutrient solution for five days (A, B, C). Total root length (L), total surface area (SA), average diameter (AD), number of root tips and leaf fresh weight in seedlings grown five days in agar medium containing or not 1 mM NO_3^- (D).

Chapter III - NO signaling is a key component of the root growth response to nitrate in *Zea mays* L.

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1. Abstract

Roots are considered to be a vital organ system of plants due to their involvement in water and nutrient uptake, anchorage, propagation, storage functions, secondary metabolite (including hormones) biosynthesis and accumulation. Crops are strongly dependent on the availability of nitrogen in soil and on the efficiency of nitrogen utilization for biomass production and yield. However, knowledge about molecular responses to nitrogen fluctuations mainly derives from the study of model species. Nitric oxide (NO) has been proposed to be implicated in plant adaptation to environment, but its exact role in the response of plants to nutritional stress is still under evaluation. Recently a novel role for NO production and scavenging, thanks to the coordinate spatio-temporal expression of nitrate reductase and non-symbiotic hemoglobins, in the maize root response to nitrate has been postulated. This control of NO homeostasis is preferentially accomplished by the cells of the root transition zone (TZ), which seem to represent the most nitrate responsive portion of maize root. The TZ is already known to function as a sensory centre able to gather information from the external environment and to re-elaborate them in an adequate response. These results indicate that it could play a central role also for nitrate sensing by roots. A lot of work is still needed to identify and characterize other upstream and downstream signals involved in the "nitrate-NO" pathway, leading to root architecture adjustments and finally to stress adaptation.

Key words: *Zea mays* L., nitrate, root, transition zone, nitric oxide

Abbreviations: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO); indole-3-acetic acid (IAA); sodium nitroprusside (SNP).

2. Introduction

To search for nutrients and water, roots need to efficiently explore large soil volumes. To this aim they generate complex root systems, allowing them to maximize their resource allocation efficiency.[1]

Despite the vital importance of roots, the difficulty in accessing intact root systems for analysis, particularly under field conditions, have slowed down the breeding programs for plant's adaptation to environmental restrictions.[2,3] The capacity of plants to take up nutrients and water is mainly determined by changes in the architecture of the root system.[1]

Three major processes affect the overall architecture of the root system: the rate of cell division, the rate of cell differentiation, and the extent of expansion and elongation of cells.[4-6] Disturbs in any of these three processes can affect the whole root-system architecture and the capacity of plants to survive and develop in adverse environments (Giehl et al. [7] and references therein).

The root system results from the coordinated control of both genetic endogenous programs (regulating growth and organogenesis) and the action of abiotic and biotic environmental stimuli.[8,9] The dynamic control of the overall root system architecture (RSA) throughout time finally determines root plasticity and allows plants to efficiently adapt to environmental constraints.[10]

The soil-environment from which plants extract nutrients and water is extremely heterogeneous, both spatially and temporally.[11] Among the nutrients present in soil, nitrate (NO_3^-) may vary by an order of magnitude within centimetres or over the course of a day.[12] The effects of NO_3^- on the root system are complex and depend on several factors, such as the concentration available to the plant, endogenous nitrogen status and the sensitivity of the species.[10,13,14]

A considerable part of the studies aimed to unravel the mechanisms controlling RSA growth and development in response to nitrate have been focused on lateral roots (LR), [8,13,15-20] while the nitrate-regulation of the primary root growth is still unclear. Beside NO_3^-, auxin has been demonstrated to strongly affect and control the LR development, [21-24] and an increasing number of studies suggests an overlap between auxin and NO_3^- signalling pathways in controlling LR development.[25-33]

3. NO_3^- has a doubtful role in regulating the growth of primary roots

Despite the high amount of reports published on nitrate effects on root elongation, the lack of univocal results make it difficult to clearly decipher this response (Table 1). In *Arabidopsis thaliana*, inhibition of primary root growth has been observed when nitrate is applied homogeneously at high concentrations (50 mM) for seven days, but not in a range between 0.1 and 10 mM.[34] On the contrary, in this same species Linkohr et al.[35] showed an inhibition of primary root elongation with the increase of nitrate concentration already beyond 0.01 mM, but in this case seedlings were grown in the nutrient medium either for 17 or 18 days. This is in contrast with results previously obtained by Zhang and Forde, [36] which did not observe changes in primary root length in a range of nitrate concentrations from 0.01 mM to 100 mM.

However, if nitrate supply was localized only to the apex, primary root growth of a number of Arabidopsis accessions was significantly stimulated, even if to a different extent according to the line responsiveness.[13] More recently Gifford et al.[37] demonstrated a stimulatory effect on primary root elongation in Arabidopsis seedlings grown for 12 days on a nitrate concentration ranging from 0 to 20 mM. Conversely, a reduction of primary root growth has been observed in both *Capsicum chinense* Jacq.,[14] and *Medicago truncatula*[38] in response to a prolonged exposure to nitrate.

In maize (*Zea mays* L.) a consistent inhibitory effect on primary root length was observed by Tian and co-authors after 12 days of growth at a nitrate concentration of 20 mM.[39] Few years later a more detailed study was published by the same authors who demonstrated that nitrate concentrations lower than 0.5 mM had no effect on elongation of primary, seminal and crown roots, while concentrations above 5 mM affected more significantly the root elongation after 12 days of treatment.[40] Moreover, by investigating the effect of different nitrate concentrations on root cell sizes, they found that high concentrations of nitrate had no effect on the length of the meristem, but did result in reduced cell elongation in the root elongation zone. Interestingly, the different types of roots considered in this study displayed different sensitivities to high nitrate, suggesting a specific regulation for each of them.[40]

Unlike what is known on the nitrate regulation of lateral root development, the mechanisms underlying the nitrate effects on primary root elongation are still controversial and poorly known. Future studies are thus needed to try to shed light on this aspect that

could highly affects plant adaptation to an external environment characterized by a spatio-temporal non constant nutrient accessibility

Table 1. Overview of the papers reporting results on primary root (PR) response to nitrate treatments.

Authors	Species	Treatments	Effect on PR length
Zhang and Forde[36]	*Arabidopsis thaliana*	Seedlings were grown on agar plates containing a range of NO_3^- concentration (0.01-100mM) and the lengths of the primary roots were measured after 14d.	No effects
Signora et al.[34]	*Arabidopsis thaliana*	Seedlings were grown on agar plates containing a range of NO_3^- concentrations (0.1-50mM). The lengths of the primary roots were recorded after 7d.	No effects (0.1-10mM) Inhibition (>50mM)
Linkohr et al.[35]	*Arabidopsis thaliana*	Seedlings were grown either for 17 or 18d on agar plates containing a range of NO_3^- concentrations (0.01-1.0mM). The lengths of the primary roots were collected after the treatments.	Inhibition
Walch-Liu & Forde[13]	*Arabidopsis thaliana*	Primary root growth was measured 9d after transfer of 5-d-old seedlings to segmented plates where NO_3^- (0.05-5mM) was present only in the bottom segment (localized treatments).	Stimulation
Gifford et al.[37]	*Arabidopsis thaliana*	Seedlings were grown on agar plates containing a range of NO_3^- concentration (0-20mM). The primary root lengths were measured after 12d.	Stimulation
Celis-Arámburo et al.[14]	*Capsicum chinense* Jacq.	Seedlings were grown on agar plates with 0.01mM NO_3^- and transferred to segmented. NO_3^- concentrations in the middle segment were adjusted to 0.01-10mM (localized treatments). For the homogeneous treatment the concentration was 1mM NO_3^-. The primary root lengths were recorded after 10d.	Inhibition
Yendrek et al.[38]	*Medicago truncatula*	Plants were grown on a N-free medium for 1 week, transferred to plates with increasing concentrations of NO_3^- (1-20-50mM) and grown for 3 weeks. The lengths of the primary roots were recorded after the treatments.	Inhibition
Tian et al.[39]	*Zea mays* L.	Plants were grown in nutrient solution containing several NO_3^- concentration (0.05-20mM). The lengths of the primary roots were recorded after 12d.	Inhibition (> 5mM)
Tian et al.[40]	*Zea mays* L.	Seedlings were incubated in the solutions containing different concentrations of NO_3^- (0.05-20mM) and the root length was measured after 12d of incubation.	No effects (0- 0.5mM) Inhibition (> 5mM)
Zhao et al.[63]	*Zea mays* L.	Seedlings were grown in varying concentrations of NO_3^- (0.1-10mM) for 7d and then exposed to 0.1 and 1mM NO_3^- for 48h. The root length was measured after the incubation.	Inhibition
Manoli et al.[68]	*Zea mays* L.	Primary root growth of 8-d-old seedlings grown in six different solutions (1mM NO_3^-, - NO_3^- and NO-donors/scavengers) were monitored for 24-48h.	Stimulation

4. The root transition zone

The root apex represents the first part of the plant getting in touch with unknown regions of the soil, and it functions as a dynamic sensory organ, able to both perceive the external environment and to adequately reorganize the root growth in response to the stimuli received.[41]

In 1990 Baluška et al.[42] invented the term transition zone to describe a unique part of the maize root apex, in which cells after leaving the meristem and before entering the elongation zone undergo slow isotropic-like growth, but do not still elongate, in fact resembling meristematic cells in many aspects. In particular, the apical part (distal) of this region seems to be characterized mainly by cells that optionally can reenter the cell cycle, whereas cells of the basal (proximal) part of this zone are able to readily enter into the fast cell elongation region.[41,43,44] This developmental feature could be differentially regulated at the opposite root flanks, providing the root apices with an effective mechanism to re-orientate growth in response to environmental stimuli.[43]

The transition zone is a unique zone being competent for integration of diverse endogenous and exogenous signals, and translating them into adaptive differential growth responses. It plays crucial functions for the perception and response to a range of external factors, as for example mechanical stimuli[41] and aluminium toxicity.[45-48]

This capability seems to be, at least in part, linked to the complex system of a polar auxin transport circuit.[41] Actually, since 1993 it has been evidenced that cells belonging to this zone are strongly auxin-responsive and accomplish dramatic rearrangements of the cytoskeleton, being subjected to a series of fundamental changes in their cytoarchitecture.[49]

A recent study conducted on maize demonstrated that the transition zone plays central roles in both sensing and adapting to root hypoxia.[50] The authors also observed that the oxygen deprivation of roots induces local NO emission in the TZ, that is essential for the successful acclimation of the entire maize root to oxygen deprivation.[50]

A number of experimental data globally indicate that the transition zone of the root may be considered as a sort of sensory center, enabling the root apex to continuously monitor environment parameters and to trigger appropriate responses.[50-62] Future studies will be needed to deepen the role of this unique root zone in translating the external stimuli in motoric responses.

