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Nitrate affects transcriptional regulation of UPBEAT1 and ROS localisation in roots of *Zea mays* L.

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1 Nitrogen (N) is an indispensable nutrient for crops but its availability in agricultural soils is subject to
2 considerable fluctuation. Plants have developed plastic responses to external N fluctuations in order to
3 optimise their development. The coordinated action of nitric oxide and auxin seems to allow the cells of the
4 transition zone (TZ) of the root apex of N-deprived maize to rapidly sense nitrate. Preliminary results support
5 the hypothesis that reactive oxygen species (ROS) signalling might also have a role in this pathway, probably
6 through a putative maize orthologue of UPBEAT1 (UPB1). To expand on this hypothesis and better
7 understand the different roles played by different root portions, we investigated the dynamics of ROS
8 production, and the molecular and biochemical regulation of the main components of ROS production and
9 scavenging in tissues of the Meristem, Transition Zone, Elongation Zone and Maturation Zone of maize
10 roots. The results suggest that the inverse regulation of *ZmUPB1* and *ZmPRX112* transcription observed in
11 cells of the TZ in response to nitrogen depletion or nitrate supply affects the balance between superoxide
12 ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in the root apex and consequently triggers differential root growth. This
13 explanation is supported by additional results on the overall metabolic and transcriptional regulation of ROS
14 homeostasis.

15
16 *Abbreviations* – DAB, diaminobenzidine tetrahydrochloride; NBT, nitroblue tetrazolium; ROS, reactive
17 oxygen species.

18 19 **Introduction**

20 The root system is essential for providing water, minerals and anchorage to the plant. Roots have to quickly
21 sense and respond to changes in the physical and chemical features of the soil environment (Philip et al.
22 2013), and attempts to understand how they do this have focussed in particular on the striking effects of
23 nitrate on root growth and branching (Bhardwaj et al. 2015). Nitrate (NO_3^-) has a dual nutritional/signalling
24 function and can exert a profound impact on the root system architecture (RSA) by altering the number,
25 length, angle and diameter of the roots and root hairs (Zhang et al. 1999, Shahzad and Amtmann, 2017, Sun
26 et al. 2017).

27 In maize, nitrate seems to be perceived by a specialised portion of the root apex, namely the transition zone
28 (TZ) (Manoli et al. 2014, 2016, Trevisan et al. 2014, 2015). When the TZ perceives external environmental
29 stimuli, it translates them into motoric responses and releases cells into the elongation region, thereby
30 providing the growing root apices with an effective mechanism for reprogramming root growth in response
31 to environmental stimuli (Baluška et al. 2010).

32 Two hours of nitrate supply to nitrogen-deprived maize seedlings triggered production of a nitrate reductase
33 (NR)-dependent nitric oxide (NO) localised in the TZ cells (Manoli et al. 2014) and the accumulation of
34 auxin (Indole-3-acetic acid, IAA) at cross wall in the root-apex TZ (Manoli et al. 2016). Both NO and IAA
35 are closely connected to the modulation of cell division-differentiation processes which regulate RSA
36 (Correa-Aragunde et al. 2016). Confocal analyses showed fewer and larger cortical cells in the TZ of roots
37 supplied with nitrate for 2 h, supporting the suggestion that early nitrate supply could locally stimulate apex

growth by activating cellular expansion rather than by accelerating cell division. However, prolonged nitrate supply seems to negatively influence primary root growth, which is in turn induced by nitrogen deprivation (Manoli et al. 2016), highlighting the existence of different and sometimes antagonistic mechanisms governing localised and systemic responses to nitrogen availability.

Several authors have reported that reactive oxygen species (ROS) distribution plays a pivotal role in regulating cell-state decisions in animals (Owusu-Ansah and Banerjee 2009, Sauer et al. 2001, Menon and Gozwami 2007), while in plants, the root tip is a zone of active ROS production (Liszkay et al. 2004). Differences in superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) accumulation in the root tip have been shown to significantly affect primary root growth and differentiation (Dunand et al. 2007) and to control the transition of cells from a zone of cell division to a zone of cell elongation and differentiation (Tsukagoshi et al. 2010).

In *Arabidopsis thaliana* (Arabidopsis), the balance between cell proliferation and elongation seems to depend on UPBEAT1 (UPB1), a bHLH transcription factor repressing peroxidase expression, thus affecting the relative distribution of $O_2^{\cdot-}$ and H_2O_2 in the tip (Tsukagoshi et al. 2010).

Using an RNAseq-based approach, Trevisan et al. (2015) identified a putative maize orthologue of UPB1, which was highly transcribed in the TZ of roots grown for 24 h in an nitrogen-free solution and was rapidly down-regulated by nitrate provision. This finding suggests that different nitrogen (N) availabilities affect the root architecture, at least in part, through UPB1-dependent modulation of the balance between $O_2^{\cdot-}$ and H_2O_2 .

Besides acting as a signal, increased ROS production also causes oxidative damage (Del Rio, 2015). To counteract these detrimental effects, plants have evolved an efficient antioxidant defence system with both enzymatic and non-enzymatic components, the most abundant of these being ascorbate and glutathione (Foyer and Noctor 2011), both of which react with H_2O_2 , $OH^{\cdot}$ and $O_2^{\cdot-}$ (Shao et al. 2005) preventing their deleterious effects. Moreover, because of its high reductive potential, reduced glutathione (GSH) is able to regenerate ascorbate and change to oxidised glutathione (GSSG), which is reduced back to GSH by the enzyme glutathione reductase. Under physiological conditions, glutathione is 95-99% in its reduced state and a correct balance between GSH and GSSG is necessary for maintaining a proper cell redox state (Noctor et al. 2017).

In order to better understand the role played by the putative maize orthologue of UPB1 in regulating ROS balance in the pathway leading to root adaptation to N fluctuations, we investigated the dynamics of ROS production, molecular regulation of the main components of ROS production and scavenging, and activation of the redox system constituted by ascorbate and glutathione in the various regions of the root apex under N-deprivation and after nitrate supply.

We hypothesise that coordinated regulation of *ZmUPB1* and of a class III peroxidase (*ZmPRX112*, Wang et al. 2015) transcription in TZ cells of roots elicits divergent H_2O_2 / $O_2^{\cdot-}$ profiles of accumulation along the first few millimetres of the primary root (PR) in response to nitrogen availability, thus modulating PR growth.

Furthermore, N depletion seem to form specific profiles of accumulation of transcripts and metabolites involved in ROS signalling along primary roots. However, two hours of early nitrate supply to N-depleted roots affects these molecular and metabolic patterns mostly in the meristem and in the TZ of the primary root.

This work advances our understanding of the signal transduction pathways that shape the root architecture in response to variations in the external N supply.

