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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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**Abstract**

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

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 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.

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 ($Na_2WO_4 \bullet 2H_2O$; Tungstate).

## 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

1 cause low yields and food insecurity, while in rich nations intensive fertilization leads to
2 leaching of nutrients and/or greenhouse gas emission (Donner and Kucharik, 2008). The
3 development of new crop cultivars with enhanced soil resource acquisition is therefore
4 an important strategic goal for modern agriculture (Lynch, 1998; Vance *et al*., 2003;
5 Lynch, 2007). Understanding nutrient responses at the organism level will be useful to
6 modify plant metabolism, physiology, growth and developmental programs to improve
7 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.
30 To trigger adaptive responses and to induce fast switching from starvation metabolism
31 to nutrient assimilation, the nitrate itself or its primary assimilation products serve as
32 signalling molecules (Schulze *et al*., 1994; Crawford, 1995; Scheible *et al*., 1997; Stitt,
33 1999; Gojon et al., 2010). Significant advances have been made during the recent period
34 concerning the molecular mechanisms of $NO_3^-$ 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).
3 A prolonged nitrate starvation was demonstrated to largely affect gene transcription,
4 producing effects on the early nitrate signalling mechanisms. Transcriptomic analyses
5 evidenced co-regulated transcriptional patterns in maize root epidermal cells for genes
6 putatively involved in nitric oxide synthesis/scavenging (Trevisan *et al*., 2012).
7 Nitric oxide is a free radical that is considered to be a general plant signal, since it
8 regulates both normal developmental processes and biotic or abiotic stress responses
9 involving cross-talk with phytohormones (for reviews, see: Durner and Klessig, 1999;
10 Wojtaszek, 2000; Beligni and Lamattina, 2001; Lamattina *et al.,* 2003).
11 NO has been reported to be required for root organogenesis (Pagnussat *et al.,* 2002),
12 formation of adventitious roots (Pagnussat *et al.,* 2003), lateral root (LR) development
13 (Correa-Aragunde *et al.,* 2004) and root hair formation (Lombardo *et al*., 2006).
14 Recently Correa-Aragunde *et al.,* (2004) suggested the possibility that auxin and NO
15 might be on a linear signalling pathway in the process of LR formation in tomato.
16 However, our knowledge of the molecular mechanisms by which NO regulates growth
17 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 Río *et al*.,
22 2004). Interestingly, it was recently shown that non-symbiotic haemoglobin 1 enzyme
23 could reduce $NO_2^-$ 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*., 2003*a; b*, 2004; Perazzolli *et al*., 2004; Hebelstrup *et al*., 2006; 2012).
29 The nitrate-regulated expression and spatial distribution in epidermal cells of NR and
30 Hb transcripts which have been recently evidenced in maize roots, strongly suggests
31 that they could play an important role during the early perception and signalling of
32 nitrate in the rhizosphere (Trevisan *et al*., 2011). Moreover the co-localization of
33 mRNAs for NR and Hb observed in the root apex matches with the major sites of NO
34 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. Finally, 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 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.

## Materials and Methods

### 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 $KNO_3$ 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

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.
3 Seedlings of the same age were also utilized to evaluate the expression of selected genes
4 in four different portions of roots, as indicated by Baluška *et al.* (2010), after nitrate
5 supply. The four zones sampled were: the root meristem (M, 4 mm), the transition zone
6 (TZ, 1 cm), the elongation zone (EZ, 1 cm) and the maturation zone (MZ, residual
7 portion). Roots were harvested after two hours of nitrate provision and the four
8 fragments were immediately cut and frozen, both for root treated and for the negative
9 control (-$NO_3^-$).
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.
20 Roots of seedlings grown 24 h in a nitrate-depleted agar plate were transferred on an
21 identical medium to which, in correspondence of specific root regions, round slices
22 (about 1-1.5 cm in diameter) of agar were removed and substituted with new ones
23 containing nitrate 1 mM. For the negative control the slices were substituted with new
24 nitrate-depleted ones, to subject roots to a similar mechanical stress, thus avoiding false
25 positives due to the perception of the discontinuance of agar and not to the nitrate
26 presence.
27
28 **Morphological analyses**
29 For the analysis with WinRhizo, germinated seeds of maize inbred line B73 were
30 transferred to 2-l-tanks containing five different aerated nutrient solutions (changed
31 every two days) according to the treatments: a) + $NO_3^-$, b) – $NO_3^-$, c) + $NH_4^+$, d) – $NH_4^+$,
32 e) +$NH_4NO_3$ and then placed in a growth chamber for eight days. The morphological
33 analyses including total root length (cm), total surface area ($cm^2$), average root diameter
34 (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).

