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

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1	NO homeostasis is a key regulator of early nitrate perception and root elongation				
2	in maize				
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1 Abstract

2 Crop plant development is strongly dependent on the nitrogen availability in the soil 3 and on the efficiency of its recruitment by roots. For this reason, the understanding of 4 the molecular events underlying the root adaptation to nitrogen fluctuations is a primary 5 goal to develop biotechnological tools for sustainable agriculture. However, knowledge 6 about molecular responses to nitrogen availability derives mainly from the study of 7 model species.

8 Nitric oxide (NO) has been recently proposed to be implicated in plant response to 9 environmental stresses, but its exact role in the response of plants to nutritional stress is 10 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 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.
This system, allowing to perform localized treatments on specific root portions, gave us
the opportunity to discern between localized and systemic effects of nitrate supply to
roots.

23

Keywords: Maize, Nitrate (NO₃⁻), Nitrate reductase (NR), Nitric oxide (NO), Nshaemoglobin (Hb), Root, Transition zone (TZ).

26

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 (Na₂WO₄•2H₂O; Tungstate).

31

32 Introduction

Soil nutrient 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
leaching of nutrients 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.

8 The macronutrient nitrogen is essential for plant growth and development as it is a 9 component of proteins, nucleic acids and many co-factors and secondary metabolites. In 10 aerobic soils nitrate is the major source of nitrogen for most plant species (Ahmad *et al.*, 11 2007; Nischal *et al.*, 2012).

12 Plants have the potential for adaptation to dramatic fluctuations of nitrogen availability 13 by modulating their capacity for nutrient acquisition and by alteration of whole-plant 14 morphology and metabolism, such as increasing the root/shoot ratio (Rubio et al., 2009). 15 Developmental adaptive mechanisms stimulate growth in organs that directly participate 16 in nutrient acquisition, such as primary roots (Walch-Liu and Forde, 2008). A dual 17 effect of external nitrate on root system architecture (RSA) development has been 18 depicted in the model species Arabidopsis thaliana: (i) a systemic inhibition of lateral 19 primordia by uniformly high nitrate concentrations at a post-emergence stage and (ii) a 20 localized stimulation of elongation on N-starved roots at the site of contact with a 21 nitrate rich supply, known as the foraging capacity (Zhang and Forde, 1998; Zhang et 22 al., 1999; Linkohr et al., 2002; Zhang et al., 2007; Ruffel et al., 2011). Apart from a 23 few known pathways that involve transcription factors, micro-RNAs, hormonal signals 24 and, more recently, nitrate transporters with dual affinity for nitrate and auxin (Little et 25 al., 2005; Remans et al., 2006; Miller et al., 2007; Chiou, 2007; Gifford et al., 2008; 26 Krouk et al., 2010; 2011; Vidal et al., 2010; Castaings et al., 2011; Rubio-Somoza and 27 Weigel, 2011; Ruffel et al., 2011; Trevisan et al., 2012; Xu et al., 2011), our 28 understanding of sensing external nitrate conditions and of the signal transduction 29 system that leads to an altered development of roots 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; Gojon et al., 2010). Significant advances have been made during the recent period concerning the molecular mechanisms of NO₃⁻ sensing and signalling in *Arabidopsis*, 1 and the striking action of NO_3^- as a signal in regulating genome expression has been 2 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.

18 NO is produced in plant tissues by two major pathways, one enzymatic and the other 19 non enzymatic (Wendehenne et al., 2004). The NO-producing enzymes identified in 20 plants are nitrate reductase (NR), and several NO synthase-like proteins, including one 21 localized in peroxisomes which has been biochemically characterized (del Rio et al., 22 2004). Interestingly, it was recently shown that non-symbiotic haemoglobin 1 enzyme 23 could reduce NO_2^{-1} to NO with a constant rate that was far in excess of that reported for 24 haemoglobins (Hbs) (Sturms et al., 2011). Plant Hbs are able to regulate several NO 25 effects, as recently reviewed by Hill (2012). Class II nsHbs contributes to NO removal 26 when over-expressed (Hebelstrup et al., 2006; 2012). Moreover, several studies have 27 demonstrated a role for plant Hbs in catalysing the turnover of nitric oxide to nitrate 28 (Dordas et al., 2003a; 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.