Materials and methods

Maize growing conditions

After 3 days of germination, *Zea mays* L. (B73) plants were hydroponically grown in 450 ml glass boxes containing 10 plants per box. These were placed in a growth chamber where the plants received an 8-h photoperiod under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR; daylight and warm white 1:1, LF-40W) at day/night temperatures of 21/18°C (Quaggiotti et al. 2003). The hydroponic boxes were filled with nutrient medium, which was constantly aerated and renewed twice a week. The composition of the nutrient medium was as previously described in Quaggiotti et al. (2003), and the nitrogen source was 1 mM KNO_3 , as previously used by Manoli et al. (2014). The pH of the medium was checked during the growth period and remained at a stable level of around pH 6.5. In the first growth phase, the plants were cultured for 24 h on nitrogen-free nutrient medium (composed of the standard nutrients excluding NO_3^- and NH_4^+) and thereafter were supplied with KNO_3 (1 mM) or a nitrogen-depleted solution (-N). The growth medium was changed after 2 h into the light phase (10:00). Samples were generally collected from the plants after 120 min incubation with NO_3^- (12:00 h). In order to compare the effects of 24 h vs 2 h treatments, plants were additionally sampled after 24 h of treatment. For the analyses of RNA, enzymes and metabolites, 2 h-treated plants were collected and roots were sampled as reported in Manoli et al. (2016). The root segments were as follows: zone 1: the meristem (0.5–2 mm from the root cap tip, M), zone 2: the Transition Zone (2–4 mm from the root cap tip, TZ), zone 3: the rapid elongation zone (4–8 mm from the root cap tip, EZ) and zone 4: the maturation zone (1 cm of the residual portion, MZ) (Fig. 1). Samples were frozen in liquid nitrogen and stored at -80°C . For histochemical staining for ROS detection, plants were analysed after 2 h and after 24 h of nitrate provision.

Histochemical staining for ROS detection

To detect the presence of superoxide ($\text{O}_2^{\bullet-}$) in the root apex of plants grown in the presence or absence of nitrate, seedlings were incubated for 30 min in a staining solution of 0.2% nitroblue tetrazolium salt (NBT) dissolved in 50 mM of sodium phosphate buffer (pH = 7.5). The seedlings were then rinsed in ethanol and preserved in sodium phosphate buffer.

The presence of H_2O_2 in the roots was detected by the 3,3'-Diaminobenzidine (DAB) staining method (1 mg ml^{-1} solution), as described in Kumar et al. (2014).

The seedlings were subsequently observed and imaged under a light stereomicroscope.

1 The mean intensity of NBT and DAB staining was determined with the ImageJ histogram function
2 (Schneider et al. 2012) reading grey intensity in inverted pictures where black has the maximum value. The
3 area measured extended from the quiescent centre to 5 mm shootward in the root.

4
5 **RNA extraction and cDNA synthesis**

6 Total RNA was extracted using the TRIzol reagent (Invitrogen, San Giuliano Milanese, Italy), as previously
7 described by Trevisan et al. (2011). DNase digestion was performed with RQ1 RNase-free DNase
8 (Promega, Milan, Italy) on an aliquot of total RNA, as described by Trevisan et al. (2011). DNA-free RNA
9 was eluted in 20 µl of RNase-free water. RNA concentrations were determined with a Nanodrop1000
10 (Thermo Scientific, Nanodrop Products, Wilmington, DE) and cDNA was synthesised from 500 ng of total
11 RNA mixed with 1 µl of 10 µM oligo-dT, as described by Manoli et al. (2012). RNA integrity was further
12 validated by gel electrophoresis. RNA was extracted from the nitrate-supplied and N-deprived root portions
13 of 15-20 plants sampled in three independent experiments.

14
15 **Real-time qPCR**

16 Relative quantification of transcripts by real-time PCR (RT-qPCR) was performed with a StepOne Real-
17 Time PCR System (Applied Biosystems, Monza, Italy), as described by Nonis et al. (2008) and Manoli et al.
18 (2014).

19 For each reaction, 2.5 ng of retrotranscribed RNA was used as a template. Three technical replicates were
20 performed on three independent biological replicates under the conditions described by Trevisan et al.
21 (2011). Melting curve analysis was performed to confirm the absence of multiple products or primer dimer
22 formation. Data were collected and analysed according to the Livak and Schmittgen (2001) method using
23 LUG (Zm00001d011309, Leunig) and MEP (Membrane protein PB1A10.07c, Zm00001d018359) as
24 reference genes, according to Manoli et al. (2012). For each transcript, the ratio between the expression
25 measured from the nitrate-supply treatment and that from the nitrogen-deprivation treatment was used to
26 estimate up- or down-regulation of the genes. All the primers used in these assays are listed in Table S1.

27
28 **In situ hybridisation**

29 RNA in situ hybridisation was performed as described by Trevisan et al. (2011), with minor modifications.
30 The ZmUPB1 template for probe synthesis was selected by PCR from the cDNA of the maize roots, and
31 sense and antisense riboprobes were transcribed in vitro using T7 and SP6 RNA polymerases (Roche, Basel,
32 Switzerland). Digoxigenin-labelled RNA probes were prepared using a DIG RNA labelling mix (cat. no.
33 11277073910, Roche) according to the manufacturer's instructions. Tissues were fixed in a solution of 50%
34 ethanol, 5% acetic acid and 3.7% formaldehyde, dehydrated in a graded series of ethanol, infiltrated with
35 paraffin (Paraplast X-tra; Sigma-Aldrich, St Louis, MO) and then segmented. Cross-sections were cut at 7
36 µm, longitudinal sections at 5 µm.

37 The sections were deparaffinised with HistoClear and rehydrated with an ethanol series.

The slides were incubated for 30 min in pre-hybridisation buffer (100 mM Tris-HCl, pH 7.5, and 50 mM EDTA) containing 2 to 10 $\mu\text{g ml}^{-1}$ proteinase K (Roche Diagnostics, Indianapolis, IN), and blocked in PBS containing 2 mg ml^{-1} Glycine. The sections were post-fixed in 3.7% (v/v) formaldehyde (in PBS1X), incubated in 100 mM TAE, pH 8.0, containing 0.5% (v/v) acetic anhydride, and rinsed in PBS1X. The slides were covered with 80 μl of hybridisation buffer (300-600 ng ml^{-1} digoxigenin-labelled RNA, 10 mM Tris-HCl, pH 7.5, 10 mM sodium phosphate buffer, pH 6.8, 50% (v/v) deionised formamide, 1 $\times$ Denhardtts, 10% (w/v) dextran sulphate, 10 mM DTT, 300 mM NaCl, 1mM EDTA, 1 mg ml^{-1} yeast tRNA) and incubated overnight at 50°C. After hybridisation, the cover slips were removed in 1xSSC at room temperature, then immersed in 0.1xSSC and washed 3 $\times$ 50 min in 0.1 $\times$ SSC at 65°C. The slides were rinsed in B1 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% v/v Triton X-100), blocked for 1 h in B2 (B1 plus BSA 2% w/v) and covered with 100 μl of a goat anti-DIG alkaline phosphatase (AP) conjugate (Roche Diagnostics, Indianapolis, IN) diluted 1:700 in B2, then incubated for 2 h at room temperature. Colour development was performed in AP buffer containing X-phosphate and nitroblue tetrazolium for 24 h. The slides were observed under bright field through a microscope (Zeiss) and photographed with a Micro Color charge-coupled device camera (Apogee Instruments, Logan, UT).