**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).

**Selection of genes to be evaluated, maize sequences identification and primer 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

1 nitrate perception. The *NOA1* sequence was identified based on its similarity with the
2 *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; http://prato.daapv.unipd.it).
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 primers, forward 5'-TCCAGTGCTACAGGGAAGGT and reverse 5'-
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 μM 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.

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 from 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.

## Results

### 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.

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 Figure 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.

**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 five days (Table 1 and Fig. S4). 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%) than 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, 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**

3 To better understand the role of NO in nitrate signalling, its production was monitored
4 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).

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

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

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

28 The same observations were performed in the presence of tungstate, NOR and cPTIO.
29 Results obtained on five biological repetitions are reported in Fig. 5C. Data were
30 expressed as relative fluorescence increase/decrease after 30 min of observation. Results
31 showed a significant increase of fluorescence for nitrate supplied and for NOR-treated
32 roots. On the contrary, when seedlings were supplied with a $–NO_3^-$-solution (negative
33 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 NR-dependent NO burst occurred immediately after nitrate supply to roots.

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

Due to the size of the mini-chamber utilized for both stereomicroscope and confocal analyses, it was necessary to work with roots sampled from younger seedlings. For this reason we decided to shift the experimental plan to younger seedlings also for the following expression analyses 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.

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.

**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.

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
5 the growth of primary root was monitored for 24 h (Fig. 8). Nitrate-supplied seedlings
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
13 to nitrate depleted roots stimulated the root growth to levels comparable to those
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.

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

22

23 **The nitrate-induced NO signalling pathway is a localized effect**

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

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

29 As a preliminary experiment, to test the validity of this method as an alternative to
30 hydroponics, seedlings were grown in the agar plates which were nitrate-supplied or
31 nitrate-deprived, by using the same timing and concentrations described for experiments
32 in hydroponics and the expression of the previously selected genes was evaluated. RT-
33 qPCR were carried out on both roots and shoots and for all the genes and the nutritional
34 conditions described in the first paragraph of the Results (data not shown), but for

1 simplicity in Fig. 9A we decided to show only those closely related to the induction of
2 NO pathway in roots after nitrate supply. Results fully confirmed those obtained for
3 seedlings grown in hydroponics. Furthermore, the root growth, analyzed by means of
4 WinRhizo software, evidenced the same behaviour of plants grown in nutrient solution
5 (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).
24 Nitrate is the main nitrogen source for plants in regular agricultural systems and, acting
25 also as a signal, triggers a number of molecular and physiological events leading to the
26 overall plant's response to its availability (Gojon *et al*., 2010 and references therein).
27 The control of nitric oxide homeostasis through the spatio-temporal coordination of
28 nitrate reductase and haemoglobin gene expression has been recently hypothesised to
29 participate to nitrate sensing in maize roots (Trevisan *et al*., 2011). In the present work
30 we tried to more deeply characterize the role of nitric oxide in the maize root response
31 and adaptation to nitrate fluctuations.
32 To better discriminate nitrate specific effects from those more generally N-dependent,
33 the expression of a list of previously selected genes (Quaggiotti *et al*., 2003; Trevisan *et*
34 *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 $NO_3^-$ 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.
25 Nitrate reductase is involved in the NO production during bacteria induced defence
26 (Modolo *et al*., 2005), disease development in certain pathogenic interactions (Shi and
27 Li, 2008), drought (Freschi *et al*., 2010), cold (Zhao *et al*., 2009), osmotic stress
28 response in roots of *Arabidopsis* (Kolbert *et al*., 2010), stomatal regulation (Srivastava
29 *et al*., 2009) and many developmental processes as, for example, the initiation of
30 flowering (Seligman *et al*., 2008).
31 The parallel strong increase of the expression of both the *nsHbs* genes observed already
32 after 30 min of nitrate supply, is not surprising if considering the high reactivity of NO,
33 which besides serving as a signal in regulating several physiological events, must also
34 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 the already hypothesised involvement of nitric oxide control homeostasis in the maize root response to nitrogen (Trevisan *et al*., 2011), demonstrate also that this is an exclusive prerogative of $NO_3^-$-signalling. In fact, when ammonium was supplied to nutrient solution as the sole nitrogen source, no significant effects were measured on the transcription of genes involved in the NO production and scavenging. On the contrary, the expression of the other genes here analyzed did not show a similar specific nitrate responsiveness. In addition, data obtained by analyzing 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 (table 1).