6 The present study is focused on both the characterization of the expression profiles of 7 selected genes putatively involved in nitric oxide homeostasis and the determination of 8 NO production by roots in response to different N treatments. In addition, since the 9 genes therein selected have proved to be very good candidates for monitoring nitrate 10 sensing in maize roots, we propose them as early physio-molecular markers for the 11 response to this anion. Furthermore, the effect of nitrate on root growth and especially 12 on root elongation was also deepened. Finally, an improved agar-plate culture system 13 for studying the Zea mays L. root response to nutrients has been developed. Thanks to 14 this system it has been possible to discriminate between localized and systemic effects 15 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 its steady-state level. The control of the NO production by the synergic action of NR and nsHbs would seem, moreover, to participate to the complex signalling network involved in the modulation of the root growth in response to nitrate.

21

22 Materials and Methods

23 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. Nitrate, ammonium or ammonium nitrate were supplied at a concentration of 1 mM. In the nitrogen depleted nutrient solution KNO₃ was replaced by 1 mM KCl and NH₄SO₄ by MgSO₄, respectively.

30 For nitric oxide content measurement, for subsequent expression analyses and for the

31 analysis of root elongation rate, seedlings were grown only 24 h in the nutrient solution,

32 to allow the manipulation of younger roots. To deepen the role of NO in the maize root

33 response to nitrate Tungstate (1 mM), cPTIO (1 mM), L-NAME (0.2 mM), SNP (0.01

1 mM) and NOR (1 mM) were supplied to the nutrient solution (either NO_3^- -supplied or 2 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 Baluška *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 (-NO₃⁻).

10

11 Growth of maize seedlings in agar medium

12 An improved method was developed to grow maize seedlings on agar. To this aim 13 specific plastic boxes (17.9 x 12.9 x 2.6 cm) modified with suitable holes on one side 14 were utilized (Figure S1). This system permits the insertion of young roots, which can 15 grown vertically along the agar medium allowing the shoot to develop outside of the 16 box, and enabled us to perform localized treatments to single portion of roots, as 17 described in Fig. 2. The agar concentration utilized was 1%, after a preliminary test with 18 concentrations ranging from 0.8 to 1.2%. The nutrients were supplied as indicated for 19 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.

27

28 Morphological analyses

For the analysis with WinRhizo, germinated seeds of maize inbred line B73 were transferred to 2-1-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 (cm²), 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).

4 For the analysis of primary root elongation rate, seedlings were grown 24 h in a 500-ml 5 beaker and subjected to six different treatments according to the growing medium, as 6 follows: a) $+NO_3^{-}$, b) $+NO_3^{-}$ +cPTIO, c) $+NO_3^{-}$ +Tungstate, d) $-NO_3^{-}$, e) $-NO_3^{-}$ +SNP, f) 7 $+NH_4^+$. The measures of primary root length were made with a ruler on sixteen 8 seedlings for each group, in four independent biological repetitions. To investigate 9 possible effects of toxicity due to the use of chemicals, both total root weight and leaf 10 weight were also measured. Statistical analyses were performed by using R software 11 (version 2.14.2).

12

13 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 RNAsefree DNAse (Promega, Milano, Italy) as described by Falchi *et al.* (2010). One μ l 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 μ l of Oligo dT 10 μ M as described by Manoli *et al.* (2012).

23

24 Selection of genes to be evaluated, maize sequences identification and primer 25 design

26 The list of genes analyzed is reported in Table S1, together with the primers utilized for 27 RT-qPCR expression analysis. They were chosen according to previously published 28 results (Trevisan et al., 2011; 2012). The Hb (NCBI: AF236080.1), the NR1 (NCBI: 29 AF153448.1) were then chosen for further more detailed analysis and the analysis was 30 extended to the expression of Hb2 (NCBI: NM_001112349.1), NiR (NCBI: 31 ACG29734.1), NOA1 (NCBI: NM_001174573) genes which were selected by screening 32 the B73 genome database (http://www.maizesequence.org/index.html) and to NRT2.1 33 (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.

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

6

7 Real time qPCR

8 Relative quantification of transcripts by Real-Time PCR (RT-qPCR) was performed in 9 a StepOne Real-Time PCR System (Applied Biosystems, Monza, Italy) as described by 10 Nonis et al. (2007). Experiments were conducted using SYBR Green chemistry 11 (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions. For 12 each reaction 2.5 ng of retrotranscribed RNA were used as template. Three technical 13 replicates were performed on six independent biological replicates using the conditions 14 described by Trevisan et al. (2011). Melting curve analysis was performed to confirm 15 the absence of multiple products or primer dimers formation. Data were exported and 16 analyzed according to the Livak and Schmittgen (2001) method using LUG (leunig 17 5'-TCCAGTGCTACAGGGAAGGT 5'primers, forward and reverse 18 GTTAGTTCTTGAGCCCACGC) and *MEP* (Membrane protein PB1A10.07c, primers: 19 forward 5'-TGTACTCGGCAATGCTCTTG and reverse 5'-20 TTTGATGCTCCAGGCTTACC) as reference genes according to Manoli et al. (2012). 21 For each transcript, the ratio between the expression measured for a given treatment and 22 that of its own control was used to estimate up or down-regulation of genes. The ratios 23 obtained were then expressed as base-2 logarithm to build the graphs.