Metabolite analysis and gamma-glutamyl-transpeptidase (GGT) activity

Frozen root samples (250 mg) from at least four biological replicates were ground in a mortar and pestle with metaphosphoric acid 1.5% and EDTA 1 mM buffer (1:4 buffer ratio) to extract soluble antioxidants. Following centrifugation at 10 000 g for 10 min (4°C), the extracts were rapidly tested for ascorbate. Following the decrease in absorbance at 265 nm, the levels of the reduced (AsA) and oxidised (DHA) forms of ascorbate were measured according to the spectrophotometric method developed by Hewitt and Dickes (1961).

The levels of reduced and oxidised thiols in the extracts were determined according to methods reported by Masi et al. (2002), with some modifications.

Low-molecular-weight thiols were separated and quantified by isocratic HPLC after reduction with tri-n-butyl phosphine and derivatisation with 4-fluoro-7-sulphobenzofurazan ammonium salt fluorophore (SBD-F) (Dojindo, Japan).

The mobile phase was 75 mM ammonium-formiate, pH 2.9, and 3% methanol (97:3 v/v). Oxidised thiols were determined by pre-treating samples with 2-vinylpyridine according to Griffith (1980). To protect the free thiol moieties, samples were buffered to basic pH and treated with 2-vinylpyridine for 1 h. After 1 h incubation the samples were washed to remove the resulting complexes. The unreacted samples (containing the oxidised thiols) were derivatised and analysed by HPLC.

For the enzyme histochemical analysis of GGT activity, maize roots were rapidly embedded in optimal cutting temperature medium (OCT, Cellpath, Newtown, UK) with no chemical fixation, placed in cryomoulds and snap frozen. Sections were collected on glass slides and air dried overnight. Sectioning and staining were performed essentially according to the procedure described elsewhere (Masi et al. 2007, Destro

et al. 2011). Images were acquired with a Leica DM4000B digital microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

The effects of nitrate on relative H_2O_2 / $\text{O}_2^{\cdot-}$ contents, and Asc, GST, Cys, Cys-Gly, γ GT, DHA, GSSG and CGox contents were subjected to a one-way analysis of variance (ANOVA). All the experiments were performed in triplicate. Details of the individual sample sizes for each analysis are given in the figure legends.

Results

UPBEAT1 identification in the *Zea mays* L. genome

Using high-resolution expression data from the Arabidopsis root, Hironaka Tsukagoshi identified UPBEAT1 (AtUPB1, At2g47270, <http://www.arabidopsis.org>) as a transcription factor belonging to the bHLH-subfamily 14 (Toledo-Ortiz et al. 2003), which regulates the first stages of the transition from cellular proliferation to differentiation (Tsukagoshi et al. 2010). A previous screening of the TZ RNAseq database gene expression data (Trevisan et al. 2015) resulted in the identification of a maize orthologue of *AtUPB1* (*Zm00001d037569*, <http://www.phytozome.net>) encoding a putative bHLH DNA-binding domain superfamily protein. This transcript exhibited a strong repression of expression (0.15-fold change) in response to 2 h of nitrate provision (Fig. 2A). *Zm00001d037569* has a unique transcript (558bp) with 1 exon (345bp).

The predicted gene product of *Zm00001d037569_T001* comprises 114 amino acids with a calculated molecular weight of 12.282 kDa and a theoretical isoelectric point of 9.96. Analysis of the deduced amino acid sequence revealed the presence of a basic helix-loop-helix (bHLH) DNA-binding domain (pfam00010) (Fig. 2C). Apart from the bHLH domain, no other functional domains were predicted in *Zm00001d037569* (Fig. 2B).

Nitrate provision to N-deprived roots alters the balance between H_2O_2 and $\text{O}_2^{\cdot-}$ in the root apex and inhibits primary root growth

In light of the previously described role of AtUPB1 in regulating the equilibrium between $\text{O}_2^{\cdot-}$ and H_2O_2 , the levels of these were measured in both N-depleted and nitrate-supplied roots.

Basing on previous findings (Trevisan et al. 2015), a preliminary staining was performed to compare N-deprived roots with roots supplied with nitrate for two hours. We found no evident differences in the relative distributions of H_2O_2 and $\text{O}_2^{\cdot-}$ (data not shown), leading us to hypothesise that a more prolonged nitrate treatment is needed to induce appreciable effects on ROS distribution. In fact, a significant difference in the staining distribution did appear 24 h after nitrate provision (Fig. 3).

In N-depleted roots, light NBT staining was observed, mainly in the meristem tissue (Fig. 3A, J), but it was already decreasing in the transition-elongation zone (Fig. 3A, J). In contrast, nitrate-supplied root tips

exhibited diffused NBT staining, which reached the columella, lateral root cap and epidermal layer of the meristem, the transition zone and the elongation zone up to 4-6 mm (Fig. 3B, J). Quantification of the signal intensity in the first 5 mm from the root cap confirmed the qualitative results, showing a 30% stronger NBT staining (which reflects the $O_2^{\cdot-}$ level) in the nitrate-supplied root (Fig. 3I).

As for H_2O_2 detected by DAB, a specular pattern was observed (Fig. 3C, D, J). Here, too, the staining tended to be localised in the root tip, but in the case of nitrogen-depleted roots it spread throughout the first 2 mm from the tip (Fig. 3C), while nitrate-supplied plants exhibited a lower signal (-30%), which was confined to the meristem (Fig. 3D, I).

H_2O_2 and $O_2^{\cdot-}$ were also found to be present in mature LRPs of both nitrate-supplied (Fig. 3F, H) and nitrate-depleted (Fig. 3E, G) seedlings.

Furthermore, when 24 h N-deprived seedlings were supplied with nitrate for an additional 24 h a significant decrease in primary root length compared with -N seedlings was observed (Fig. 3K).

***ZmUPBI* and *ZmPRX112* expression are specularly regulated by N deprivation and nitrate supply**

In order to gain new insights into the regulation of *ZmUPBI* gene expression by N deprivation and early nitrate supply, we analysed by zone (1, 2, 3 and 4) its transcript accumulation in 24 h nitrate-depleted roots and in seedlings of the same age after two hours in a 1 mM nutrient solution supplied with nitrate (Fig. 4).

Q-PCR analysis of the nitrate-depleted seedlings showed that the *ZmUPBI* transcript was almost absent from the meristem (zone 1). Maximum *ZmUPBI* expression was detected in zone 2 (2/1 ratio = 10.5 f.c.), while transcript accumulation drastically decreased in zone 3 and finally returned to steady-state levels in zone 4. Two hours of nitrate supply induced a significant decrease in *ZmUPBI* expression in the TZ cells (2.5 f.c.), but no significant differences were observed in the other root zones. Overall, the distribution of *ZmUPBI* transcript along the primary root was consistent with its known expression pattern in Arabidopsis.