5. Nitrate affects root elongation through NO-elicited actions

Recently nitric oxide (NO) was proposed to be involved in the regulation of the nitrate-dependent primary root growth. [63] The authors showed that high nitrate supply may reduce IAA levels and subsequently inhibits NO synthase activity, leading to a decrease in the endogenous NO level, which serves as a trigger to elicit nitrate-dependent root growth. A regulatory role for NO in the inhibition of primary root growth has also been suggested in tomato[64] and Arabidopsis.[65,66]

Furthermore, a recent study carried out in maize provided evidences that NO is produced by nitrate reductase (NR) as an early response to nitrate supply and that the coordinated induction of non-symbiotic hemoglobins (nsHbs) could finely regulate the NO steady state.[67,68] Both nitric oxide biosynthesis and gene regulation were preferentially accomplished by cells of the transition zone of roots, which would seem the most nitrate responsive portion of maize root. [68] NsHbs play important roles in plant physiology by regulating a number of downstream physiological events involved in plant developmental processes and stress responses, also interacting with many hormonal signalling (for a review see refs 69,70). They catalyse the conversion of nitric oxide to nitrate, contributing to the control of nitric oxide homeostasis in plant cells. They should be considered to be as important as NO generation in regulating *in planta* NO signalling.[70]

Moreover, in this same study,[68] a stimulatory effect of a low concentration of nitrate (1 mM) on root elongation after one/two days of treatment was measured in very young seedlings. Nevertheless, when an inhibitor of nitrate reductase activity (tungstate) or a nitric oxide scavenger (cPTIO) were supplied together with the nitrate, no effects on root elongation were observed. On the contrary the treatment of nitrate-depleted roots with a low concentration (10 μM) of a nitric oxide donor (SNP) stimulated root elongation to an extent similar to that measured after nitrate supply. These results strongly suggest that the mechanism through which nitrate affects root elongation is dependent on nitric oxide, as also observed by Zhao et al.[63] even if these authors found some different and in some way opposite results. This apparently contradictory finding could derive from the very different experimental plan and growth conditions utilized in these two works, making difficult to compare results obtained. Furthermore, our unpublished results suggest that nitrate is able to affect the root elongation in a contrasting mode according to their concentration, acting as a stimulatory signal for concentration equal or below 1 mM, and as a negative regulator

at higher concentration, suggesting the existence of a multifaceted concentration/time-dependent mechanism of regulation of root elongation by nitrate availability.

NO is considered a key regulator of plant developmental processes and defence (for a reviews see refs 70-74), although the mechanism and direct targets of NO action remain largely unknown (for a review see ref 75 and references therein).

In the case of NO-dependent nitrate regulation of root elongation, the downstream events triggering the root to elongate have still to be identified. Cytoskeletal proteins seem to represent a highly probable molecular target for NO signal[76-78] and accumulating evidences place NO among the key elements in the control of a number of cytoskeleton-mediated processes in plants, such as root growth and development,[79] guard cell dynamic,[80] vesicle trafficking,[76] pollen[81] and root hair tip growth[82] or gravitropic bending.[83] In particular, Kasprowicz et al.[76] demonstrated that the actin-dependent endocytosis and organization of the actin cytoskeleton are modulated by NO levels in maize root apices, according to cell-type and developmental stage with the most remarkable effects noticed at level of the transition zone. Thus, the involvement of cytoskeletal rearrangements in the NO-mediated nitrate regulation of primary root elongation is highly conceivable.

Moreover, since NO and auxin act synergically to control diverse aspects of root biology (for a review see Freschi et al.[84]) and lateral root development in response to nitrate is strongly auxin dependent,[85] a role of NO as a coordinator of nitrate and auxin signaling to control the overall root response to the anion cannot be excluded. The involvement of nitric oxide homeostasis control in the root elongation response to nitrate[68] adds a novel component to the complicated puzzle of the root adaptation to nitrate fluctuations in soil (Fig. 1). Furthermore, the prominent role of the maize transition zone in the accomplishment of this sensing pathway widens the range of signal/molecules which are sensed and decoded by this particular region of root, which seems to transversally operate to translate in motoric behaviour a large number of endogenous and exogenous clues.

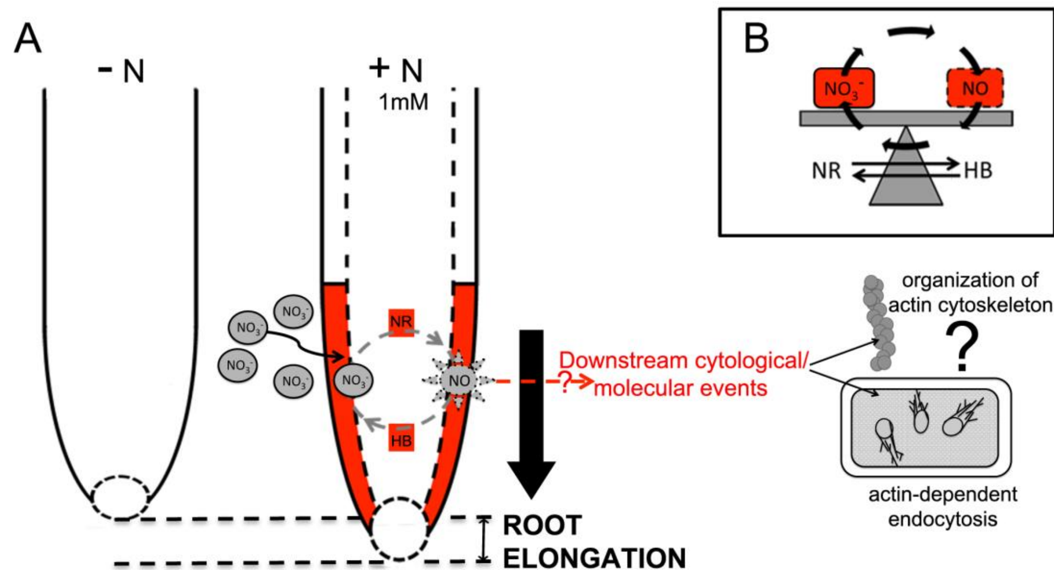

Figure 1. Model of the NO-mediated nitrate regulation of primary root elongation. A) The transfer of seedlings from a NO_3^--deprived media to a NO_3^--supplied solution results in a elongation of the primary root. The stimulatory effect of NO_3^- (1mM) was demonstrated to be dependent on the control of nitric oxide (NO) homeostasis thank to the coordinate regulation of cytosolic nitrate reductase (NR) and non-symbiotic hemoglobins (nsHbs) (B).[67,68] The preferential localization and the strong transcriptional responsiveness of both NR and nsHbs in the transition zone of the apex straightened the hypothesis of a role of this root portion in translating the environmental stimuli in developmental response.[67,68] Because of the role of NO in several cytoskeleton-mediated processes in plants,[76-83] the actin-dependent endocytosis and the organization of the actin cytoskeleton are proposed as candidates in transducing the NO-dependent nitrate regulation of root elongation.

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7. References

1. Lòpez-Bucio J, Cruz-Ramírez A, Herrera-Estrella L. The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 2003; 6:280-7.
2. de Dorlodot S, Forster B, Pages L, Price A, Tuberosa R, Draye X. Root system architecture: opportunities and constraints for genetic improvement of crops. Trends Plant Sci 2007; 12:474-81.
3. Lynch JP. Roots of the second green revolution. Aust J Bot 2007; 55: 493-512.
4. Benfey PN, Linstead PJ, Roberts K, Schiefelbein JW, Hauser MT, Aeschbacher RA. Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis. Development 1993; 119:57-70.

5. Scheres B, Benfey P, Dolan L. Root development. The Arabidopsis book 2002. Somerville CR, Meyerowitz EM, eds. Rockville: America Society of Plant Biologists.

6. Jiang K, Feldman LJ. Regulation of root apical meristem development. Annu Rev Cell Dev Biol 2005; 21:485–509.

7. Giehl RF, Gruber BD, von Wirén N. It's time to make changes: modulation of root system architecture by nutrient signals. J Exp Bot 2013; doi: 10.1093/jxb/ert421.

8. Malamy JE. Intrinsic and environmental response pathways that regulate root system architecture. Plant Cell Environ 2005; 28:67-77.

9. Hodge A, Berta G, Doussan C, Merchan F, Crespi M. Plant root growth, architecture and function. Plant Soil 2009; 321:153-87.

10. Gruber BD, Giehl RFH, Friedel S, von Wirén N. Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Phys 2013; 163: 161-79.

11. Bloom AJ, Frensch J, Taylor AR. Influence of inorganic nitrogen and pH on the elongation of maize seminal roots. Ann Bot 2006; 97:867-73.

12. Jackson LE, Bloom AJ. Root distribution in relation to soil nitrogen availability in field-grown tomatoes. Plant Soil 1990; 128:115-26.

13. Walch-Liu P, Forde BG. Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. Plant J 2008; 54:820-8.

14. Celis-ArámburoTde J, Carrillo-Pech M, Castro-Concha LA, Miranda-Ham Mde L, Martínez-Estévez M, Echevarría-Machado I. Exogenous nitrate induces root branching and inhibits primary root growth in *Capsicum chinense* Jacq. Plant Physiol Biochem 2011; 49:1456-64.

15. Forde BG. Local and long-range signaling pathways regulating plant responses to nitrate. Annu Rev Plant Biol 2002; 53:203-24.

16. Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A. The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci USA 2006; 103:19206-11.

17. Benkova E, Bielach A. Lateral root organogenesis from cell to organ. Curr Opin Plant Biol 2010; 13:677-83.

18. Bennett T, Scheres B. Root development-two meristems for the price of one? Curr Top Dev Biol 2010; 91:67-102.

19. DeSmet I. Lateral root initiation: one step at a time. New Phytol 2012; 193:867-73.

20. Jung JK, McCouch S. Getting to the roots of it: genetic and hormonal control of root architecture. Front Plant Sci 2013; 4:186.
21. Teale WD, Paponov IA, Palme K. Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 2006; 7:847-59.
22. Vieten A, Sauer M, Brewer PB, Friml J. Molecular and cellular aspects of auxin transport-mediated development. Trends Plant Sci 2007; 12:160-8.
23. De Smet I. Lateral root initiation: one step at a time. New Phytol 2012; 193:867-73.
24. Van Norman JM, Xuan W, Beeckman T, Benfey PN. To branch or not to branch: the role of pre-patterning in lateral root formation. Development 2013; 140:4301-10.
25. Zhang HM, Jennings A, Barlow PW, Forde BG. Dual pathways for regulation of root branching by nitrate. Proc Natl Acad Sci USA 1999; 96: 6529-34.
26. Forde BG. Local and long-range signaling pathways regulating plant responses to nitrate. Annu Rev Plant Biol 2002; 53:203-24.
27. Guo Y, Chen F, Zhang F, Mi G. Auxin transport from shoot to root is involved in the response of lateral root growth to localized supply of nitrate in maize. Plant Sci 2005; 169:894-900.
28. Beeckman T, Friml J. Nitrate contra auxin: nutrient sensing by roots. Dev Cell 2010; 18:877-8.
29. Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K et al. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Dev Cell 2010; 18:927-37.
30. Liu J, An X, Cheng L, Chen F, Bao J, Yuan L, Zhang F, Mi G. Auxin transport in maize roots in response to localized nitrate supply. Ann Bot 2010; 106:1019-26.
31. Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutierrez RA. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 2010; 107:4477-82.
32. Kiba T, Kudo T, Kojima M, Sakakibara H. Hormonal control of nitrogen acquisition: roles of auxin, abscisicacid, and cytokinin. J Exp Bot 2011; 62:1399-409.
33. Mounier E, Pervent M, Ljung K, Gojon A, Nacry P. Auxin-mediated nitrate signalling by NRT1.1 participates in the adaptive response of Arabidopsis root architecture to the spatial heterogeneity of nitrate availability. Plant Cell Environ 2014; 37:162-74.
34. Signora L, De Smet I, Foyer CH, Zhang H. ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J 2001; 28:1-9.