24

25 NO detection

26 Germinated seeds were transferred to a nitrogen-depleted nutrient solution, and after 24 27 h root apices of 2 cm length ca. were excised and incubated for 30 min in 2 ml detection 28 buffer (10 mM Tris-HCl, pH 7.4) added with 15 μ M of DAF-2DA. Subsequently the 29 apices were washed twice for 5 min with fresh detection buffer and placed on a 30 microscope slide fixed with a Secure-Seal[™] hybridization chamber gasket (Life 31 Technologies, Carlsbad, CA, USA) (20-mm diameter, 0.8-mm deep) and analysed for 32 NO production by stereo- and confocal microscopy. For each chamber one apex was 33 incubated as described below.

1 For stereomicroscope analyses the chambers were immediately filled with nutrient 2 solution containing 1 mM KNO₃ (+NO₃⁻), or nitrogen-depleted nutrient solution containing 1 mM KCl (negative control, -NO3⁻), and examined by epi-fluorescence with 3 4 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 5 6 Adobe Photoshop CS4 (Adobe, San Jose, CA, USA). 7 Confocal NO measurements were carried out filling the chambers alternatively with: a) 8 the $+NO_3$ solution; b) $-NO_3$ solution; c) $+NO_3$ nutrient solution supplied with the NO 9 scavenger cPTIO; d) $-NO_3$ nutrient solution supplemented with the NO donor NOR-3;

e) +NO₃⁻ solution with sodium tungstate. The incubation in DAF-2DA was carried out
as previously described.

12 All apices were observed with a Leica TCS-SP2 confocal microscope (Leica 13 Microsystems CMS, Mannheim, Germany) and images were acquired every five min 14 for 45 min from the beginning of the incubation. Images were then analysed using the 15 Leica Confocal Software application. Normalization of the data and ratios of average 16 fluorescence intensities were calculated as described by Calcagno *et al.* (2012). Five 17 root pieces were tested for each condition and five independent repeats were analyzed 18 for each treatment.

19

20 Results

21 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 a specific nitrate responsiveness.

- 31 The expression of the same set of genes was also assessed on root and leaf tissues of
- 32 five-days old seedlings, but after only 30 min, 2 and 6 h of nitrate/ammonium provision
- 33 or depletion. The time-course of the expression of the five nitrate specific targets in both

1 roots and leaves after few hours of nitrate/ammonium supply/starvation is shown in Fig.

2 4 (the expression patterns of the other genes tested is reported in Figure S2).

3 The nitrate supply induced a significant increase of transcript accumulation for all the 4 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 NO₃⁻ supply, to 8-5 6 >100 fold after six hours). Conversely, the transcription of the five genes did not show a 7 similar increase when ammonium was supplied as unique nitrogen form, neither in roots 8 nor in leaves (Fig. 4A and 4B, lower side), confirming the specificity of responsiveness 9 to nitrate. Also in the case of N-deprivation all five genes displayed a more evident 10 response (decrease of expression) to nitrate deprivation in comparison to that measured 11 for ammonium removal (Fig. 4A and 4B, left column), both in leaves and in roots. 12 These five genes specifically nitrate inducible were thus selected for the subsequent and

- 13 more detailed expression analyses.
- 14

15 Root growth responds specifically to nitrate availability

16 The effect of nitrate supply on root development was evaluated in comparison to that of 17 both ammonium and NO₃NH₄ in plants grown in nutrient solution for five days (Table 1 18 and Fig. S4). The analysis of root length, root surface area and number of tips evidenced 19 a similar pattern, showing the strongest root growth stimulation in seedlings grown with 20 nitrate 1 mM (treatment 1). Values measured for these three parameters in plants grown 21 with ammonium (treatment 3) were significantly lower (50-60%) than those observed 22 for nitrate-supplied roots and closest to rates observed for NO₃-depleted roots 23 (treatment 2, nitrate negative control). Furthermore, an inhibitory effect of ammonium 24 supply was visible for both root length and tips number, which showed values even 25 lower with respect to negative control (treatment 4). The supply of NO_3NH_4 (treatment 26 5) slightly stimulated these three parameters, even if to a significantly lower extent with 27 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 nitratedepleted roots (treatment 2). These observations, besides 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. 1

2 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.