Among other putative targets, UPBEAT1 in Arabidopsis regulates three peroxidases, which are highly expressed at the boundary of the meristem and the elongation zone in the Arabidopsis Root Map (Tsukagoshi et al. 2010). We selected three maize orthologues of these peroxidases with high expression levels in the TZ (*Zm00001d017696*; *Zm00001d024119*; *Zm00001d014467*; Trevisan et al. 2015) (Fig. 5A) and assessed their expression along the root and in response to nitrate.

Nitrate induces only modest changes in transcript abundances of both *Zm00001d014467* and *Zm00001d017696* (Supplementary Fig. S1).

In contrast, we found the expression profile of *Zm00001d024119* (the peroxidase superfamily protein *ZmPRX112*, orthologue of *AtPer40*) to be highly regulated by the presence of nitrate (Fig. 5B). In N-depleted plants, transcript accumulation progressively decreased from the meristem to the maturation zone. The supply of nitrate resulted in a significant induction of *ZmPRX112* transcript accumulation (2.5 f.c.) in zone 2, and the opposite profile in zone 1 ("mirror" pattern). However, no significant differences were noticed in zones 3 and 4. These results show that *ZmPRX112* was dysregulated in the opposite direction to *ZmUPBI* in cells of the TZ.

ZmUPB1 in situ hybridisation (ISH)

In situ hybridisation analysis revealed that *ZmUPB1* was expressed in the primary root mainly in the transition zone (zone 2) (Fig. 6A). In the root apex longitudinal section, comprising the root cap and the meristematic area (Fig. 6A section II), a *ZmUPB1* probe showed a diffuse but weak hybridisation signal in the calyptrogen and in the meristematic area (Fig. 6A, sections I and II). Moving along the primary root, the intensity of the probe signal increases, and the *ZmUPB1* probe preferentially localises in the epidermis and in the differentiating vascular tissues of the central cylinder (phloem pole) of the TZ (Fig. 6A, section III). Localisation is maintained in the same tissues of the EZ (Fig. 6A, sections I and VI), but the intensity of the signal is lower. In the mature zone, transcript accumulation slightly decreases and its localisation becomes diffuse (Fig. 6A, sections I and V). To analyse the changes in the *ZmUPB1* spatial distribution in greater depth, additional ISH experiments were carried out on the transverse sections of different regions of the primary root (Fig. 6B). These sections included the root cap (section I), the QC (section II), domains with initial regions (section III) and advanced regions (sections IV-V-VI) of cellular differentiation, and fully-differentiated mature tissues (sections VII-VIII). In the meristem itself, the probe was detected at a very low intensity close to the tip (Fig. 6B, sections I-II). The signal density in the cells of the epidermis and in a ring of cells at the interface between the cortex and the stele, which probably represent the maturing endodermis/pericycle, was higher than in the cortical cells (Fig. 6B, sections III-IV). In the transverse sections of the elongation zones of maize seedling roots, we observed phloem pericycle-specific expression of the UPB1 gene (Fig. 6B, sections V-VI). In the longitudinal sections, *ZmUPB1* mRNA was specifically localised in the phloem element (Fig. 6A, section I), reaching a maximum in the phloem companion cells and the cells of the phloem pole pericycle and the phloem pole endodermis, which are committed to initiate primordia (Fig. 6A, sections IV to V). A diffuse signal was detected in the cortex cells and in the pith of the meristem and the transition zone (Fig. 6B, sections I to IV). The staining in the elongation zone was weaker than in the TZ (Fig. 6B, sections V-VI), while in the differentiated zone it was weak and non-specific (Fig. 6B, sections VII-VIII). A hybridisation signal was detected during lateral root (LR) formation (Fig. 6C): in 5-day-old seedlings *ZmUPB1* expression was weakly detected in the early stage of LR formation and its accumulation increased during LR emergence.

Expression analysis of genes related to ROS homeostasis

Q-PCR analyses were carried out to establish whether nitrate affects ROS distribution by differentially regulating the expression of key genes involved in controlling ROS homeostasis. According to previous RNAseq data (Trevisan et al. 2015), nitrate induces moderate regulation of a large set of genes that are involved in ROS metabolism (Fig. 7). Enrichment analysis on an enlarged data set (Trevisan et al. 2015, Supplementary Fig. S2) revealed slight but evident regulation on genes with the molecular function associated with the term ‘oxidant activity’ (GO:0016209). This term is applied to components that can trap

free radicals, thereby breaking the chain reaction that normally leads to extensive biological damage (The Gene Ontology Consortium, 2017). Catalases (CATs), superoxide dismutases (SODs) and respiratory burst oxidase homologues (RBOHs) are among these annotations.

RBOHs

Plant RBOHs (also known as NADPH oxidases) (Suzuki et al. 2011) are among the most widely-studied enzymatic sources of ROS. In our experiments, all four maize genes encoding RBOH (A, B, C, D) (Lin et al. 2009) displayed different expression profiles, according to both root segment (zone 1, zone 2, zone 3, zone 4) and the response to nitrogen deprivation or nitrate supply (Fig. 7A). *ZmRbohA* in N-depleted roots was mainly expressed in the meristematic cells (zone 1) and did not respond to nitrate provision, whereas *ZmRbohB*, which was expressed to a similar extent in all four root zones, showed a clear reduction of transcription in the TZ cells after early nitrate supply. The most striking effect was observed in the case of *ZmRbohC* transcription, which was noticeably induced in the meristem after two of hours nitrate supply (7 f.c.). In contrast, the *ZmRbohD* gene was more abundantly transcribed in the older zones (3 and 4) of N-depleted roots, but was clearly down-regulated by nitrate provision, especially in the TZ.

Superoxide dismutase and catalase

Superoxide dismutases and catalases play a crucial role in controlling ROS production.

ZmSOD4 (*Zm00001d029170*) was expressed along the entire primary root, but transcripts were more abundant in the meristematic cells. However, its expression was not affected by two hours of nitrate supply (Fig. 7B). On the other hand, *ZmCAT2* was transcribed more abundantly in the mature zones (3 and 4) and was almost absent or only slightly expressed in the meristematic tissues and TZ cells, although in this last root portion *ZmCAT2* transcription received early stimulation from nitrate supply. Significant differences were also observed in the root maturation zone (4), where a 5-fold increase in its expression was observed. No effects were found in the meristem and only minor differences were observed in the elongation zone (Fig. 7B).