35. Linkohr BI, Williamson LC, Fitter AH, Leyser O. Nitrate and phosphate availability and distribution have different effects on root system architecture of Arabidopsis. Plant J 2002; 29:751-60.

36. Zhang H, Forde BG. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 1998; 279:407-9.

37. Gifford ML, Banta JA, Katari MS, Hulsmans J, Chen L, Ristova D, Tranchina D, Purugganan MD, Coruzzi GM, Birnbaum KD. Plasticity regulators modulate specific root traits in discrete nitrogen environments. PLoS Genet 2013; 9:e1003760.

38. Yendrek CR, Lee YC, Morris V, Liang Y, Pislariu CI, Burkart G, Meckfessel MH, Salehin M, Kessler H, Wessler H, et al. A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. Plant J 2010; 62:100-12.

39. Tian QY, Chen FJ, Zhang FS, Mi GH. Possible involvement of cytokinin in nitrate-mediated root growth in maize. Plant Soil 2005; 100:1-12.

40. Tian Q, Chen F, Liu J, Zhang F, Mi G. Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. J Plant Physiol 2008; 165:942-51.

41. Baluška F, Mancuso S, Volkmann D, Barlow P. Root apex transition zone: a signalling-response nexus in the root. Trends Plant Sci 2010; 15:402-8.

42. Baluška F, Kubica S, Hauskrecht M. Postmitotic “isodiametric” cell growth in the maize root apex. Planta 1990; 181:269-74.

43. Verbelen JP, De Cnodder T, Le J, Vissemberg K, Baluška F. The root apex of *Arabidopsis thaliana* consists of four distinct zones of growth activities. Plant Signal Behav 2006; 1:296-304.

44. Baluška F, Mancuso S. Root apex transition zone as oscillatory zone. Front Plant Sci 2013; 4:354.

45. Sivaguru M, Horst WJ. The distal part of the transition zone is the most aluminum-sensitive apical root zone of maize. Plant Physiol 1998; 116:155-63.

46. Sivaguru M, Baluška F, Volkmann D, Felle HH, Horst WJ. Impacts of aluminum on the cytoskeleton of the maize root apex. Short-term effects on the distal part of the transition zone. Plant Physiol 1999; 119:1073-82.

47. Kollmeier M, Felle HH, Horst WJ. Genotypical differences in aluminum resistance of maize are expressed in the distal part of the transition zone. Is reduced basipetal auxin flow involved in inhibition of root elongation by aluminum? Plant Physiol 2000; 122:945-56.
48. Illéš P, Schlicht M, Pavlovkin J, Lichtscheidl I, Baluška F, Ovečka M. Aluminium toxicity in plants: internalisation of aluminium into cells of the transition zone in Arabidopsis root apices relates to changes in plasma membrane potential, endosomal behaviour, and nitric oxide production. J Exp Bot 2006; 57:4201-13.
49. Ishikawa H, Evans ML. The role of the distal elongation zone in the response of maize roots to auxin and gravity. Plant Physiol 1993; 102:1203-10.
50. Mugnai S, Azzarello E, Baluška F, Mancuso S. Local root apex hypoxia induces NO-mediated hypoxic acclimation of the entire root. Plant Cell Physiol 2012; 53:912-20.
51. Ishikawa H, Evans ML. Induction of curvature in maize roots by calcium or by thigmo stimulation: role of the postmitotic isodiametric growth zone. Plant Physiol 1992; 100:762-8.
52. Winch S, Pritchard J. Acid-induced cell wall loosening is confined to the accelerating region of the root growing zone. J Exp Bot 1999; 50:1481-7.
53. Baluška F, Volkmann D, Barlow PW. Specialized zones of development in roots: view from the cellular level. Plant Physiol 1996; 112:3-4.
54. Wu Y, Cosgrove DJ. Adaptations of roots to low water potentials by changes in cell wall extensibility and cell wall properties. J Exp Bot 2000; 50:1543-53.
55. Mancuso S, Boselli M. Characterisation of the oxygen fluxes in the division, elongation and mature zones of Vitis roots: influence of oxygen availability. Planta 2002; 214:767-77.
56. Ober ES, Sharp RE. Electrophysiological responses of maize roots to low water potentials: relationship to growth and ABA accumulation. J Exp Bot 2003; 54:813-24.
57. Mancuso S, Barlow PW, Volkmann D, Baluška F. Actin turnover-mediated gravity response in maize root apices: gravitropism of decapped roots implicates gravisensing outside of the root cap. Plant Signal Behav 2006; 1:52-8.
58. Mancuso S, Marras AM. Adaptative response of Vitis root to anoxia. Plant Cell Physiol 2006; 47:401-9.
59. Amenòs M, Corrales I, Poschenrieder C, Illès P, Baluška F, Barcelò J. Different effects of aluminum on the actin cytoskeleton and brefeldin A-sensitive vesicle recycling in root

apex cells of two maize varieties differing in root elongation rate and aluminum tolerance. Plant Cell Physiol 2009; 50:528-40.

60. Sobol M, Kordyum E. Distribution of calcium ions in cells of the root distal elongation zone under clinorotation. Microgr Sci Technol 2009; 21:179-85.

61. Marciano DPRO, Toledo-Ramos F, Neiva-Alvim M, Magalhaes JR, Costa França MG. Nitric oxide reduces the stress effects of aluminum on the process of germination and early root growth of rice. J Plant Nutr Soil Sci 2010; 173:885-91.

62. Mugnai S, Marras AM, Mancuso S. Effect of hypoxic acclimation on anoxia tolerance in Vitis roots: response of metabolic activity and K^+ fluxes. Plant Cell Physiol 2011; 52:1107-16.

63. Zhao DY, Tian QY, Li LH, Zhang WH. Nitric oxide is involved in nitrate-induced inhibition of root elongation in *Zea mays*. Ann Bot 2007; 100:497-503.

64. Correa-Aragunde N, Graziano M, Lamattina L. Nitric oxide plays a central role in determining lateral root development in tomato. Planta 2004; 218:900-5.

65. Méndez-Bravo A, Raya-González J, Herrera-Estrella L, López-Bucio J. Nitric oxide is involved in alkamide-induced lateral root development in Arabidopsis. Plant Cell Physiol 2010; 51:1612-26.

66. Fernández-Marcos M, Sanz L, Lewis DR, Muday GK, Lorenzo O. Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. Proc Natl Acad Sci USA 2011; 108:18506-11.

67. Trevisan S, Manoli A, Begheldo M, Nonis A, Enna M, Vaccaro S, Caporale G, Ruperti B, Quaggiotti S. Transcriptome analysis reveals coordinated spatiotemporal regulation of hemoglobin and nitrate reductase in response to nitrate in maize roots. New Phytol 2011; 192:338-52.

68. Manoli A, Begheldo M, Genre A, Lanfranco L, Trevisan S, Quaggiotti S. NO homeostasis is a key regulator of early nitrate perception and root elongation in maize. J Exp Bot 2014; 65:185-200.

69. Hill RD. Non-symbiotic hemoglobins-What's happening beyond nitric oxide scavenging? AoB Plants 2012; 2012:pls004.

70. Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJ, Hebelstrup KH, Gupta KJ. Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants 2013; 5:pls052.

71. Durner J, Klessig DF. Nitric oxide as a signal in plants. Curr Opin Plant Biol 1999; 2:369-74.

72. Wojtaszek P. Nitric oxide in plants. To NO or not to NO. Phytochemistry 2000; 54:1-4.
73. Beligni MV, Lamattina L. Nitric oxide: a non-traditional regulator of plant growth. Trends Plant Sci 2001; 6:508-9.
74. Lamattina L, García-Mata C, Graziano M, Pagnussat G. Nitric oxide: the versatility of an extensive signal molecule. Annu Rev Plant Biol 2003; 54:109-36.
75. Simontacchi M, García-Mata C, Bartoli CG, Santa-María GE, Lamattina L. Nitric oxide as a key component in hormone-regulated processes. Plant Cell Rep 2013; 32:853-66.
76. Kasprowicz A, Szuba A, Volkmann D, Baluška F, Wojtaszek P. Nitric oxide modulates dynamic actin cytoskeleton and vesicle trafficking in a cell type-specific manner in root apices. J Exp Bot 2009; 60:1605-17.
77. Wang Y, Chen T, Zhang C, Hao H, Liu P, Zheng M, Baluška F, Šamaj J, Lin J. Nitric oxide modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes. New Phytol 2009; 182:851-62.
78. Yao LL, Pei BL, Zhou Q, Li YZ. NO serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton during responses to VD-toxins in Arabidopsis. Plant Signal Behav 2012; 7:174-7.
79. Kolbert Z, Bartha B, Erdei L. Exogenous auxin-induced NO synthesis is nitrate reductase-associated in *Arabidopsis thaliana* root primordia. J Plant Physiol 2008; 165:967-75.
80. Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I. Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot 2008; 59:165-76.
81. Prado AM, Porterfield DM, Feijó JA. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 2004; 131:2707-14.
82. Lombardo MC, Graziano M, Polacco JC, Lamattina L. Nitric oxide functions as a positive regulator of root hair development. Plant Signal Behav 2006; 1:28-33.
83. Hu X, Neill SJ, Tang Z, Cai W. Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol 2005; 137:663-70.
84. Freschi L. Nitric oxide and phytohormone interactions: current status and perspectives. Front Plant Sci 2013; 4:398.
85. Laskowski M. Lateral root initiation is a probabilistic event whose frequency is set by fluctuating levels of auxin response. J Exp Bot 2013; 64:2609-17.

Chapter IV - Immunofluorescence labeling of maize root cells reveals effects on cell wall deposition and PIN1-mediated auxin accumulation in the transition zone in response to nitrate.

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1. Introduction

In the last few years, the transition zone (TZ) of root is gradually becoming established as a central regulator of root growth, since this unique zone, located between the apical meristem and the fast elongation zone, is thought to be responsible for integrating several endogenous and exogenous signals, translating them into adaptive differential root phenotypes (Baluška and Mancuso, 2013). In fact, a number of investigations suggest that the transition zone of the growing root apex is some kind of sensory centre, enabling the growing apex to continuously monitor diverse environmental parameters and to effect appropriate responses (Baluška *et al*., 2010). For instance, cells of the TZ are very sensitive to touch and extracellular calcium, gravity and auxin, water and salt stress, as well as, to aluminium (Baluška *et al*., 2001a and references therein). Moreover, the transition zone is also critical with respect to the “steering” of root extension, enabling the advancing root tip to “navigate” towards nutritionally rich areas of soil and, otherwise, to avoid unfavourable areas (Barlow and Baluška, 2000; Verbelen *et al*., 2006). The high sensitivity of transition zone cells, which are not engaged in mitotic divisions, seems to be related to their specific cyto-architecture, in which post-mitotic nuclei occupy a central position within the cell, suspended in networks of F-actin and radial arrays of perinuclear microtubules extending to the cell periphery (Baluška *et al*., 2001a). The centred cell bodies (*i.e* nuclei surrounded by microtubules and actin filaments) of TZ in fact, in contrast to the mitotically active cell bodies of meristematic root cells, which are continually assembling and disassembling mitotic spindles, are not engaged in such activities and thus, are free to pursue new activities, such as environmental sensing (Baluška *et al*., 2000). Conversely, during cell elongation, cells become filled with vacuoles and the metabolically less active nuclei become appressed against the cell walls, not allowing efficient interactions with environmental signals and developmental cues (Baluška *et al*., 1998; Baluška and Mancuso, 2013), as showed in Fig. 1.