According to these results it would seem that nitric oxide may 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 (Fig. 5). 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, 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 nitrate-depleted 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.

The NR-dependent NO production observed after nitrate supply, was then corroborated by the expression analyses performed on roots of one day olds seedlings (Fig. 6). 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 exclude 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) encoding 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 (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 nitrate-depleted and in nitrate-treated (1 mM) seedlings (Fig. 7). In N-starved roots all five

1 transcripts evidenced their maximum accumulation at the meristem level. This pattern
2 radically changed when nitrate was furnished to roots with a very significant increase of
3 transcript abundance in the transition zone (TZ), that is located between the meristem
4 (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
33 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).
16 Besides this, NO is involved in the regulation of actin cytoskeleton, endocytosis, vesicle
17 trafficking and the polarity of growing tip cells (Prado *et al*., 2004; Lombardo *et al*.,
18 2006; Salmi *et al*., 2007; Prado *et al*., 2008; Kasprowicz *et al*., 2009; Wang *et al*., 2009),
19 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

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 the rapid increase of nitrate availability and that it could mediate the root elongation observed after nitrate provision (Fig. 10).

**Supplementary material**

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.

Supplementary Fig. S2. Time course of the expression of genes following short-term nitrate/ammonium treatments in maize roots and leaves.

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

Supplementary Fig. S4. Root and leaf fresh weight and relative root/shoot ratio in seedlings grown in nutrient solution for five days (A, B, C).

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 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
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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 [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] |
| **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 ($T_0$-T50') on apices treated for 30 min with 1 mM KCl (negative control, -$NO_3^-$) (A-H) and 1 mM $KNO_3$ solution (I-P). Scale bar 500 $\mu$m. B) Confocal detection of DAF-2T in the transition zone of nitrate treated and untreated apices at $T_0$ 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 $\mu$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**

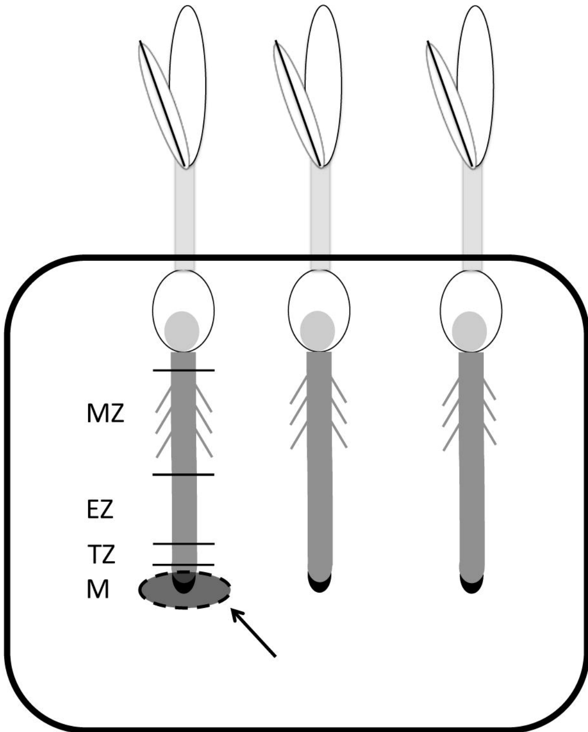
Expression analysis of NO and nitrate metabolism related genes *NR1*, *Hb*, *Hb2*, *NiR*, *NRT2.1* on roots of 24 h old seedlings grown on nitrate-depleted agar medium and treated in a fresh medium added with nitrate in the whole plate or locally at the root tip. In panel A the RT-qPCR on roots at two (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 ± 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

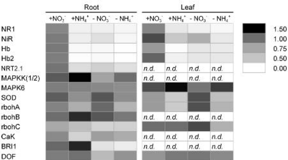
with nitrate. The size of the different zones (Meristem; TZ transition zone; EZ elongation zone; MZ maturation zone) doesn't reflect the real values of length; please refer to the material and methods for the exact measures. Values of fold change are expressed by means of a grey scale. The authors arbitrarily 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.