MPK7, MKK2, 6PGDH and G6PD

We also assessed the expression of two previously identified genes (Trevisan et al. 2015) encoding two maize orthologues (*Zm00001d036215* and *Zm00001d045359*) of ATMAPK6 (AT2G43790) and ATMKK2 (AT4G29810) of Arabidopsis, in light of the crucial and transversal role that these two MAPK play in the signalling leading to the response to oxidative stress (Jalmi and Sinha, 2015). In nitrate-deprived roots, both these genes were highly expressed in the meristem (zone 1), where transcript amounts were 4-6 times more abundant than in the other three zones. However, after two hours of nitrate supply their expression appeared significantly reduced in the meristem (zone 1) (0.7-0.6 f.c) and clearly induced in the transition zone (zone 2) (2-4 f.c.) (Fig. 7C).

Finally, we evaluated the expression of two additional genes (*Zm00001d025015* and *Zm00001d049187*) previously identified as nitrate responsive (Trevisan et al. 2015) and encoding, respectively, Phosphogluconic acid dehydrogenase (6PGDH) and Glc-6-phosphate dehydrogenase (G6PD) (Fig. 7D). The transcription of both was highly induced by nitrate in all four root zones. However, despite zone 1 (M) exhibiting the highest transcript accumulation, the effect of nitrate was particularly evident in zone 2 (TZ), which had the highest up-regulation (13 f.c.), while the increases in the other two root portions ranged from 6 to 8 (Fig. 7D).

Changes in the total amount of ascorbate and glutathione in response to nitrate provision

There were no significant differences in the amount of total Ascorbate (Asc) accumulation along the root, as all four zones had similar contents. Nor did we observe any effects on Asc content after nitrate was supplied to N-deprived roots (Fig. 8A).

On the other hand, whereas there was no significant change in total glutathione (GSH) content along N-depleted roots, when nitrate was supplied there was a significantly increase in its content in the meristem (zone 1) and a considerable decrease in the elongation zone (zone 3). The content of the glutathione precursor cysteine (Cys) exhibited increasing accumulation from the distal to the proximal regions of the root in both nutritional conditions. However, the amount of Cys was significantly higher in the elongation zone (zone 3) of N-deprived roots than in the same zone of nitrate-supplemented plants (Fig. 8C). Cys-gly (the product of glutathione degradation by gamma-glutamyl transpeptidase (GGT) activity) was present in the four zones of the maize roots, and nitrate supply did not affect its spatial distribution (Fig. 8D), suggesting that availability of the ion does not affect the GGT degradation process in the primary root. As far as g-EC was concerned (Fig. 8E), it was found only in the mature zone (zone 4) of the root and its accumulation was not affected by the nitrate. These results indicate that 2 h of nitrate provision does not affect the distribution of ascorbate but is sufficient to redistribute thiols along the primary root.

Effects of N deprivation on the balance between the oxidised and reduced forms of ascorbate and glutathione

Although there was no appreciable difference in the total amount of ascorbate along the root nor in response to nitrate availability, its oxidised form, dehydroascorbic acid, (DHA) was detectable only in the apical region of the root (zone 1), mainly in the meristem, and to a lesser extent in the TZ cells (zone 2). It accumulated significantly in both these portions following nitrate provision, with values 2.5 times higher in the meristem and 4 times higher in the TZ than the values in N-depleted roots (Fig. 9A, B). The oxidised form of glutathione (GSSG) was detected in all four root portions and was always the minor fraction of total glutathione. However, the oxidised glutathione content (GSSG) was three times higher in meristematic tissues of N-depleted roots (zone 1) than in the same root zone of seedlings supplied with nitrate for two hours (Fig. 9A, B). No significant differences were observed in the TZ (zone 2) and MZ (zone 4), while in the EZ (zone 3) the opposite trend was observed. As far as the oxidised form of the dipeptide Cys-Gly is

concerned, a trend similar to that of DHA was observed in the meristem (zone 1) and in the transition zone (zone 2), with nitrate-supplied roots having significantly higher amounts. On the other hand, in the elongation zone (zone 3) a reversion of the accumulation was noticed, with a larger amount of oxidised Cys-Gly in -N roots. No differences were found in the mature zone (zone 4) of the root.

The opposite trends observed in the amounts of GSSG and oxidised Cys-Gly in the meristematic region (zone 1) may be explained by the presence of intense gamma-glutamyl-transpeptidase (GGT) activity, which is specifically localised in this region, as revealed by enzyme-histochemical detection (Fig. 9C).

Discussion

Primary root growth is determined by the rate of cell division in the apical meristem and cell elongation in the elongation zone. The transition from proliferation to elongation in the primary root occurs in a specific zone, defined as the transition zone (Baluska et al. 2010), which marks the initial stage of the differentiation processes. The location of this zone depends on each cell type, and exogenous and endogenous stimuli can change it. Previous studies have shown that 2 hours of nitrate provision stimulates maize root growth, which in turn is associated with increased cell size, but a more prolonged exposure to nitrate (24 and 48 h) triggers the primary root to slow down its growth compared with N-deprived plants (Manoli et al. 2016 and Fig. 3K). Moreover, NO, auxin and Strigolactones (SLs) seem to act in coordination in cells of the TZ to regulate the early response of the maize root apex to nitrate (Manoli et al. 2016). Preliminary results suggest that ROS metabolism might also take part in the signalling leading to maize root adaptation to N fluctuations. In fact, an untargeted approach aimed at characterising early TZ transcriptomic response to nitrate (Trevisan et al. 2015) identified a gene encoding a putative bHLH DNA-binding domain superfamily protein (Zm00001d037569, orthologue of AtUPB1) among the transcripts present in the TZ of N-deprived roots and down-regulated by early nitrate provision.

In Arabidopsis root elongation zone UPBEAT1 (UPB1) negatively regulates the expression of class III peroxidases leading to an increase in H₂O₂ accumulation (Tsikagoshi et al. 2010), which, according to the author's model, is fundamental for inducing cells to differentiate. In plant roots, superoxide is mainly produced in the apoplastic space by RBOHs (also known as NADPH oxidases). The superoxide is then converted to H₂O₂ and O₂^{•-} spontaneously or enzymatically (Bowler et al. 1992) and hydrogen peroxide is then degraded by the class III peroxidases, which are also secreted in the apoplast (Cosio 2009).

In the present work, we found an accumulation of H₂O₂ in the cells overlying the meristem in N-deprived roots (24 and 48 h) (Fig. 3). The supply of nitrate, instead, triggered a different pattern of O₂^{•-} and H₂O₂ accumulation, lowering the spread of H₂O₂ and switching the equilibrium in favour of O₂^{•-} species, which are consequently more abundant and more widely diffused in nitrate-supplied roots.

This profile of ROS distribution is consistent with the observed patterns of expression of *ZmUPB1* and peroxidase (*ZmPRX112*). Indeed, the *ZmUPB1* transcript exhibited the highest amount of mRNA accumulation in the TZ-enriched segment (zone 2), where it also greatly increased following nitrogen depletion (Fig. 4). Moreover, in the same root segment the expression of a gene encoding a class III

peroxidase (*Zm00001d024119*, *ZmPRX112*; Wang et al. 2015) was significantly down-regulated by nitrogen deprivation, thus displaying a specular transcriptional pattern compared with *ZmUPB1*. This result seems to suggest a conserved function of UPB1 also in maize.