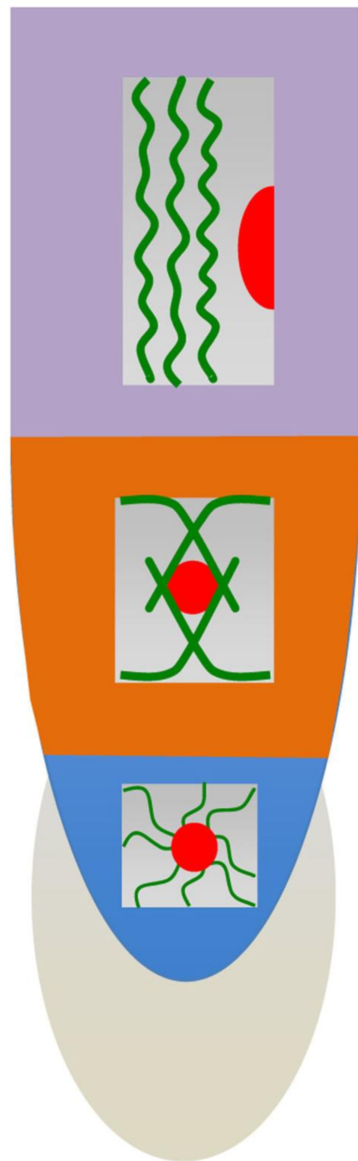


Figure 1. Schematic views of cellular architecture in meristem, transition zone and elongation region. Cells in the meristem are characterized with centrally positioned nuclei suspended in networks of F-actin and radial arrays of perinuclear microtubules. In the transition zone, nuclei still keep their central position, but fine F-actin networks are replaced by bundles of F-actin organized via the nuclear surface and the end-poles enriched with myosinVIII. In the elongation region, cells start to elongate very rapidly and develop their central vacuole which is pushing their nuclei toward the side walls. F-actin bundles obtain longitudinal and wrinkled/loosened appearances, from Baluška and Mancuso, 2013.

In addition to that, also the establishment of cell polarity has unique features in the transition zone (Baluška *et al*., 2001a and references therein). In the transition zone in fact, the cells that have ceased mitotic division continue to expand laterally and longitudinally. This post-mitotic cell growth terminates with the onset of rapid cell elongation; after passing this developmental stage, the cellular root-growth machinery is focused exclusively on rapid and polarized cell elongation. In this context, the switch-point in cell growth polarity and accelerated root elongation are two key events succeed within the transition zone, driven by unique configuration of cortical microtubules and actin filaments in this root zone (Baluška *et al*., 2003). The establishment of cell polarity is an essential feature not only for plant cells but also for almost all prokaryotic and eukaryotic cells (Huang and Ingber, 1999). In regard to

plants, organ polarity is closely related to the properties of cell walls (Fowler and Quatrano, 1998; Wojtaszek, 2000). The mechanical robustness of the plant cell walls constrains expansion of the cytoplasm and thus, cell walls not only set, but also maintain, the growth polarity of plant cells (Baluška *et al*., 2001a). Interestingly in this scenario, cell wall acidic pectins undergo internalization in meristem and transition zone cells of maize root apices, suggesting that internalization of cell wall pectins could play a key role in the dynamic turnover of pectins in dividing and fast elongating cells (Baluška *et al*., 2002). Moreover, data obtained both in maize and *Arabidopsis* also showed in root transition zone cells the highest activities of xyloglucan endotransglycosylase (XET) (Pritchard *et al*., 1993 and Vissenberg *et al*., 2000, respectively). This enzyme cleaves xyloglucan chains and seems to play a predominant role in cell wall expansion. Similarly to pectins, also XETs could be involved in cell wall loosening during cell expansion (Vissenberg *et al*., 2000).

In contrast with most of eukaryotic cells, in plants polarity results from the establishment of non-growing domains, which are actively maintained at opposite end-poles (known also as cross-walls or transverse walls) of the cell (Baluška *et al*., 2003). In fact, whereas the cells in the meristem grow diffusely around the whole of their perimeter, the end-poles portions of their perimeter progressively cease to expand in the basal part of the transition zone to cease definitely to expand at all in the elongation zone (Baluška *et al*., 2001a). Interestingly, the available data indicate that these non-growing end-pole domains of plant cells are sites of intense endocytosis and recycling (Baluška *et al*., 2010). In this scenario, it is not surprising that the cells of the transition zone have the highest rate of vesicle recycling activity and of auxin transport activity (Schlicht *et al*., 2006; Baluška *et al*., 2010). A number of papers reported the localization of the auxin efflux transporter PIN1 and auxin influx transporter AUX1 at the plasma membrane of root apical and basal end-poles, respectively (for PIN1 see Steinmann *et al*., 1999; Geldner *et al*., 2001, 2003; Grebe *et al*., 2002; for AUX1 see Swarup *et al*., 2001). In addition to that, cells of the root transition zone are unique also because they assemble F-actin enriched plasma membrane domains at these end-poles (Schlicht *et al*., 2006). This in turn serve as dynamic platforms for rapid endocytosis and high rate of vesicle recycling, allowing this root zone to be more sensitive not only to internal developmental cues, but, importantly, to environmental inputs, including nutrient availability in the soil. Very interestingly, cell wall pectins and xyloglucans

of maize root cells seem to use the same endocytosis-based recycling pathway during their cell wall remodelling (Baluška *et al*., 2005).

Consequently, in order to shed light on these key biological aspects and on the role of the transition zone in maize response to nitrate, here we reported a series of confocal laser scanning microscope analysis by using immunofluorescence microscopy. In this regard, embedding techniques using Steedman's wax permit to monitor diverse antigens both in cytoplasm and within cell walls of maize root apices. Combined with the currently available antibodies we first tested antibodies against the cell wall constituent xyloglucans. Moreover, monoclonal antibodies against IAA as well as against the efflux IAA carrier PIN1 were used in our investigation. In addition, as vesicle trafficking inhibitor, the fungal metabolite Brefeldin A (BFA) was used. Our preliminary data suggest that nitrate has an important role in modifying cell wall recycling in the transition zone. Also PIN1-mediated auxin accumulation seems to be interfered in response to nitrate.

2. Materials and methods

2.1 Plant material, chemicals and experimental layout

Maize grains (*Zea mays* L., inbred line B73) were soaked for 6 hour and germinated in well moistened rolls of filter paper for three days in darkness at 25°C, and then transferred to different nutrient solution, according to the plot reported in Fig. 1. Young seedlings with straight primary roots, 50–70 mm long, were selected for inhibitor treatments and subsequent immunolabeling studies. Unless stated otherwise, all chemicals were obtained from Sigma Chemicals (St. Louis, Mo, U.S.A.). For Brefeldin A (BFA) treatment, we used a diluted in in phosphate-buffered saline (PBS) solution to achieve an effective working solution of 1mM immediately before submergence of root apices for 2 h.

2.2 Indirect immunofluorescence labeling

Apical root segments (7 mm) encompassing the major growth zones were excised into 3.7% formaldehyde prepared in stabilizing buffer (SB) (50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 5 mM MgSO4, 5mM EGTA, pH 6.9) and fixed for 1 h at room temperature. Following rinsing in SB, the root apices were dehydrated in a graded ethanol series diluted with PBS. They were then embedded in Steedman's wax and processed for

immunofluorescence (for details, see Baluška *et al*., 1992). To enable efficient penetration of antibodies, sections were dewaxed in absolute ethanol, passed through a graded ethanol series diluted with PBS, and then placed in SB for 30 min. After a 10 min rinse with absolute methanol at -20°C, the sections were transferred to SB containing 1% BSA for 30 min at room temperature.

Sections were then incubated with the following primary antibodies: anti-XG antibodies diluted 1:200, anti-IAA monoclonal antibodies diluted 1:20 and anti-PIN1 polyclonal antibodies diluted 1:40. All primary antibodies were diluted in PBS. The buffers were supplemented with 1% BSA. Sections were incubated in primary antibody for 1 h at RT. After rinsing in PBS, the sections were incubated for 1 h with anti-rabbit IgGs, each raised in goat and diluted 1 : 100 in appropriate buffer containing 1% BSA. A further rinse in PBS (10 min) preceded a 10 min treatment with 0.01% Toluidine Blue to diminish autofluorescence of the root tissues. The sections were then mounted using an anti-fade mounting medium containing *p*-phenylenediamine (Baluška et al., 1992). Sections were examined with an Axiovert 405M inverted microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard fluorescein isothiocyanate excitation and barrier filters.

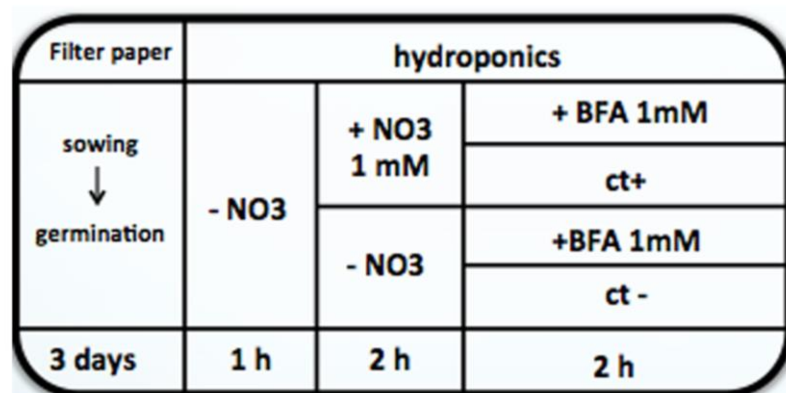


Figure 2. Workflow model of the experimental conditions. Seeds were sowed on filter paper, and 3 days after germination seedlings were transferred for 1 h to a nitrate-depleted hydroponic nutrient solution (for details see Trevisan *et al*., 2011). Then, the seedlings were divided into two groups and transferred for 2 h to two different solutions (with or without nitrate). Finally, some seedlings from both the nitrate-supplied group and the nitrate-depleted one were treated with BFA for 2 h.

2.3 Measurements and statistical analysis

For measurements and statistical analysis images were analysed with ImageJ software. For statistical analysis, measurements for each experimental variant were performed in duplicate on 50 randomly selected cortex cells from the root transition zone.

The areas of cells and BFA compartments were free-hand traced and measured, and number of compartments per cell was counted. All vesicular structures visible after labelling with anti-XG antibodies under fluorescence microscope were considered as BFA compartments. Finally, for each cell, the percentage of cell area covered by BFA compartments was estimated. The statistical analyses were performed by using R version 2.14.2. Due to non-normality within treatments and to variance inequality among treatments, data were analysed by the non-parametric Kruskal–Wallis test. All data given are means ± SE.