3 days
germination
5 days
Nutrient Solution
30'-2h-6h
Nutrient Solution
LATE EFFECTS
of $NO_3^-/NH_4^+$
supply or
deprivation
$+NO_3^-$
$-NO_3^-$
$+NH_4^+$
$-NH_4^+$
$+NO_3^-$
$-NO_3^-$
$+NO_3^-$
$-NO_3^-$
$+NH_4^+$
$-NH_4^+$
$+NH_4^+$
$-NH_4^+$
EARLY EFFECTS
of $NO_3^-/NH_4^+$
supply or
deprivation


MZ
EZ
TZ
M



Root
Leaf
+NO3-
+NH4+
- NO3-
- NH4+
NR1
NiR
Hb
Hb2
NRT2.1
MAPKK(1/2)
MAPK6
SOD
rbohA
rbohB
rbohC
CaK
BRI1
DOF
n.d.
1.50
1.00
0.75
0.50
0.00

A

− +

$NO_3^-$

■30' □2h ■6h

8 6 4 2 0 -2 -4 -6

NR1 NiR Hb Hb2 NRT2.1

NR1 NiR Hb Hb2 NRT2.1

$NH_4^+$

NR1 NiR Hb Hb2 NRT2.1

NR1 NiR Hb Hb2 NRT2.1

B

$NO_3^-$

■30' □2h ■6h

5 2.5 0 -2.5 -5

NR1 NiR Hb Hb2

NR1 NiR Hb Hb2

$NH_4^+$

NR1 NiR Hb Hb2

NR1 NiR Hb Hb2

$T_0$ $T_{5'}$ $T_{10'}$ $T_{15'}$ $T_{20'}$ $T_{30'}$ $T_{40'}$ $T_{50'}$

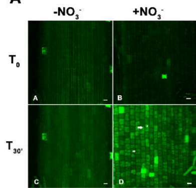
$-NO_3^-$

A B C D E F G H

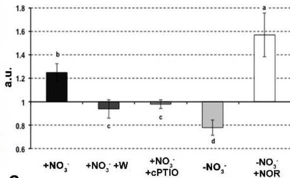
$+NO_3^-$

I J K L M N O P

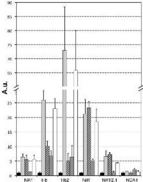
**A**



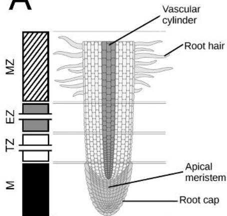

**B**

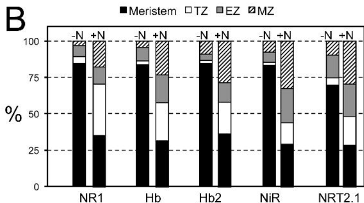


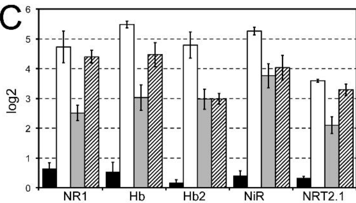

**C**



-NO3-
+NO3-
+NO3- + cPTIO
+NO3- + W
+NO3- + L-NAME
A.U.
90
85
80
75
70
65
60
55
25
20
15
10
5
0
NR1
Hb
Hb2
NiR
NRT2.1
NCA1


A
MZ
EZ
TZ
M
Vascular cylinder
Root hair
Apical meristem
Root cap


B
Meristem
TZ
EZ
MZ
-N
+N
%
100
75
50
25
0
NR1
Hb
Hb2
NiR
NRT2.1


C
log2
6
5
4
3
2
1
0
NR1
Hb
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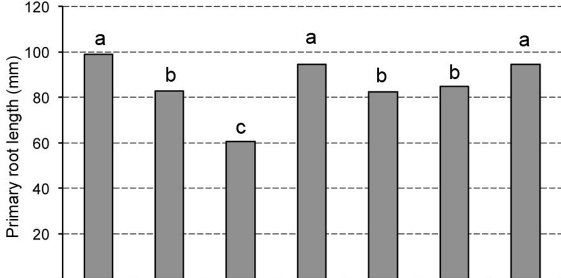
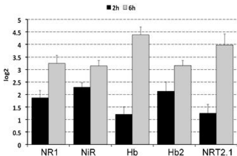
NiR
NRT2.1