Tsukagoshi et al. (2010) demonstrated that in *Arabidopsis* the spatial distribution of superoxide and hydrogen peroxide is critical in determining where cells in the root tip transit from a zone of proliferation to a zone of elongation and differentiation, and that peroxidase activity regulation by UPB1 is a key element in maintaining this equilibrium. Our results suggest that nitrate may affect the primary root architecture (Fig. 3K) by disturbing this balance and that the molecular regulation of this process primarily occurs in a small root portion between the meristem and the elongation zone, namely the transition zone.

Moreover, in situ localisation of *ZmUPB1* transcripts showed clear evidence of a significant amount of mRNA in the TZ and EZ, and in particular in the epidermis and at the interface of the cortex and the stele in cells which are probably maturing (zone 2) or mature (zone 3) endodermis/pericycle cells (Fig. 5). As such, the spatial expression pattern of *ZmUPB1* supports the hypothesis that UPB1 plays a role in regulating cell cycle activity (Wells et al. 2010) in root pericycle cells. Lateral root primordia (LRP) are initiated from the founder cells in the root pericycle, and in maize this occurs in phloem pericycle cells (Jansen et al. 2012, Casero et al. 1995, Yu et al. 2016). Localisation of ROS during LRP development has recently been reported in *Arabidopsis* (Manzano et al. 2014, Orman-Ligeza et al. 2016) and maize (Orman-Ligeza et al. 2016). Orman-Ligeza also detected H_2O_2 in the middle lamellae of cell walls of *Arabidopsis*.

Using a cell-sorting approach aimed at isolating cells of the LRP, Manzano et al. (2014) found that in *Arabidopsis* *UPB1* and some of its peroxidase targets were highly represented in their dataset and concluded that *UPB1*-dependent ROS accumulation is also important for LR emergence. In the present work, a clear *ZmUPB1* signal was also detected in the emerging lateral root primordia (Fig. 6C). Reyt et al. (2015) suggested that in *Arabidopsis* UPB1 is important in preserving ROS balance to reduce LR emergence in conditions of iron deficiency. Our results seem to indicate that in maize, too, UPB1 should be part of the equipment necessary to control lateral root development and that it could be transcriptionally regulated by nitrate availability. This suggestion is strengthened by the detection of H_2O_2 and $O_2^{\cdot -}$ staining in the emerged LRP (Fig. 3E-H).

Changes in redox homeostasis usually entail alteration of the entire machinery involved in the control of ROS production and scavenging. To gain a more complete picture of the redox status of N-depleted roots, we studied the expression of a number of crucial genes involved in ROS signalling. N depletion triggered specific profiles of *ZmRbohs* (A, B, C and D) transcript accumulation along the primary root, which were altered by nitrate provision, especially in zones 1 and 2, except for *ZmRbohA* whose expression was unaffected by the anion (Fig. 7A). The most conspicuous effect was, however, on *ZmRbohC* transcription which was remarkably up-regulated in meristematic tissues (zone 1) when nitrate was supplied to N-deficient roots. In *Arabidopsis*, *RbohC* is crucial for root hair development (Foreman et al. 2003) and also for hydrotropic root bending (Krieger et al. 2016). Moreover, its expression is strictly correlated with ROS

1 levels and cell length in the root (Zhang et al. 2014), so it is not unexpected that it could also take part in the
2 signalling leading to nitrate regulation of the root in maize.

3 SOD and catalase are important enzymes for ROS detoxification, catalysing the enzymatic dismutation of
4 superoxide to H_2O_2 and their subsequent degradation. The expression profiles of two genes encoding a maize
5 SOD and a member of the catalase gene family (CAT2) differed according to primary root zone and
6 nutritional status (Fig. 7B), suggesting the existence of sophisticated mechanisms of ROS homeostasis
7 control in response to endogenous developmental signals, but also as a response to nutritional stimuli.

8 Oxidative burst induces downstream events involving transduction mechanisms, including the activation of
9 specific MAPK cascades (Jalmi and Sinha, 2015, Lin et al. 2009, Huang et al. 2014). The expression of two
10 genes encoding key components of MAPK signalling (MPK7 and MKK2) in N-depleted roots showed
11 accumulation of transcripts prevalently in the meristem (zone 1), which was significantly reduced in
12 response to nitrate supply, which in turn led to a notable increase in mRNAs in the other three portions, in
13 particular the TZ (zone 2), a trend similar to that observed with *ZmPRX112*. This result lends support to the
14 hypothesis that TZ cells function as a specialised centre for NO_3^- sensing (Trevisan et al. 2015) and, in
15 general, for interpreting environmental stimuli and translating them into plastic responses. The idea that
16 normal root morphogenesis could, at least in part, depend on ROS homeostasis in the root apex has been
17 already put forward and discussed (Kagenishi et al. 2016, Tsukagoshi 2012, 2016). The involvement of ROS
18 during the gravitropic response has also been proposed by other authors (Mugnai et al. 2014).

19 The expression of two genes previously identified by Trevisan et al. (2015), one encoding Phosphogluconic
20 acid dehydrogenase (6PGDH), the other encoding Glc-6-phosphate dehydrogenase (G6PD), also displayed
21 high responsiveness to nitrate provision, which strongly induced their transcription in all the root portions
22 examined, although more markedly in cells of the transition zone (zone 2). They encode enzymes of the
23 pentose phosphate pathway (PPP) (Nancy R. Hofmann, 2012) and their activities produce NADPHs, which
24 serve as the main form of reducing power used for the detoxification of reactive oxygen species, but also as a
25 substrate for NADPH oxidases.

26 Glutathione and its derived compounds are crucial molecules for controlling the oxidative status of living
27 tissues (Hernandez et al. 2015). In N-deprived roots, glutathione was similarly distributed along the primary
28 root, but the presence of nitrate led to an increase in its content in the meristematic zone (zone 1) and to a
29 parallel decrease in the elongation zone (zone 3) (Fig. 8B). Furthermore, the meristems (zone 1) of roots
30 grown under N deficiency had a high content of the oxidised form of glutathione, but it was significantly
31 lower after two hours of nitrate provision (Fig. 9A). This result seems to indicate that N-deficiency induces a
32 redox shift towards more oxidative conditions in the meristematic zone (zone 1), an observation that is also
33 corroborated by the pattern of MAPK transcript accumulation described above.