5 Seedlings grown for 24 h without nitrate, were supplied with 1mM nitrate and the 6 fluorescence produced was observed (Fig. 5A, panels I-P) in comparison to that 7 measured in negative control (Fig. 5A, panels A-H). The nitrate supply caused a slight 8 but consistent increase in DAF fluorescence since the first minutes after treatment 9 (panels J and K). No fluorescence increase was induced by NO_3 -deprived control 10 treatments, where by contrast a signal decrease was observed after ten minutes, 11 probably due to the decay of the probe (panels A-H). Based on these observations the 12 increment of fluorescence was mainly localized immediately above the meristematic 13 apex and more precisely in the transition zone, as defined by Verbelen et al. (2006) and 14 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 shows a difference between the two treatments, with a strong increase in the DAF fluorescence in response to nitrate provision (panel D), that was not observed in the case of negative control ($-NO_3^-$ roots) (panel C).

Moreover, higher magnification analyses (Figure 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

- 1 fluorescence did not increase throughout the experiment. These results globally suggest
- 2 that a NR-dependent NO burst occurred immediately after nitrate supply to roots.
- 3

4 Genes putatively involved in the control of NO homeostasis are involved in the 5 early response to nitrate

6 Due to the size of the mini-chamber utilized for both stereomicroscope and confocal 7 analyses, it was necessary to work with roots sampled from younger seedlings. For this 8 reason we decided to shift the experimental plan to younger seedlings also for the 9 following expression analyses and to focus only on the early events after nitrate 10 provision. Plants were, thus, grown 24 h in a $-NO_3^-$ solution and then transferred to a 11 $+NO_3$ medium for two hours. The transcript accumulation of the previously selected 12 genes (NR1, Hb, Hb2, NiR) together with a new one (NOA1) encoding a putative 13 AtNOA1 orthologous was examined after nitrate supply and in the presence of cPTIO, 14 tungstate and L-NAME. The expression of NRT2.1 was also included among the 15 analyses, as a positive control of the nitrate perception.

16 The nitrate addition induced strong increments of transcription for all the genes 17 analyzed, except for *NOA1* (Fig. 6, first two columns of each gene). The expression of 18 nitrate reductase gene reached rates six/nine fold higher in comparison to that measured 19 in $-NO_3^-$ roots, whereas the two isoforms of ns-haemoglobin increased their 20 transcription even by 27-72 fold. The *NiR* and the *NRT2*.1 showed an induction of their 21 expression of 21 and six fold respectively.

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.

33

1 The transcription for genes involved in NO production and scavenging is 2 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 nitratedepleted roots and after 2 h of nitrate provision.

10 All the five genes considered evidenced a significant change of localization when 11 seedlings grown without nitrate were treated with the anion (Fig. 7B). In fact, in nitrate-12 starved root (left columns of Fig. 7B) the 70-80% of the mRNA was concentrated in the 13 meristematic cells (M) for all five genes, with the remaining 20-30% of mRNAs 14 prevalently localized in the elongation (EZ) and maturation zones (MZ). In these 15 conditions the amount of transcript detected at the transition zone level (TZ) was 16 extremely low or even negligible. On the contrary, in seedlings supplied with 1 mM 17 nitrate for two hours (after being grown 24 h in a $-NO_3^-$ solution), the transcripts of all 18 five genes were more equally distributed between the apical meristem (M) and the 19 transition zone (TZ), with a significant increase of accumulation in the TZ which 20 showed an amount of mRNA for each gene ranging from 20 to 40% of the total. 21 Moreover, after nitrate supply the maturation zone also evidenced an increase in terms 22 of gene expression if compared with nitrate-depleted roots.

23 Fig. 7C describes the increases of transcription for each gene in each of the four 24 portions, independently from their relative abundance. All five genes evidenced an 25 induction of their expression in all the four portions sampled, with the maximum in the 26 TZ, that showed a transcription rate more then 30 times higher if compared with that 27 measured in the same portion of nitrate-depleted roots (except the case of NRT2.1 that 28 increased more then 10 times). In the MZ and EZ of nitrate-supplied roots the amount 29 of mRNAs increased by around 8-20 and 4-8 times respectively. On the contrary, in the 30 meristematic cells the increase of gene transcription measured was very low or 31 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.