3. Results

3.1 Xyloglucans immunolocalization

Previous works have shown as xyloglucan endotransglycosylase, a key enzyme in cleaving XGs chains, had the most prominent activity in the transition zone in both maize and *Arabidopsis* (Pritchard *et al*., 1993 and Vissenberg *et al*., 2000, respectively). Beside this, root transition zone also shows the highest rate of vesicle recycling activity in removing XGs from cell walls into BFA-compartments, as demonstrated by Baluška *et al*. (2005). Xyloglucans visualized by labelling with antibodies were abundant, especially at the cross wall (end-poles) of xylem elements and of cells.

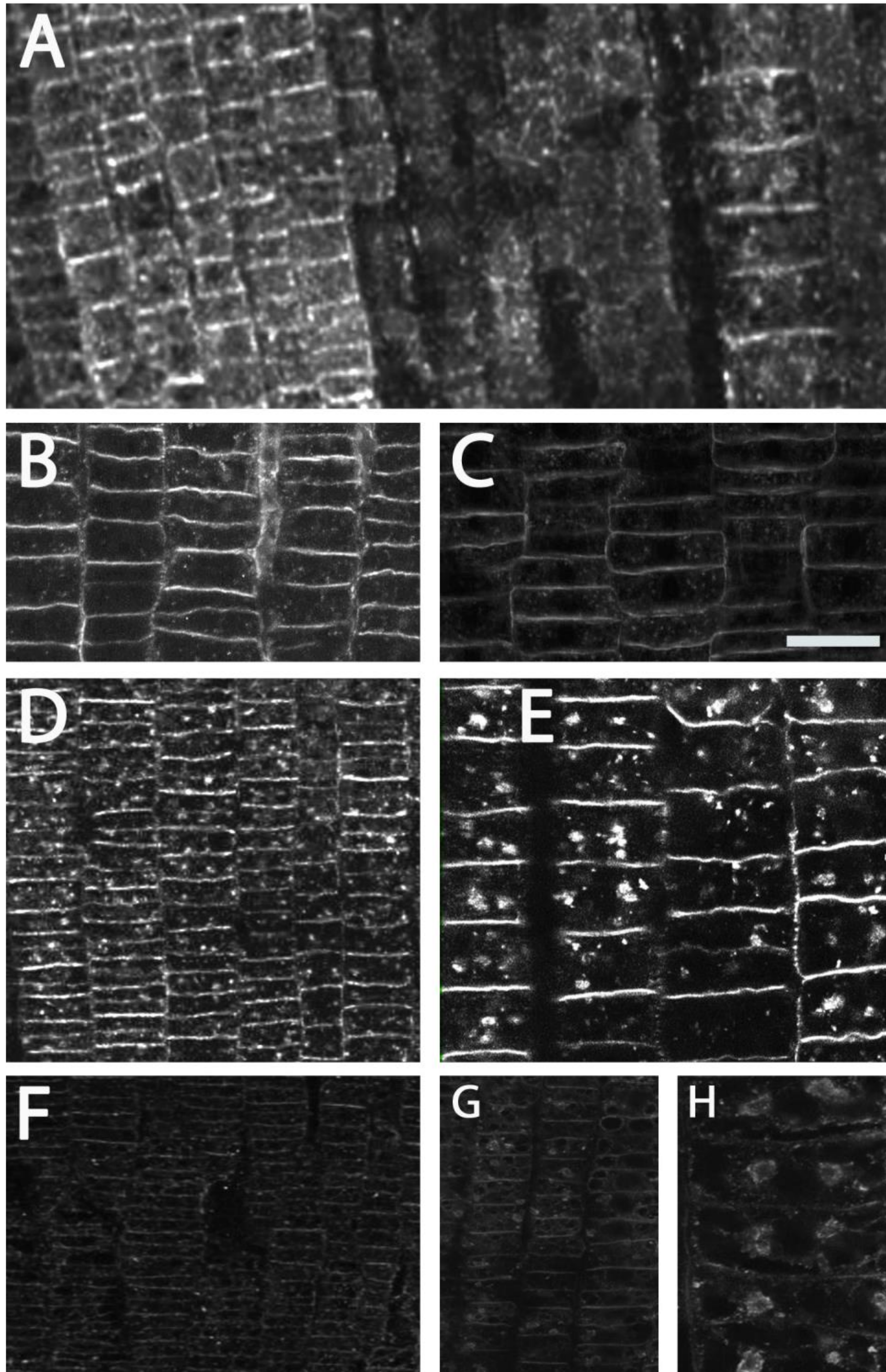


Figure 3. Immunolocalization of xyloglucans in cells of root transizione zone. **A** Xyloglucans are very abundant at the cross wall of xylem elements and of cells in the middle and outer cortex of maize root transition. **B** Nitrate treatment results in very abundant accumulation of XGs, especially in cross walls, in comparison with the negative control (**C**). **D-H** In BFA-treated cells, almost all XGs internalize into BFA compartments. **D-E** Roots grown in a nitrate-resupply or (**F-H**) nitrate-depleted solution. Bar in C: for A, 40 µm; for B, C and G, 25 µm; for D and F, 30 µm; for E, 20 µm; for H, 8 µm.

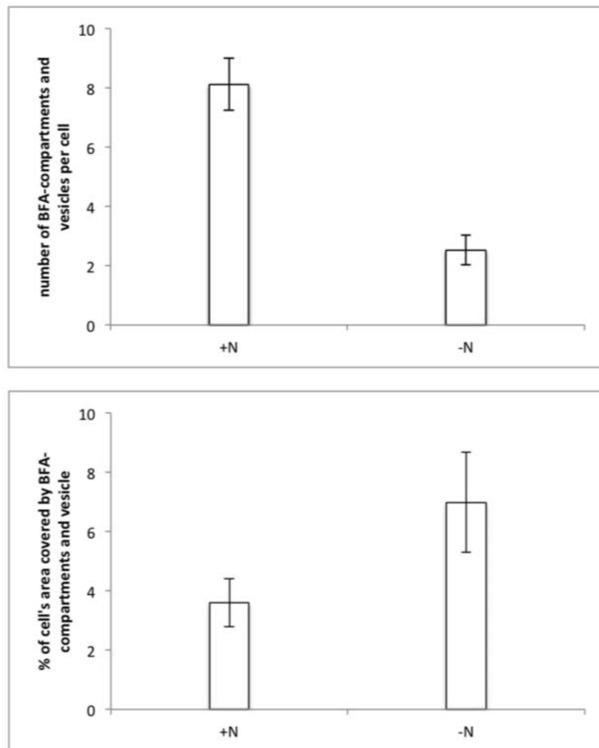


Figure 4. The presence of nitrate affects vesicle formation and trafficking in cortex cells in the transition zone of maize root apices. The treatment of root apices was identical to that described in Fig. 1. **A** The number of BFA compartments and veiscle per cell. **B** Total size of BFA compartments and vesicles expressed as a percentage of the cell area. For statistical analysis, measurements for each experimental variant were performed in duplicate on 50 randomly selected cortex cells from the root transition zone. The areas of cells and BFA compartments were free-hand traced and measured, and number of compartments per cell was counted. All vesicular structures visible after labelling with anti-XG antibodies under fluorescence microscope were considered as BFA compartments. Finally, for each cell, the percentage of cell area covered by BFA compartments was estimated. The statistical analyses were performed by using R version 2.14.2. Due to non-normality within treatments and to variance inequality among treatments, data were analysed by the non-parametric Kruskal–Wallis test. All data given are means ± SE.

3.2 Auxin and PIN1 immunolocalization

Similarly to previous XG immunolabeling observations, we focused our attention in analysing auxin and PIN1 proteins on root transition zone cells, according to several papers that reported the highest degree of auxin transport and PIN1 activity in the transition zone (Schlicht *et al.*, 2006, Baluška *et al.*, 2010, Baluška and Mancuso, 2013). As far as auxin immunolabeling is concerned, the localization of IAA in -N maize roots showed that a prominent IAA signal was visible at the cytoplasm while weaker signal was also localized within nuclei (Fig. 4A). Exposure of root apices to nitrate resulted in a slightly increased signal within nuclei on one hand, and in a strong immunofluorescence at the cross walls (end-poles), on the other hand, as showed in Fig. 4B. Regarding PIN1 immunolocalization, a prominent PIN1 signals was scored within nuclei in -N maize root TZ cells (Fig. 4C). In the nitrate treated roots, auxin and PIN1 seem to co-localize, since PIN1 labeling within nuclei slightly vanished while almost all end-poles were strongly enriched with PIN1. Finally, to test the polar auxin transport (PAT) via vesicle recycling of PIN1 proteins, according to the endosomal model of PAT proposed by Schlicht *et al.* (2006), we also performed maize PIN1

antibody in root samples treated with BFA. In this regard, BFA-treatments unfortunately failed in inducing BFA compartments and for this reason no images are shown.

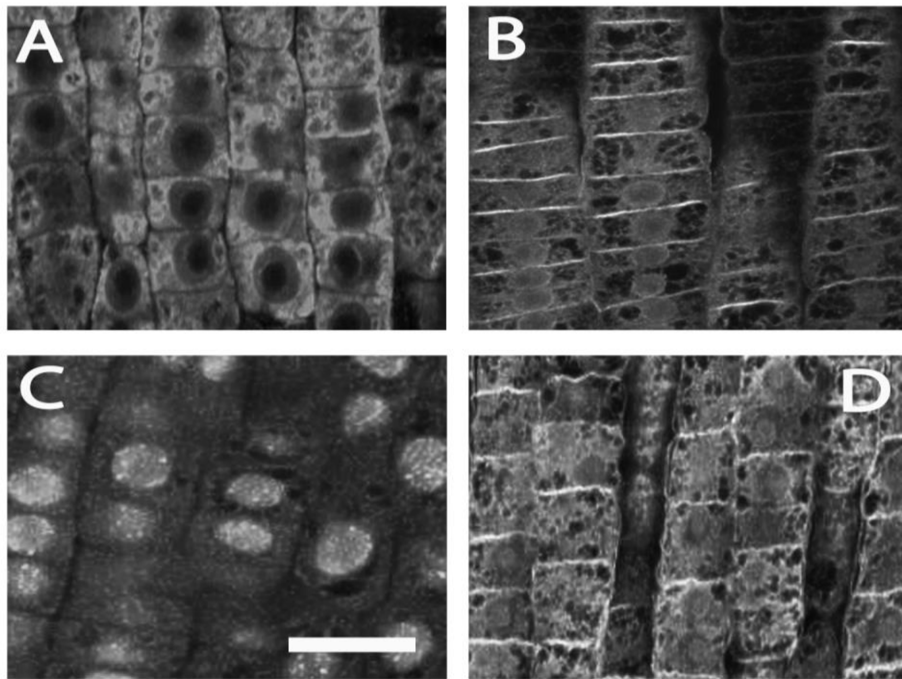


Figure 5. Immunolocalization of auxin (A-B) and PIN1 (C-D) in cells of root transition zone. A The localization of IAA in -N maize roots showed that a prominent IAA signal was visible at the cytoplasm while weaker signal was also localized within nuclei. B Exposure of root apices to nitrate resulted in a slightly increased signal within nuclei and in a strong immunofluorescence at the cross walls (end-poles). C A prominent PIN1 signals was scored within nuclei in -N maize roots. D In the nitrate treated roots, PIN1 labeling within nuclei slightly vanished while almost all end-poles were strongly enriched with PIN1. Bar in C: for C 13 µm; for A,D 16 µm; for B 24 µm.