34 Plants with a mutation on the RML1 gene, which encodes the first enzyme of glutathione biosynthesis, are
35 unable to form an active root meristem (Vernoux et al. 2000, Yu et al. 2013). This suggests that the correct
36 functioning of the glutathione cycle, resulting in a suitable equilibrium between the oxidised and reduced
37 forms of glutathione, should be crucial to guaranteeing correct root apex growth. We may, therefore,

hypothesise that N deprivation affects primary root growth by forcing the accumulation of oxidised glutathione in meristematic cells. Furthermore, the presence of intense gamma-glutamyl-transpeptidase (GGT) activity in this region (Fig. 9C) and its greater affinity with the oxidised form of glutathione (Ohkama-Ohtsu 2007) seems to explain the opposite trend observed for GSSG and cys-gly accumulation. The presence of GGT in this region has been related to the maintenance of a proper redox balance and/or redox sensing (Masi et al. 2015).

Overall, our results lead us to hypothesise (Fig. 10) that N-deprived maize roots have a typical redox profile characterised by the prevalence of H_2O_2 spread. This seems to depend on the up-regulation of *ZmUPBI* transcription occurring in cells of the TZ (zone 2), which in turn negatively regulate the expression of *ZmPRXI12*. The consequent increase in H_2O_2 accumulation along the first mm of the root apex (zones 1 and 2) probably triggers the root to grow in search of nitrogen. On the other hand, nitrate provision to N-depleted roots rapidly switched off *ZmUPBI* transcription in the TZ (zone 2), with a consequent induction of *ZmPRXI12*, thereby reinstating the equilibrium between H_2O_2 and $O_2^{\cdot -}$ and probably triggering the reduction of primary root growth observed after 24 h of nitrate supply.

N-deprived roots also seem to be characterised by a higher oxidative status, which is fine-tuned in response to the early supply of nitrate, as a result of the concomitant regulation of many factors controlling redox homeostasis in plant cells and conceivably affecting the redox state of ascorbate and glutathione, the main redox couple in plant cells. ROS scavenging results in the formation of dehydroascorbic acid, which is transported into cells where it is reduced to ascorbic acid by GSH. Following NO_3^- supplementation, the antioxidant cascade restores the glutathione redox state to levels more commonly observed (95-99% reduction) by increasing the total glutathione content and degrading its oxidised form.

Other players that would add greater detail to the present picture needs to be identified. Overall, this complex scenario highlights the fact that the interplay between ROS and antioxidant reactions may control the response to N supplementation and confirms the crucial role of TZ (zone 2) cells in sensing and translating nitrate availability in the soil.

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Supporting information

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

Fig.S1. Q-PCR analyses of two maize peroxidases (Zm00001d017696 and Zm00001d014467).

Fig. S2. Enrichment analysis of differentially expressed genes identified by RNAseq analysis (Trevisan et al. 2015).

Table S1. List of primers used in the Q-PCR experiments.

Figure captions

Fig. 1. Primary roots of maize seedlings exposed to the presence or absence of a nitrate source (1 mM KNO₃, 2 h) were sampled as illustrated. The primary root was divided into four portions (zones 1, 2, 3 and 4) at fixed distances from the root tip (0-2mm, 2-4mm, 4-8mm and 8-18mm, respectively).

Fig. 2. Identification of a maize orthologue of AtUPB1 (AT2G47270.1). An RNA-seq experiment (Trevisan et al. 2015) revealed a cluster of genes that are strongly down-regulated by short-term nitrate exposure. Zm00001d037569 was identified among the negatively-regulated DEGs (A). Panel A shows the presence of ZmUPB1 together with the main characteristics of the genes, panel B reports the features of the sequence, and panel C shows the 2d protein structure prediction.

Fig. 3. Light Stereomicroscope pictures of NBT (A, B, E and F) and DAB (C, D, G and H) staining to detect superoxide and H₂O₂. The brownish colour represents accumulated H₂O₂, the bluish colour accumulated O₂⁻. Seedlings were grown in a nitrate-depleted solution for 24 h and were then transferred to a nitrate-supplied solution (+NO₃⁻) or a nitrate-depleted solution (-N). After 24 h the plants were stained. The most representative picture is shown here (n = 20). Bars = 2mm.

Images show NBT and DAB staining of primary root apices of nitrogen depleted (A and C) and nitrate-supplied (B and D) maize seedlings.

ROS were localised in mature zones of nitrogen-depleted (E and G) and nitrate-supplied (F and H) roots.

Comparison of NBT and DAB staining intensities (arbitrary units) are shown in panel I. Error bars represent SEs from triplicate experiments. Asterisks indicate means differing significantly from the control group -N (*P* < 0.01) based on the ANOVA

A working model of ROS redistribution in response to 24 h of nitrate provision is shown in panel J.

Panel K shows the repression of primary root growth exerted by 24 h of nitrate provision.

Fig. 4. QPCR analysis of the levels of ZmUPB1 expression in the four portions (zone 1, zone 2, zone 3 and zone 4) of maize primary roots. Three-day-old seedlings were grown in control conditions without nitrate (-N) and then transferred to a nitrate-supplied solution (+1mM KNO₃, black square) or a nitrate-depleted solution (-N, grey circle) for 2 h. At the end of the treatment, the primary roots were sectioned (zone 1, zone 2, zone 3 and zone 4) and harvested. The expression values were normalised to the endogenous housekeeping genes (Manoli et al. 2012). The relative quantities of each mRNA were calibrated against the amount in -N root section 2, used as the reference sample. Three biological replicates were performed. Error bars represent SEs.

Fig. 5. Identification of maize orthologues of the Arabidopsis direct targets of UPB1 (A) and qPCR analysis of ZmPRX112 expression levels (B) in the four root portions (zone1, zone 2, zone 3 and zone 4) of 3-day-old maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrogen-depleted solution (-N, grey circle) or a nitrate-supply solution (1mM KNO₃, black square) for 2 h. The relative quantities of each mRNA were calibrated against the amount in -N root section 2, used as the reference sample. Error bars represent SEs from triplicate experiments.

Fig. 6. In situ hybridisation of maize root apices with specific probes for transcript ZmUPB1 (A). Longitudinal sections of the primary roots of plants grown in nitrate-deprived solution were allowed to hybridise with digoxigenin-labelled RNA probes (sections I-V). Hybridisation signals are visible as brownish-purple staining. Hybridisations with sense probes are shown in sections VI and VII. Bar = 100 µm. The separation between zones 1, 2, 3 and 4 of the primary root are shown in section I (panel A). In situ hybridisation of cross-sections of nitrate-depleted maize roots with specific probes for transcript ZmUPB1 (panel B). Cross-sections were taken from each part of the root; numbers indicate the positions they were taken from (from I, the quiescent centre, to VII, 1 cm shootward in the root). Panel C shows in situ hybridisation of ZmUPB1 antisense probe at different developmental stages in maize seedlings grown in the absence of nitrogen sources.

Fig. 7. QPCR analyses of genes involved in ROS generation (*Rbohs*, panel A), homeostasis (*CATs*, *SOD*, panel B) and signalling (MAPKs, panel C; *6PGDH* and *G6PD*, panel D). Their relative abundances in the four root portions (zones 1, 2, 3 and 4) of seedlings grown for 24 h in a nitrogen-depleted solution then transferred to a nitrate-supplied solution (+KNO₃ 1mM, black bars) or a nitrogen-deprived solution (-N, grey bar) for 2 h are shown in the histograms. Error bars represent SEs from triplicate experiments.