1 2

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

3 After germination seedlings were transferred to six different nutrient solutions $(+NO_3^-, -$ 4 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 5 6 and $-NO_3$ +SNP-seedlings showed the more elevated rate of root elongation, with 7 values significantly higher in comparison to all the remaining treatments. The supply of 8 ammonium did not produce any increase in the elongation rate, which was similar to 9 that measured for negative control plants, as already observed also for older seedlings. 10 The provision of tungstate together with nitrate inhibited even more significantly the 11 root growth in comparison with nitrate-depleted roots. A similar decrease was also 12 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 13 14 measured for positive control. Conversely, as also observed in the case of gene 15 transcription, the supply of L-NAME, that inhibits the NOS activity, did not produce 16 significant effects on root lengthening.

17 These results besides suggesting the involvement of NO in the regulation of nitrate 18 induced root elongation, clearly confirm the key role of nitrate reductase for this 19 signalling pathway.

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

22

23 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 between local and systemic effects on gene expression.

As a preliminary experiment, to test the validity of this method as an alternative to hydroponics, 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. RTqPCR 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 (Fig. S4), further confirming the validity of this method.

6 Seedlings were then submitted to a treatment with nitrate localized only to the 7 meristematic apex (4 mm, for details see the M&M). The transcription of the five genes 8 previously chosen was evaluated independently in the four different root zones (M, TZ, 9 EZ, MZ) in both seedlings whose tip was treated with 1 mM nitrate and negative control 10 (Fig. 9B). NR1, Hb1, Hb2 strongly increased their expression in apex of nitrate supplied 11 roots, whereas NiR transcription increased to a lesser extent. On the contrary, the 12 mRNA abundance of NRT2.1 did not evidence any increase, indicating that this high 13 affinity nitrate transporter is not involved in the influx of nitrate by root meristem. 14 Furthermore, in all the other three root zones (TZ, EZ and MZ) no differences in terms 15 of transcript accumulation were detected after local nitrate provision to apex, suggesting 16 that the NO signalling activation by nitrate should represent a localized effect of nitrate.

17

18 Discussion

19 Nitrogen is a major element for plant life and crops strongly depend on intense 20 fertilization programmes throughout the world, thus affecting environment quality. The 21 identification of crop cultivars with improved nutrient acquisition efficiency in low-22 input farming systems continues to be a real priority for plant scientists (Robertson and 23 Vitousek, 2009; Xu *et al.*, 2012).

Nitrate is the main nitrogen source for plants in regular agricultural systems and, acting
also as a signal, triggers a number of molecular and physiological events leading to the
overall plant's response to its availability (Gojon *et al.*, 2010 and references therein).

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

1 deprivation (Fig. 3). This first screening allowed us to focus later in this work only on 2 genes responding exclusively to nitrate (and not to ammonium), which coincided with 3 those involved in the control of NO biosynthesis and scavenging. In particular, genes 4 encoding the cytosolic nitrate reductase and two different ns-haemoglobins, together 5 with a gene encoding nitrite reductase evidenced both in short-term and long-term 6 experiments a clear and noticeable responsiveness to nitrate supply or starvation, but did 7 not change their expression in response to ammonium (Fig. 3 and 4). A gene encoding a 8 high affinity root nitrate transporter was also used as internal control, in light of its 9 putative role in the nitrate influx and of its transcriptional inducibility during the first 10 phases of nitrate supply (Quaggiotti et al., 2003). The expression profile recovered for 11 this gene provided indirect evidence of the entry of nitrate into the root epidermal cells, 12 hence enabling the activation of the signalling pathways in which nitrate is involved.

13 Besides being the first enzyme of nitrate assimilation, NR represents also one of the 14 most important sources of NO in plants (Mur et al., 2012). It is a cytosolic enzyme that 15 could both reduce nitrate to nitrite and nitrite to nitric oxide, even if it shows a better 16 affinity for nitrate than for nitrite. However, NR seems to be switched to the latter 17 reaction when high nitrite levels are produced (Gupta et al., 2011; Mur et al., 2012). 18 This occurs, for example, when the external nitrate rapidly increases after a nitrate 19 starvation leading, as a consequence, to a strong increase of the NO₃⁻ influx inside cells, 20 as it might be happened in this case study. Once inside the root cells, nitrate is promptly 21 converted to nitrite by NR leading to nitrite accumulation. Besides serving as substrate 22 for NiR, nitrite accumulation could also shift the NR equilibrium toward its second 23 mode of action, promoting thus the biosynthesis of nitric oxide in response to nitrate. 24 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

1 studies have indicated a role for haemoglobins in the detoxification from high 2 intracellular NO concentrations (Dordas et al., 2003a, b; Perazzolli et al., 2004; Vieweg 3 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. 4 5 (2004) provided evidence that Arabidopsis non-symbiotic haemoglobin AtHb1 6 functions as a NO-dioxygenase, metabolizing NO to nitrate. Moreover plant 7 haemoglobins seem to be involved in the control of NO accumulation during rhizobial 8 and mycorrhizal symbioses (Vieweg et al., 2005) and in the response to hypoxia in 9 different tissues such as seeds, roots, and stem tissue (Dordas et al., 2003a, b). Plant 10 Hbs can control developmental and physiological reactions by modulating cellular NO 11 levels (Hill 2012) and should be considered to be as important as NO generation in 12 regulating in planta NO signalling (Mur et al., 2012).