4. Discussion

Root apex of higher plants shows very high sensibility to a number of environmental stimuli; however, the motoric responses to these stimuli do not succeed in the root apex but in the adjacent elongation zone (Baluška *et al*., 2010). This spatial discrepancy was explained after the discovery and characterization of the transition zone, which has unique role as the determiner of cell fate and root growth due to a very high degree of activity in cytoskeletal rearrangements, endocytosis and endocytic vesicle recycling, as well as, high rates of auxin fluxes (Baluška *et al*., 2010). In this scenario, the root transition zone perceives and integrates diverse external and internal inputs to translate them into motoric outputs in the elongation zone. Regarding environmental stimuli, the competence of roots to efficiently respond to different nutrient availability and to develop suitable root phenotypes is vital to allow effective soil exploitation in searching for nutrients. Here, in order to shed light on

some aspects relating to maize responses to nitrate, a series of confocal laser scanning microscope analyses by using immunofluorescence microscopy were carried out in the maize root transition zone. According to some authors (Pritchard *et al*., 1993 and Vissenberg *et al*., 2000) xyloglucan endotransglycosylase (XET), a key enzyme in XGs cleaving that plays an important role in cell wall expansion, show its highest activity in cells of root transition zone of both maize and *Arabidospis.* We used specific antibodies against XGs and Brefeldin A (BFA) drug to study nitrate responses in maize transition zone.

Xyloglucans are hemicellulosic polysaccharides, found in dicots and monocots, they locate in primary cell walls and firmly associate with cellulose microfibrils through hydrogen bonds to maintain the cell wall architecture (Fry, 1989a; Hayashi, 1989; Sonobe *et al*., 2000). In addition to such structural role, physiological and molecular studies suggested that cell wall XGs should have certain regulatory functions in elongation of the cell walls (Baluška *et al*., 2005). In contrast to cellulose, which is synthesized on the plasma membrane, XGs are synthesized in the Golgi apparatus (Moore and Staehelin, 1988) and they can be actively internalized in root apex cells since, after BFA treatment, almost all XGs were removed from cell walls into BFA-compartments, revealing a high rate of XGs recycling in the root apex cells (Baluška *et al*., 2005). Additionally to that, xyloglucans accumulated abundantly in the early cell plates of cytokinetic maize root cells (Sonobe *et al*., 2000). As suggested by these authors, one possible role of internalized cell wall XGs is to serve as a ready source of material for the rapid cell plate formation that occurs during plant cytokinesis. Beside plant cytokinesis, Baluška *et al*. (2005) suggested that these internalized cell wall XGs could be also considered as storage compartments temporarily placed within the cytoplasm, allowing rapid recruitment of ready-to-use cell wall material in situations where rapid secretion is needed, such as root cell elongation that occurs after ceasing mitotic division. In this scenario, looking for a connection between xyloglucans and nitrate is exceedingly promising, considering that the molecular and physiological effects of nitrate in modifying root system architecture are well known in many plant species, including the model plant *Arabidopsis* (Walch-Liu P and Forde, 2008; Gifford *et al*., 2013) and maize (Tian *et al*., 2005, 2008 and references therein).

Interestingly in this work, xyloglucans visualized by labelling with antibodies were very abundant, especially in maize roots cross walls (end-poles) of xylem elements and of cells in the middle and outer cortex of maize root transition zone cells (Fig. 2A).

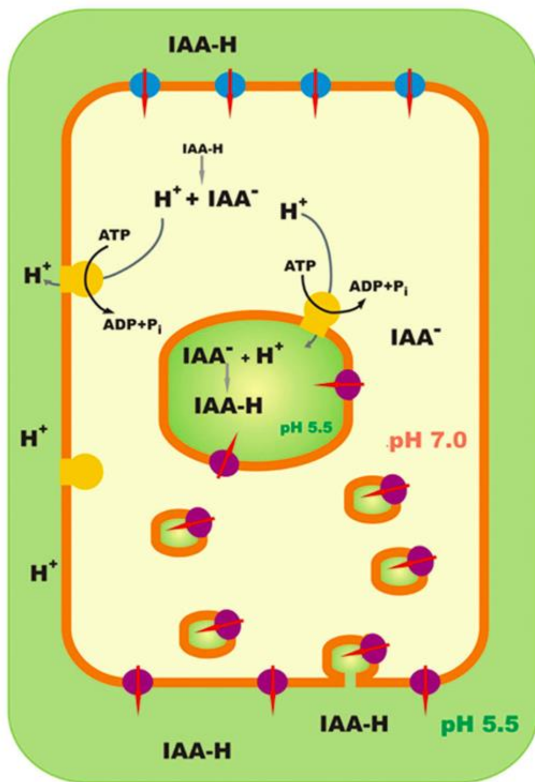
Immunofluorescence signal in fact, was generally stronger in the sample subjected to nitrate treatment (Fig. 2B), when compared to the negative control (Fig. 2C), suggesting a higher rate of XGs synthesis in response to the anion in maize root transition zone. After BFA treatment, cross walls of root transition zone cells showed very weak signal in the -N samples (Fig. 2F-H), since almost all XGs were removed from cell walls into BFA-compartments, according to BFA action (reviewed by Nebenführ *et al*., 2002; Geldner *et al*., 2003; Šamaj *et al*., 2004), which prevents vesicle formation in the exocytosis pathway while allowing endocytosis, thus resulting in the cytoplasmic accumulation of all recycling molecules. Intriguingly, BFA treatment partially failed in removing all XGs from cell walls in +N samples, since a marked immunofluorescence was still visible at cross walls (Fig. 2D-E), despite the strong effect of the drug that resulted in the abundance of BFA-compartments also within these cells (Fig. 3A). Taken together, these data open a fascinating scenario in which nitrate might act in promoting rapid cell elongation of root apex by regulating, in a mechanism as yet unknown, the synthesis or the turn-over (or both) of xyloglucans within root transition cells, and some evidences support this hypothesis.

For instance, immunolocalization of xyloglucan endotransglycosylase (XET) activity showed most prominent fluorescence in the transition zone in both maize and *Arabidopis* roots (Pritchard *et al*., 1993 and Vissenberg *et al*., 2000, respectively). Xyloglucan endotransglycosylases enzyme, as mentioned before, cleaves a xyloglucan chain (the donor substrate) endolytically and forms a covalent polysaccharide–enzyme complex (Sulová *et al*., 1998). Although the cell wall contains numerous enzymes that can modify polysaccharides, XET seem well suited to play a predominant role in cell expansion (Fry, 1995). For plant cells to expand in fact, cellulose micro-fibrils in parallel alignment need to move apart or past one another, and this movement may create the possibility for newly synthesized XG molecules to become hydrogen-bonded (Fry, 1989b). In this scenario, because XG tethers are thought to be the principal tension-bearing molecules in the cell wall, breaking of the tethers has been proposed as a mechanism for achieving reversible cell wall loosening in elongating cells without compromising strength (Fry, 1989b; Hayashi, 1989). Besides the proposed role of XETs in cell wall loosening, these enzymes may also favour integration of newly synthesized XGs into the cell wall, another necessary element for continued cell expansion. (Xu *et al*., 1996; Nishitani, 1997). In addition to that, Baluška and colleagues' data (2005) showed that cell wall pectins and xyloglucans internalize in endosome in meristem and transition zone

cells of maize root apex. According to these authors, internalize cell wall macromolecules such as xyloglucans can be related to tight control of the mechanical properties of cell walls in the transition zone. In order to maintain a loosened wall structure to enable extensive elongation after ceasing mitotic divisions, it is necessary to actively maintain low levels of pectins and XGs within cell walls. Endocytosis of pectin-xyloglucan complexes and subsequent recycling would fulfill this requirement without loss of molecules and expending energy. In this context, endocytic vesicles filled with ready-to-use cell wall macromolecules would be ideally suited to provide "building blocks" for rapid formation of cell walls in cells that have ceased mitotic division and start to elongate.

As far as the role of phytohormone auxin in regulating root system architecture in response to nitrate is concerned, a number of papers have reported a strictly regulatory connection in both the control of lateral root development and primary root growth, as summarized in the Chapter I of this work. Auxin acts as a pivotal regulator of many cellular responses crucial for plant development, including playing a key role in establishing and elaborating patterns in root meristems (Jiang and Feldman, 2003). Auxin is synthesized predominantly, even though not exclusively, in the aerial parts of plants and is redistributed within the plant body through a complex long- distance IAA transport network, mediated by IAA influx and efflux carriers (Friml, 2003; Blakeslee *et al*., 2005). These processes are essential for root cell division and elongation and, thus, for regulating root growth (Casimiro *et al*., 2001; Blilou *et al*., 2005). In this context, investigations on the root transition zone are very promising, considering that both in the model specie *A*. *thaliana* and maize transition zone cells show IAA fluxes significantly higher than those measured in the meristem and the elongation zone (Mancuso *et al*., 2005, 2007). In fact, F-actin networks at the end-poles are very abundant especially in cells of the transition zone (Baluška *et al*., 2009) and their abundance correlates closely with the amounts of auxin transported across these cell–cell adhesion domains (Schlicht *et al*., 2006). Importantly, F-actin is not essential for cell expansion in the transition zone (Baluška *et al*., 2001b), but it is critical for both endocytosis and endocytic vesicle recycling, which is inherent part of polar auxin transport (Baluška *et al*., 2008). Further studies in fact (Šamaj *et al*., 2004; Schlicht *et al*., 2006), also indicate that PIN1 proteins support IAA flux via vesicular secretion of IAA together with its transporter, since the hormone is "trapped" within the recycling vesicles together with recycling PIN1 proteins, according to the endosomal polar auxin transport

proposed by Schlicht and colleagues (Fig. 6). In this scenario, looking for a link between auxin and nitrate within the root transition zone is extremely crucial, considering the master role of the transition zone as determiner of cell fate and root growth on one hand, and the multifunctional signaling properties of IAA on the other hand. However, it remains unclear in some respects, how IAA levels in root transition zone are modulated by nitrate supply.



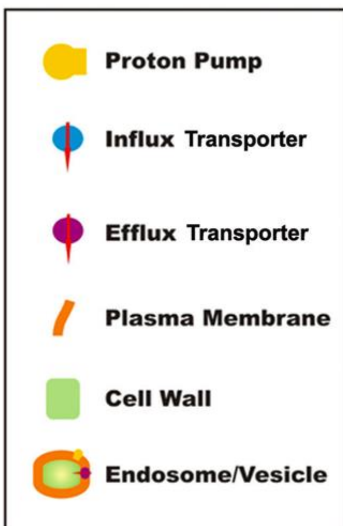


Figure 6. Schematical representation of the endosomal models for the polar auxin transport (PAT), modified from Schlicht *et al*., 2006.