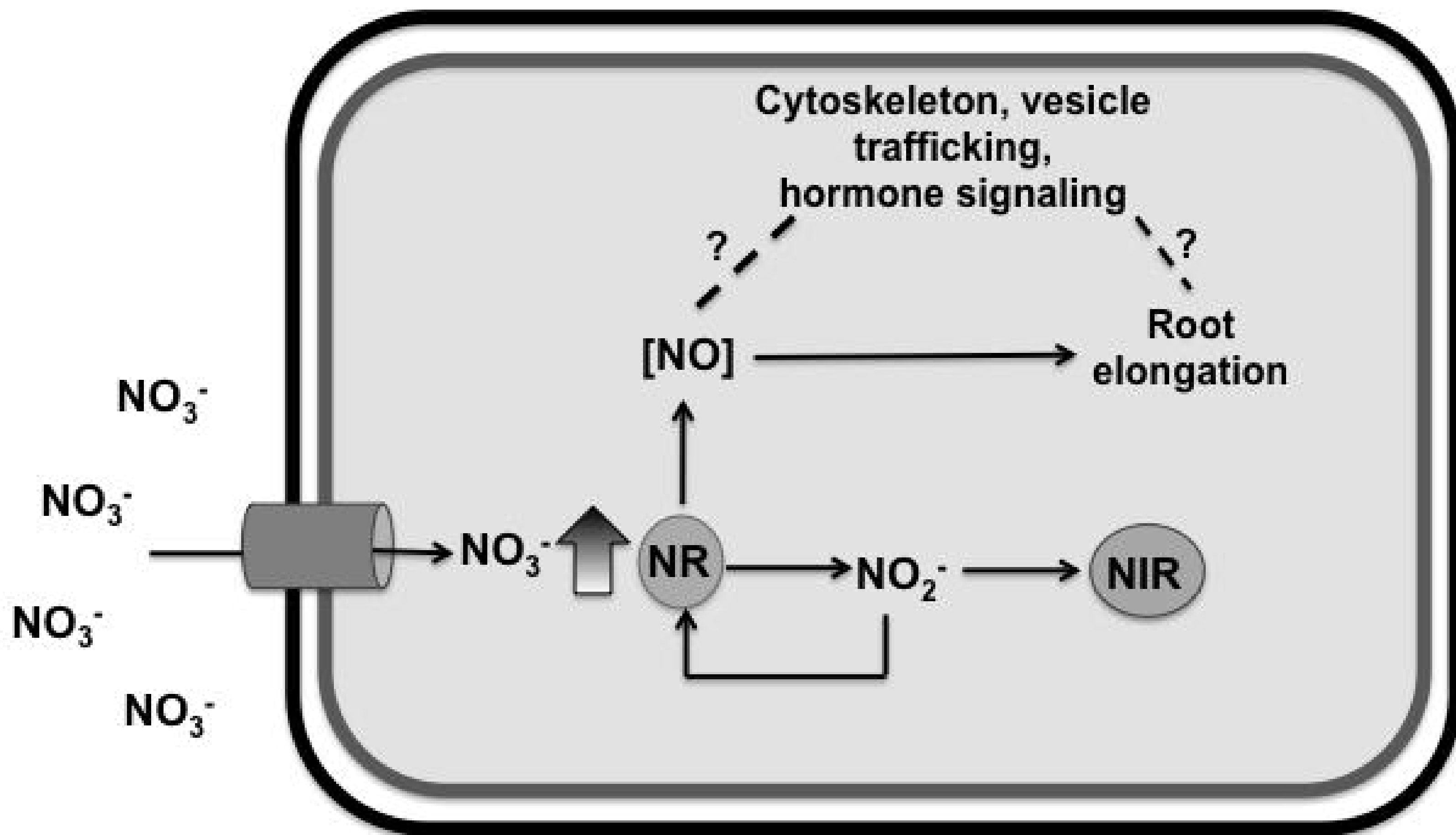

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $NO_3^-$ | + | + | + | + | - | - | - |
| cPTIO | - | + | - | - | - | - | - |
| W | - | - | + | - | - | - | - |
| L-NAME | - | - | - | + | - | - | - |
| $NH_4^+$ | - | - | - | - | + | - | - |
| SNP | - | - | - | - | - | - | + |




A



B



Cytoskeleton, vesicle trafficking, hormone signaling
?
?
[NO]
Root elongation
$NO_3^-$
$NO_3^-$
$NO_3^-$
$NO_3^-$
$NO_3^-$
NR
$NO_2^-$
NIR