13 Our results, besides confirming the already hypothesised involvement of nitric oxide 14 control homeostasis in the maize root response to nitrogen (Trevisan et al., 2011), 15 demonstrate also that this is an exclusive prerogative of NO_3 -signalling. In fact, when 16 ammonium was supplied to nutrient solution as the sole nitrogen source, no significant 17 effects were measured on the transcription of genes involved in the NO production and 18 scavenging. On the contrary, the expression of the other genes here analyzed did not 19 show a similar specific nitrate responsiveness. In addition, data obtained by analyzing 20 root morphological parameters by the WinRhizo software highlighted the same 21 specificity of nitrate, which significantly affected root growth when supplied to N-22 deprived roots, in contrast to what happens when the same concentration of ammonium 23 is given to roots (table 1).

24 According to these results it would seem that nitric oxide may be produced by roots as 25 an early signal of nitrate perception. To deepen this hypothesis an *in vivo* NO detection 26 was carried out. Results obtained by using the DAF-2DA probe (Kojima et al., 1998) at 27 both stereo- and confocal microscope evidenced a clear induction of fluorescence after 28 nitrate provision (Fig. 5). The main zone of NO production seems to be located 29 immediately above the meristematic apex. A similar localization has been recently 30 observed in this same species by Mugnai et al. (2012) as a response to hypoxic 31 conditions.

The fluorescence detected after nitrate supply was not relieved in the presence of tungstate, giving support to the role of nitrate reductase in this process. Moreover, also the addition of cPTIO suppressed the development of fluorescence, 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 nitratedepleted roots. 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.

6 The NR-dependent NO production observed after nitrate supply, was then corroborated 7 by the expression analyses performed on roots of one day olds seedlings (Fig. 6). In 8 particular, our results proved the strong induction of NR1, NiR and nsHbs transcription 9 in the early phases of nitrate perception. As also observed in the case of NO production, 10 the transcription of all genes was significantly inhibited after tungstate and cPTIO 11 addition, confirming the cooperation between nitrate reductase and haemoglobin 12 activities in the fine tuning control of NO homeostasis. However, to exclude the 13 possible involvement of sources of NO other than NR, the study was extended also to 14 the orthologous of the Arabidopsis NOA1 (Guo et al., 2003) encoding the Nitric Oxide 15 Associated 1 protein (Zemojtel et al., 2006). NOA1 was previously named AtNOS1 and 16 it has been described as a potential nitric-oxide synthase (NOS) in Arabidopsis thaliana, 17 despite lack of sequence similarity to animal NOSs. It has been, successively, 18 established to be a GTPase (Moreau et al., 2008) and not to possess NOS activity and 19 for this reason it has been renamed AtNOA1. Previous studies have shown that NOA1-20 dependent NO synthesis is involved in hormonal signaling, stomatal movement, 21 flowering, pathogen defence, and oxidative stress (Guo et al., 2003; He et al., 2004; 22 Zeidler et al., 2004; Zhao et al., 2007). The transcription of the AtNOA1 orthologous in 23 maize did not evidence any alteration neither in response to nitrate nor to the other 24 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 (Fig. 6 and 8), 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 analyzed in four different root zones (M, TZ, EZ, MZ) both in nitratedepleted and in nitrate-treated (1 mM) seedlings (Fig. 7). 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), that is located between the meristem (M) and the region of fast cell elongation (EZ).