In the present study, antibodies against IAA as well as against the efflux IAA carrier PIN1 were used in order to better understand these connections. As far as auxin immunolabeling is concerned, the most interesting result was obtained by observing IAA immunolocalization in response to nitrate treatments (Fig. 4B). In these root sections in fact, IAA signal was strongly localized at the cross wall (end-poles) of transition zone cells. In contrast, no cross wall labelling was detected in roots not treated with nitrate (Fig. 4A), thus suggesting that IAA end-poles labelling was probably due to increased IAA fluxes triggered specifically by nitrate. In support of this hypothesis we also observed that IAA and its transporter PIN1 protein colocalize in NO_3^--treated roots (see Fig. 4B and 4D, respectively) at the cross walls (end-poles), thus providing further, although preliminary, evidences that nitrate in the maize root transition zone is able to increase IAA-fluxes, in a mechanism as yet unknown that involved also PIN1 proteins. As mentioned before, auxin is asymmetrically distributed in the root tip, with the transition zone showing the most active zone with respect to auxin flux (Baluška *et al*., 2010). Moreover, it has been reported that a decrease in auxin concentration in roots alters cell growth and reduces root elongation (Jiang and Feldman, 2003). Taken together, the stimulatory effect of nitrate on primary root growth in maize (as reviewed in this work, see Chapter I, par. 2.4, and also confirmed by our data, see Chapter II, par. 4.2) might be explained by increasing auxin levels in the transition zone. Consistent with this hypothesis, Tian and colleagues (2008) found that primary root length showed a positive correlation with IAA content in roots in maize. These data are crucial because showed that nitrate resulted in modifying root cells elongation without affecting cell division. Very interestingly, xyloglucans turnover is correlated with auxin-induced elongation and the gene expression of XETs and XET-related (XTP) proteins are also regulated by auxin (Vissenberg *et al*., 2000 and references therein).

In conclusion, in this work we showed that cross walls (end-poles) of maize root transition zone were particularly active in response to nitrate both in the accumulation and turn-over of cell wall materials (*i.e.* xyloglucans) and in modifying auxin fluxes via PIN1-mediated IAA transport. These preliminary results collectively point to the hypothesis that nitrate-induced primary root elongation might involve either the synthesis or recycling of XGs (as BFA-treatments have revealed) or both, in order to provide “building blocks” for rapid cell wall formation required by cells to initiate elongation. In addition to that, nitrate seems to trigger increased auxin fluxes PIN1-mediated in the transition zone. However, to

better understand this latter data, IAA and PIN1 immunolabeling experiments in BFA-treated roots are needed, in order to clarify some aspects of PAT in response to nitrate. In this regard, our findings are very promising considering that at the end-poles of maize transition zone cells internalized cell wall materials, such as pectins and xyloglucans, accumulate within the same BFA-compartments as the auxin efflux carrier PIN1 (Baluška *et al*., 2005; Schlicht *et al*., 2006). Thus, future studies should focus on both endosomes and vesicular recycling in order to unravel further critical details of the polar auxin transport in response to nitrate in the root transition zone.

5. References

Baluška F, Parker JS, Barlow PW. 1992. Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays* L.). *Journal of Cell Science* **103,** 191-200.

Baluška F, Barlow PW, Lichtscheidl IK, Volkmann D. 1998. The plant cell body: a cytoskeletal tool for cellular development and morphogenesis. *Protoplasma* **202,** 1-10.

Baluška F, Volkmann D, Barlow PW. 2000. Actin-based domains of the 'cell periphery complex' and their associations with polarized 'cell bodies' in higher plants. *Plant Biology* **2,** 253-267.

Baluška F, Volkmann D, Barlow PW. 2001a. A polarity crossroad in the transition growth zone of maize root apices: cytoskeletal and developmental implications. *Journal of Plant Growth Regulation* **20,** 170-181.

Baluška F, Jasik J, Edelmann HG, Salajová T, Volkmann D. 2001b. Latrunculin B-induced plant dwarfism: plant cell elongation is F-actin-dependent. *Developmental Biology* **231,** 113-124.

Baluška F, Hlavacka A, Samaj J, Palme K, Robinson DG, Matoh T, McCurdy DW, Menzel D, Volkmann D. 2002. F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. *Plant Physiology* **130,** 422-431.

Baluška F, Wojtaszek P, Volkmann D, Barlow P. 2003. The architecture of polarized cell growth: the unique status of elongating plant cells. *BioEssays* **25,** 569-576.

Baluška F, Liners F, Hlavacka A, Schlicht M, Van Cutsem P, McCurdy DW, Menzel D. 2005. Cell wall pectins and xyloglucans are internalized into dividing root cells and accumulate within cell plates during cytokinesis. *Protoplasma* **225,** 141-155.

Baluška F, Schlicht M, Volkmann D, Mancuso S. 2008. Vesicular secretion of auxin: evidences and implications. *Plant Signaling and Behaviour* **3,** 254-256.

Baluška F, Schlicht M, Wan Y-L, Burbach C, Volkmann D. 2009. Intracellular domains and polarity in root apices: from synaptic domains to plant neurobiology. *Nova Acta Leopoldina* **96,** 103-122.

Baluška F, Mancuso S, Volkmann D, Barlow PW. 2010. Root apex transition zone: a signalling-response nexus in the root. *Trends in Plant Science* **15,** 402-408.

Baluška F, Mancuso S. 2013. Root apex transition zone as oscillatory zone. *Frontiers in Plant Science* **4,** 354.

Barlow PW, Baluška F. 2000. Cytoskeletal perspectives on root growth and morphogenesis. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **51,** 289-322.

Blakeslee JJ, Peer WA, Murphy AS. 2005**.** Auxin transport. Current Opinion in Plant Biology 8, 494-500.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* **433,** 39-44.

Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inzé D, Sandberg G, Casero PJ, Bennett M. 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. *The Plant Cell* **13,** 843-852.

Fowler J, Quatrano R. 1998. Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. *Annual Review of Cell Developmental Biology* **13,** 697-743.

Friml J. 2003. Auxin transport-shaping the plant. *Current Opinion in Plant Biology* **6,** 7-12.

Fry SC. 1989a. The structure and functions of xyloglucan. *Journal of Experimental Botany* **40,** 1-11.

Fry SC. 1989b. Cellulases, hemicellulases and auxin-stimulated growth: a possible relationship. *Physiologia Plantarum* **75,** 532-536.

Fry SC. 1995. Polysaccharide-modifying enzymes in the plant cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 497-520.

Geldner N, Friml J, Stierhof Y-D, Jürgens G, Palme K. 2001. Auxin-transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413, 425-428.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G. 2003. The *Arabidopsis* GNOM ARFGEF mediates endosomal reycling, auxin transport, and auxin-dependent plant growth. *Cell* **112,** 219-230.

Gifford ML, Dean A, Gutiérrez RA, Coruzzi GM, Birnbaum KD. 2008. Cell-specific nitrogen responses mediate developmental plasticity. *Proceedings of the National Academy of Science, USA* **105,** 803-808.

Grebe M, Friml J, Swarup R, Ljung K, Sandberg G, Terlou M, Palme K, Bennett MJ, Scheres B. 2002. Cell polarity signaling in *Arabidopsis* involves a BFA-sensitive auxin influx pathway. *Current Biology* **12,** 329-334.

Hayashi T. 1989. Xyloglucans in the Primary Cell Wall. *Annual Review of Plant Physiology and Plant Molecular Biology* **40,** 139-168.

Huang S, Ingber DE. 1999. The structural and mechanical complexity of cell growth control. *Nature Cell Biology* **1,** E131-E138.

Jiang K, Feldman LJ. 2003. Root meristem establishment and maintenance: the role of auxin. *The Journal of Plant Growth Regulation* **21,** 432-440.

Mancuso S, Marras AM, Magnus V, Baluska F. 2005. Non-invasive and continuous recordings of auxin fluxes in intact root apex with a carbon-nanotube-modified and self-referencing microelectrode. *Analytical Biochemistry* **341,** 344-351.

Mancuso S, Marras AM, Mugnai S, Schlicht M, Žárský V, Li G, Song L, Xue HW, Baluška F. 2007. Phospholipase Dζ2 drives vesicular secretion of auxin for its polar cell-cell transport in the transition zone of the root apex. *Plant Signaling and Behaviour* **2,** 240-244.

Moore PJ, Staehelin LA. 1988. Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.: implication for secretory pathways. *Planta* **174,** 433-445.

Nebenführ A, Ritzenthaler C, Robinson DG. 2002. Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiology* **130,** 1102-1108

Nishitani K. 1997. The role of endoxyloglucan transferase in the organization of plant cell walls. *International Review of Cytology* **173,** 157-206.

Pritchard J, Hetherington PR, Fry SC, Tomos AD. 1993. Xyloglucan endotransglycosylase activity, microfibril orientation and the profiles of cell wall properties along growing regions of maize roots. *Journal of Experimental Botany* **44,** 1281-1289.

Šamaj J, Baluška F, Voigt B, Schlicht M, Volkmann D, Menzel D. 2004. Endocytosis, actin cytoskeleton, and signaling. *Plant Physiology* **135,** 1150-1161.

Schlicht M, Strnad M, Scanlon MJ, Mancuso S, Hochholdinger F, Palme K, Volkmann D, Menzel D, Baluška F. 2006. Auxin immunolocalization implicates vesicular neurotransmitter-like mode of polar auxin transport in root apices. *Plant Signaling and Behavior* **1,** 122-133.

Sonobe S, Nakayama N, Shimmen T, Sone Y. 2000. Intercellular distribution of subcellular organelles revealed by antibody against xyloglucan during cell cycle in tobacco BY-2 cells. *Protoplasma* **213,** 218-227.

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Gälweiler L, Palme K, Jürgens G. 1999. Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286,** 316-318.

Sulová Z, Takáčová M, Steele NM, Fry SC, Farkaš V. 1998. Xyloglucan endotransglycosylase: Evidence for the existence of a relatively stable glycosyl–enzyme intermediate. *Biochemical Journal* **330,** 1475-1480.

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M. 2001. Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. *Genes and Development* **15,** 2648-2653.

Tian QY, Chen FJ, Zhang FS, Mi GH. 2005. Possible involvement of cytokinin in nitrate-mediated root growth in maize. *Plant and Soil* **100,** 1-12.

Tian QY, Chen FJ, Liu J, Zhang FS, Mi GH. 2008. Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. *Journal of Plant Physiology* **165,** 942-951.

Trevisan S, Manoli A, Begheldo M, Nonis A, Enna M, Vaccaro S, Caporale G, Ruperti B, Quaggiotti S. 2011. Transcriptome analysis reveals coordinated spatiotemporal regulation of haemoglobin and nitrate reductase in response to nitrate in maize roots. *New Phytologist* **192,** 338-352.

Verbelen JP, De Cnodder T, Le J, Vissenberg K, Baluška F. 2006. The root apex of *Arabidopsis thaliana* consists of four distinct zones of growth activities: meristematic zone, transition zone, fast elongation zone and growth terminating zone. *Plant Signaling and Behavior* **1,** 296-304.

Vissenberg K, Martinez-Vilchez IM, Verbelen JP, Miller JG, Fry SC. 2000. In vivo colocalization of xyloglucan endotransglycosylase activity and its donor substrate in the elongation zone of *Arabidopsis* roots. *The Plant Cell* **12,** 1229-1237.