5 Cells of the TZ undergo a series of fundamental changes in their cytoarchitecture and 6 physiology, and accomplish dramatic rearrangements of the actin cytoskeleton (Baluška 7 et al., 1997; 2001). This is essential for the developmental switch into rapidly 8 elongating root cells which expand strictly uniaxially (Baluška et al., 1997). The distal 9 part of this zone in characterized by a prevalence of cells that optionally can re-enter the 10 cell cycle, whereas the proximal part is equipped with cells competent to rapidly enter 11 into the fast cell elongation zone. As this developmental passage of cells can be 12 differentially regulated at the opposite root flanks, this unique zone provides the root 13 apices with an effective mechanism to re-orientate growth in response to environmental 14 stimuli (Verbelen et al., 2006). A number of experimental proofs suggest that the TZ 15 should be considered as a sort of sensory and information processing zone, enabling the 16 growing root apex to monitor environmental parameters continuously and to trigger 17 appropriate responses (Mugnai et al., 2012). If this is true and hypothesizing a role for 18 NO homeostasis control through the combined action of NR and nsHB in the early 19 perception of nitrate by roots, our results on transcript accumulation re-distribution 20 along root apex are not surprising. Based on our finding it would seem that nitrate 21 supply could activate its own sensing by stimulating the NO production by the TZ cells, 22 thus initiating a signalling pathway contributing to the physiological adaptation (e.g. 23 root growth) to nitrate fluctuations.

24 The most important example of the plasticity that plant express to fit with nutrients 25 withdrawal in soil is, in fact, represented by the capability of rearranging root 26 architecture to maximize their capture (López-Bucio et al., 2003; Hermans et al., 2006; 27 Zhang et al., 2007; Zolla et al., 2010; Giehl et al., 2012; De Pessemier et al., 2013). 28 Nitrate affects root development by finely regulating the growth of lateral roots 29 depending on its external concentration and localization, as described above (Péret et al., 30 2009; Mounier et al., 2013; Yu et al., 2013) and as also showed by our findings 31 obtained with the WinRhizo software (Table 1). 32 Based on our preliminary results showing the preferential localization of NO production

32 at the level of the transition zone, we decided to focus on nitrate effects on root 34 elongation, which takes place in the zone immediately above and neighbouring the TZ 1 (Fig. 8). Our results evidenced a strong and specific induction of root elongation of 2 young maize seedlings supplied with 1 mM nitrate and a drastic inhibition in the 3 presence of ammonium, cPTIO and tungstate. No effects were recorded in the presence 4 of L-NAME. On the contrary, when the negative control ($-NO_3^-$) was supplied with a 5 NO donor (SNP) the root length increased significantly. These results strongly suggest 6 that the NO generated through nitrate reductase should significantly contribute to the 7 root lengthening noticed after nitrate provision.

8 The involvement of NO in root development has been observed in numerous studies, as 9 for example those published by the Lamattina group (Pagnussat et al., 2002; Pagnussat 10 et al., 2003; Corre-Aragunde et al., 2004; Lombardo et al., 2006), but it had already 11 been hypothesised in 1997 by Gouêva et al., who found that NO was able to induce cell 12 elongation in a way similar to auxin. Moreover, a recent study suggested that class-2 13 non-symbiotic hemoglobins play a role in regulating the synthesis and transport of 14 auxins by altering the level of the signal molecule, NO, in specific cells (Elhiti et al., 15 2013).

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.

20 Considering also that NO is widely implicated in the plant response to environmental 21 stresses (Beligni and Lamattina 2001; Dat *et al.*, 2004), it seems to play crucial 22 functions in at the cross-roads between developmental and abiotic stress tolerance. For 23 this reason, it should also represent a very good molecular candidate to regulate root 24 development in response to abiotic stresses, as for example nutrients or oxygen 25 deprivation (Mugnai *et al.*, 2012), but also an early player in symbiotic interactions 26 establishment, which also need root architecture to be adapted to the environment.

27 In the present research, thanks to the set-up of a method allowing to grow maize 28 seedlings in vertical plates with an agar medium, some major details on NO-mediated 29 nitrate signalling have been attained. Our results suggest that the mechanism underling 30 the root response to nitrate and involving NO signalling is directly activated on cells 31 which enter in contact with external nitrate (Fig. 9). Moreover, this alert system does 32 not seem to be turned on by some nitrate derived compounds or by the nitrate that move 33 up through the root. In fact, when only the meristematic apex was treated with nitrate, 34 the induction of the transcription of NR1 and Hb was exclusively restricted to the apex 1 itself, whereas in the upper zone of the roots no differences were detected in comparison 2 with the negative control. This is even more remarkably considering that, conversely, 3 when the entire root gets in touch with nitrate, the apex is the portion that show the 4 lower responsiveness to this anion in terms of induction of gene expression, being 5 instead the transition zone the most receptive.

6 Moreover, these results indicate that nitrate transporters other than NRT2.1 should be 7 implicated in the nitrate perception at the root meristem, since the transcription of the 8 gene encoding *NRT2.1* is not activated at all by nitrate, in contrast to what observed in 9 all the other three root zones when the whole root was supplied with nitrate. Basing on 10 these data, it would seem that the NO mediated pathway here described represents an 11 early alert system for external nitrate sensing by root cells, which seem to individually 12 posses the competence to activate this pathway when external nitrate is perceived.

13 Since root growth is modulated by the convergence of multiple environmental inputs 14 which are integrated by specific signal pathways to decide how to explore the 15 surrounding environment, additional experiments will be needed to better understand 16 the functioning of this NO-mediated pathways and to identify the downstream events 17 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 the rapid increase of nitrate availability and that it could mediate the root elongation observed after nitrate provision (Fig. 10).

23

24 Supplementary material

25 Supplementary data are available at *JXB* on line.

Supplementary Fig. S1. The improved agar-plate culture system for studying the Zea
 mays L. root response to different nutrients availability.

28 Supplementary Fig. S2. Time course of the expression of genes following short-term

29 nitrate/ammonium treatments in maize roots and leaves.

30 Supplementary Fig. S3. Confocal detection of DAF-2T in the transition zone of nitrate

31 treated apices.

32 Supplementary Fig. S4. Root and leaf fresh weight and relative root/shoot ratio in

33 seedlings grown in nutrient solution for five days (A, B, C).

21

1 Total root length (L), total surface area (SA), average diameter (AD), number of root 2 tips and leaf fresh weight in seedlings grown five days in agar medium containing or not 3 $1 \text{ mM NO}_3^{-}(D).$ 4 Supplementary Table S1. List of the genes analyzed by means of Real Time qPCR. 5 Maize GDB and NCBI accessions are reported together with the gene functions and the 6 primer sequences. 7 Supplementary Table S2. Merged effects of different chemicals interfering with NO 8 biosynthesis/scavenging and nitrate supply/depletion on root and leaf fresh weight. 9 10 11 Acknowledgements 12 Funds were provided by the University of Padua, ex 60% 2010-2011, 2011-2012. 13 We thank Professor Paola Bonfante for the hospitality in her lab and Dr. Mara Novero 14 for helping in laboratory activities. We thanks also Professor Massimo Saccomani and 15 Dr. Piergiorgio Stevanato for the use of WinRhizo software. We are also grateful to Dr. 16 Benedetto Ruperti for critical reading of the manuscript.

17

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

19

20

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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 five 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 °	8.60±0.46 ^c	0.55±0.01 °	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 °
4	51.34±2.15 ^c	9.03±0.43 °	0.52 ± 0.01^{d}	72.90±2.75 ^b
5	60.08±3.56 ^b	10.42±0.55 ^b	0.61 ± 0.01^{ab}	64.23 ± 2.79 bc

Figure legends

Fig. 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 (NO_3^- and NH_4^+ - 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.

Fig. 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.

Fig. 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 three technical repetitions performed on six independent biological replicates.

Fig. 4

Time course of the expression of five genes significantly regulated by short-term nitrate/ammonium treatments in *Zea mays* L. roots (A) and leaves (B). Data are reported as base-2 logarithm of the ratio between the expression levels measured for samples subjected to the treatments, as described in fig. 1 (short-term nitrogen starvation in seedlings grown in +N conditions and short-term nitrogen provision in seedlings grown in –N conditions, respectively), and that of its own control.

The left column (-) shows the differences in gene expression in roots (A) and leaves (B) of seedlings supplied for five days with NO_3^- (upper part) or NH_4^+ (lower part) and then deprived for 30 min, 2 and 6h. On the contrary, the right column (+) shows the differences of expression measured after NO_3^- (upper part) or NH_4^+ (lower part) resupply (30 min, 2 and 6h) to seedlings grown five days in a N-deprived medium (A, roots; B, leaves).

Fig. 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, $-NO_3^-$) (A-H) and 1 mM KNO₃ solution (I-P). Scale bar 500 μ m. 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 μ m.

C) DAF-2T fluorescence intensity values at 30 min after treatment of root segments with NO_3^- ; NO_3^- and tungstate (W); NO_3^- with the NO scavenger cPTIO; KCl (- NO_3^-); KCl (- NO_3^-) 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)

Fig. 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).

Fig.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. The percentages were expressed as the ratio between the mRNA abundance measured in each specific root zone and the global amount of transcript in the overall root.

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.

Fig. 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 of four independent biological repetitions. Significantly different means at *P* <0.05 are indicated by different letters.

Fig. 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 \pm 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, which do not reflect any quantitative value.

Fig. 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 (Baluška *et al.*, 2001) and need to be further studied and characterized.

