Walch-Liu P, Forde BG. 2008. Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *The Plant Journal* **54,** 820-828.

Wojtaszek P. 2000. Genes and plant cell walls: a difficult relationship. *Biological Reviews* **75,** 437-475.

Xu W, Campbell P, Vargheese AK, Braam J. 1996. The *Arabidopsis* XET-related gene family: environmental and hormonal regulation of expression. *The Plant Journal* **9,** 879-889.

Chapter V - General conclusions

Nitrate (NO_3^-) is not only a major nutrient for plants but also acts as a signal, regulating gene expression and several physiological and developmental processes. Crops are strongly dependent on the availability of NO_3^- in soil and on the efficiency of nitrogen utilization for biomass production and yield. Roots are able to sense NO_3^- in their environment, allowing them to quickly respond to the dramatic fluctuations of its availability. However, knowledge about molecular responses to NO_3^- fluctuations mainly derives from the study of model species. Nitric oxide (NO) has been recently proposed to be implicated in plant adaptation to environment, but its exact role in the response of plants to nutritional stress is still under evaluation, as reviewed in detail in Chapter I.

Here we suggest a novel role for NO production and scavenging, thanks to the coordinate spatio-temporal expression of nitrate reductase and non-symbiotic hemoglobins, in the maize root response to nitrate. This control of NO homeostasis is preferentially accomplished by the cells of the root transition zone, which seems to represent the most nitrate responsive portion of maize root. This new signaling route seems to be an interesting case study to illustrate the master role of the root transition zone in integrating diverse inputs from exogenous and endogenous stimuli and translates them into signalling and motoric outputs as adaptive differential growth responses. Thus, we proposed here a model for the NO-mediated nitrate regulation of primary root elongation in maize, as shown in Fig. 1.

During my first part of this Ph.D. project, we demonstrated that the maize root response to NO_3^- depends, at least in part, on the control of NO homeostasis, thank to the coordinate regulation of cytosolic nitrate reductase (NR) and non-symbiotic hemoglobins (nsHbs), as discussed in Chapter II. In fact, besides being the first enzyme of nitrate assimilation, reducing NO_3^- to nitrite (NO_2^-), NR represents also one of the most important sources of NO in plants (Yu *et al*., 2014). Nitrate reductase seems to be switched to the latter reaction when high NO_2^- levels are produced (Gupta *et al*., 2011; Mur *et al*., 2013), for example, when the external nitrate rapidly increases after a nitrate starvation, thus promoting the biosynthesis of NO in response to NO_3^-. Due to its toxicity, NO is rapidly inactivated by nsHbs, a class of proteins that is well known to control developmental and physiological reactions by modulating cellular NO levels (Hill, 2012). They should be considered to be as important as NO generating enzymes in controlling *in planta* NO signalling (Mur *et al*., 2013). In this scenario, NO_3^- would seem to act as a signal to induce its

own sensing via the NR/nsHb-dependent NO fine-tuning, likely regulating down-stream root architecture adjustments (see also Chapter II, Fig. 8).

The preferential localization and the strong transcriptional responsiveness of both NR and nsHbs in the root transition zone, straightened the already hypothesized role of cells of TZ in perceiving and translating the environmental stimuli, as widely discussed in Chapter III. A number of experimental proofs suggest that the transition zone should be considered as a sort of sensory and information processing centre, enabling the growing root apex to monitor environmental parameters continuously and to trigger appropriate responses (Baluška *et al*., 2010; Baluška and Mancuso, 2013). Based on our finding, we suggest that nitrate could activate its own sensing by stimulating NO production by the transition zone cells, thus initiating a signalling pathway contributing to the physiological adaptation (*e.g.* root growth) to nitrate fluctuations.

In the case of NO-dependent nitrate regulation of root elongation, the downstream events triggering the root to elongate have still to be identified. However, cytoskeletal proteins seem to represent a highly probable molecular target for NO signal and increasing evidences place NO among the key elements in the control of several cytoskeleton-mediated processes in plants (Kasprowicz *et al*., 2009; Wang *et al*., 2009; Yao *et al*., 2012). Preliminary data showed in the last part of this work (discussed in Chapter IV) and obtained by means of immunofluorescence microscopy, point out the hypothesis that nitrate might regulate root elongation, by modulating cytoskeleton-mediated cell wall deposition and recycling in the transition zone. Additionally, also PIN1-mediated auxin accumulation seems to be affected in response to nitrate. Since it has been proposed that in the transition zone cells auxin is transported via endocytosis of IAA molecules embedded within cell wall material like pectins and xyloglucans (Baluška *et* al., 2005; Schlicht *et al*., 2006), the actin-dependent endocytosis and the organization of the actin cytoskeleton are proposed as candidates in transducing the nitrate regulation of root elongation in the transition zone. This hypothesis is also corroborated by the observation made in BFA treated roots, which showed that nitrate strongly affects the rate of xyloglucans removal into BFA-compartments, stimulating a higher recycling rate in comparison to the NO_3^--depleted roots. A schematic representations of all our finding is reported in Fig. 1.

However, a number of open questions still remain to be answered and additional microscopy experiments must be carried on to better understand the exact involvement of

auxin transport and PIN functions in this signalling pathway. In this regard, crucial confirmations could come by using NO donors/scavengers and IAA donors / inhibitors in both NO_3^--treated/untreated root apices in presence of BFA, thus allowing us to better decipher the link existing between NO_3^-, NO, auxin and cytoskeleton modifications. Furthermore, recently an untargeted approach based on both transcriptomic and proteomic analyses (data not shown) has been pursued to better characterize the overall response of the maize root transition zone to nitrate, thus enabling us to broaden the number of components involved in this scenario. The output of emerging data offers a rough snapshot of the molecular events occurring in cells of TZ after few hours of nitrate provision, thus providing an informative template on which seek for other novel components of nitrate sensing and signalling by roots.

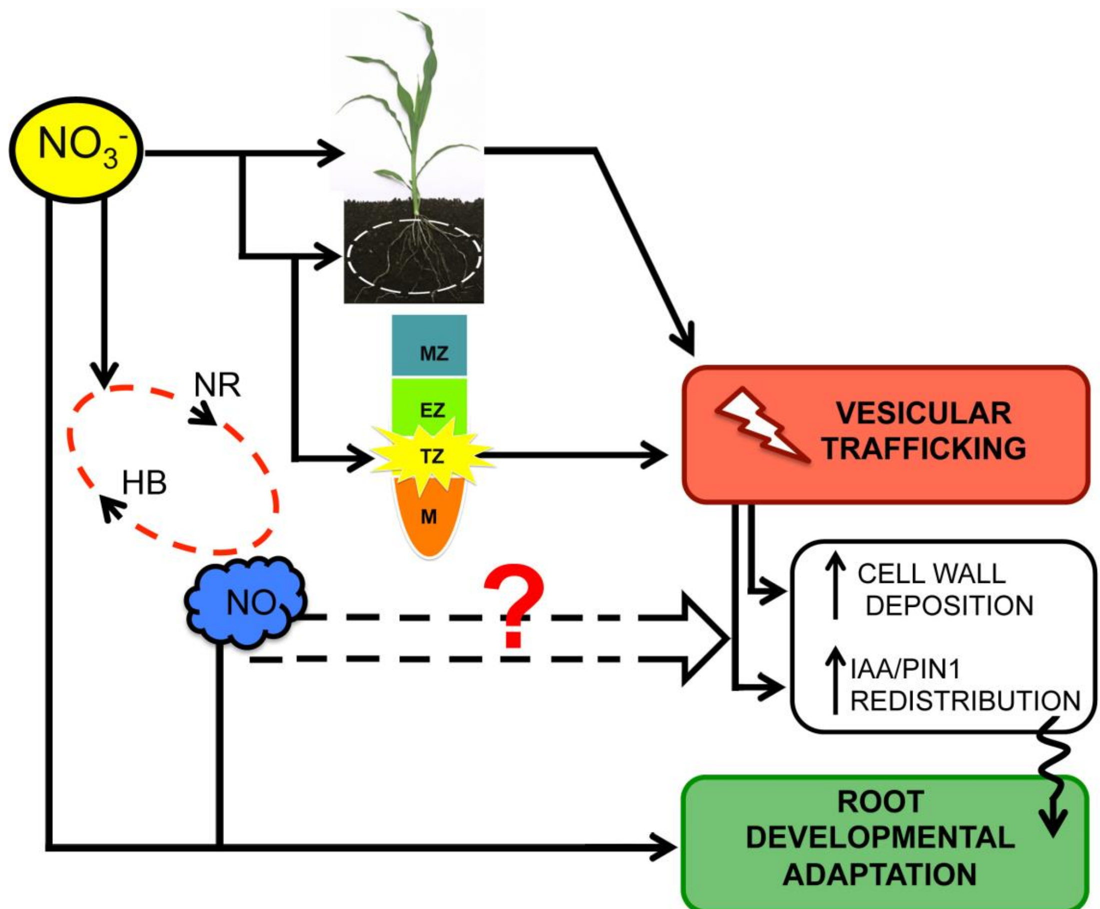


Figure 1. Schematic representation of NO-mediated nitrate regulation of maize root architecture.

References

Baluška F, Liners F, Hlavacka A, Schlicht M, Van Cutsem P, McCurdy DW, Menzel D. 2005. Cell wall pectins and xyloglucans are internalized into dividing root cells and accumulate within cell plates during cytokinesis. *Protoplasma* **225,** 141-155.

Baluška F, Mancuso S, Volkmann D, Barlow PW. 2010. Root apex transition zone: a signalling-response nexus in the root. *Trends in Plant Science* **15,** 402-408.

Baluška F, Mancuso S. 2013. Root apex transition zone as oscillatory zone. *Frontiers in Plant Science* **4,** 354.

Gupta KJ, Fernie AR, Kaiser WM, van Dongen JT. 2011. On the origins of nitric oxide. *Trends in Plant Science* **16,** 160-168.

Hill RD. 2012. Non-symbiotic haemoglobins—what's happening beyond nitric oxide scavenging? *AoB Plants*. **2012,** pls004.

Kasprowicz A, Szuba A, Volkmann D, Baluška F, Wojtaszek P. 2009. Nitric oxide modulates dynamic actin cytoskeleton and vesicle trafficking in a cell type specific manner in root apices. *Journal of Experimental Botany* **60,** 1605-1617.

Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJ, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* **5,** pls052.

Schlicht M, Strnad M, Scanlon MJ, Mancuso S, Hochholdinger F, Palme K, Volkmann D, Menzel D, Baluška F. 2006. Auxin immunolocalization implicates vesicular neurotransmitter-like mode of polar auxin transport in root apices. *Plant Signaling and Behavior* **1,** 122-133.

Wang Y, Chen T, Zhang C, Hao H, Liu P, Zheng M, Baluška F, Šamaj J, Lin J. 2009. Nitric oxide modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in Pinus bungeana pollen tubes. *New Phytologist* **182,** 851-862.

Yao LL, Pei BL, Zhou Q, Li YZ. 2012. NO serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton during responses to VD-toxins in *Arabidopsis*. *Plant Signaling and Behaviour* **7,** 174-177.

Yu M, Lamattina L, Spoel SH, Loake GJ. 2014. Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytologist* **202,** 1142-1156.