Fig. 8. Ascorbic acid (Asc), Gluthatione (GSH), cystein (cys), cysteine-glycine and γ-glutamylcysteine contents in four root portions (zones 1, 2, 3 and 4) of maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrate-supplied solution (+KNO₃ 1mM, black bars) or a nitrogen-deprived solution (-N, grey bar) for 2 h.

Data (means ± SE) are the combined results from four independent replicates with 15–20 seedlings per data point. Asterisks indicate statistically significant differences from the -N control (ANOVA, $P < 0.05$).

Fig. 9. Content analysis of oxidised ascorbate (DHA), oxidised glutathione (GSSG) and CGox in four root portions (zones 1, 2, 3 and 4) of maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrate-supplied solution (+KNO₃ 1mM, black bars) or a nitrogen-deprived solution (-N, grey bar) for 2 h. Bars indicate the means ± standard errors of four replicates (n = 15-20 seedlings). Asterisks indicate statistically significant differences from the -N control (ANOVA, $P < 0.05$).

The ratio of reduced to oxidised thiols in portions of maize seedlings are reported in panel B.

Panel C shows the enzyme histochemical detection of GGT activity in maize root tips. Red staining indicates active sites of glutathione degradation.

Fig 10. Proposed model for ROS modulation by UPBEAT1 in the primary root apex of maize.

Short-term (2 h) nitrate provision (+KNO₃, 1mM) is sufficient to inhibit the transcription of a putative orthologue of UPBEAT1 in nitrogen-deprived maize seedlings (24 h, -N).

In nitrogen-deprived plants, ZmUPB1 TF down-regulates a member of the class III peroxidases, which results in regulation of the production of H₂O₂ in the elongation zone (zone 3). At the same time, O₂^{•-} is overproduced in the meristematic zone (zone 1) of roots exposed to nitrate compared with nitrogen-deprived plants. These cellular processes meet in the transition zone (zone 2). In our work, we have demonstrated that the gradient of O₂^{•-} and H₂O₂ was controlled early on by the presence of nitrate in the nutrient solution.

The meristematic cells divide in the proliferation zone (zone 1), which has a high level of O₂^{•-}. After reaching the transition zone (zone 2), in the face of increased H₂O₂ accumulation they stop dividing and start to elongate and differentiate, ultimately reaching the differentiation zone.

In this scenario, by regulating the expression level of the bHLH transcriptional factor UPB1, nitrate also regulates the size of the primary root, thereby balancing the O₂^{•-} / H₂O₂ ratio.

Fig. 1. Primary roots of maize seedlings exposed to the presence or absence of a nitrate source (KNO_3 1mM, 2 h) were sampled as illustrated. The primary root was divided into four portions (zones 1, 2, 3 and 4) at fixed distances from the root tip (0-2mm, 2-4mm, 4-8mm and 8-18mm, respectively).

80x53mm (300 x 300 DPI)

Fig. 2. Identification of a maize orthologue of AtUPB1 (AT2G47270.1). An RNA-seq experiment (Trevisan et al. 2015) revealed a cluster of genes that are strongly down-regulated by short-term nitrate exposure. Zm00001d037569 was identified among the negatively-regulated DEGs (A). Panel A shows the presence of ZmUPB1 together with the main characteristics of the genes, panel B reports the features of the sequence, and panel C shows the 2d protein structure prediction.

124x144mm (300 x 300 DPI)

Fig. 3 Light Stereomicroscope pictures of NBT (A, B, E and F) and DAB (C, D, G and H) staining to detect O₂^{•-} and H₂O₂. The brownish colour represents accumulated H₂O₂, the bluish colour accumulated O₂^{•-}. Seedlings were grown in a nitrate-depleted solution for 24 h and were then transferred to a nitrate-supplied solution (+KNO₃ 1mM) or a nitrate-depleted solution (-N). After 24 h the plants were stained. The most representative picture is shown here (n = 20). Bars = 2mm. Images show NBT and DAB staining of primary root apices of nitrogen depleted (A and C) and nitrate-supplied (B and D) maize seedlings. ROS were localised in mature zones of nitrogen-depleted (E and G) and nitrate-supplied (F and H) roots. Comparison of NBT and DAB staining intensities (arbitrary units) are shown in panel I. Error bars represent SEs from triplicate experiments. Asterisks indicate means differing significantly from the control group -N (p < 0.01) based on the ANOVA.

166x130mm (300 x 300 DPI)

Fig. 4. QPCR analysis of the levels of ZmUPB1 expression in the four portions (zone 1, zone 2, zone 3 and zone 4) of maize primary roots. Three-day-old seedlings were grown in control conditions without nitrate (-N) and then transferred to a nitrate-supplied solution (+1mM KNO₃, black square) or a nitrate-depleted solution (-N, grey circle) for 2 h. At the end of the treatment, the primary roots were sectioned (zone 1, zone 2, zone 3 and zone 4) and harvested. The expression values were normalised to the endogenous housekeeping genes (Manoli et al. 2012). The relative quantities of each mRNA were calibrated against the amount in -N root section 2, used as the reference sample. Three biological replicates were performed. Error bars represent SEs.

82x84mm (300 x 300 DPI)

A

UPB1 Direct Target*	maize homolog (GRAMENE)
At5g17820; Per57	Zm00001d017696
At4g16270; Per40	Zm00001d024119
At4g11290; Per39	Zm00001d014467

*Tsukagoshi et al. 2010

Fig. 5. Identification of maize orthologues of the Arabidopsis direct targets of UPB1 (A) and qPCR analysis of ZmPRX112 expression levels (B) in the four root portions (zone1, zone 2, zone 3 and zone 4) of 3-day-old maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrogen-depleted solution (-N, grey circle) or a nitrate-supply solution (1mM KNO₃, black square) for 2 h. The relative quantities of each mRNA were calibrated against the amount in -N root section 2, used as the reference sample. Error bars represent SEs from triplicate experiments.

104x135mm (300 x 300 DPI)

Fig. 6. In situ hybridisation of maize root apices with specific probes for transcript ZmUPB1 (A). Longitudinal sections of the primary roots of plants grown in nitrate-depleted solution were allowed to hybridise with digoxigenin-labelled RNA probes (sections I-V). Hybridisation signals are visible as brownish-purple staining. Hybridisations with sense probes are shown in sections VI and VII. Bar = 100 μ m. The separation between zones 1, 2, 3 and 4 of the primary root are shown in section I (panel A). $\parallel$ $\perp$ In situ hybridisation of cross-sections of nitrate-depleted maize roots with specific probes for transcript ZmUPB1 (panel B). Cross-sections were taken from each part of the root; numbers indicate the positions they were taken from (from I, the quiescent centre, to VII, 1 cm shootward in the root). Panel C shows in situ hybridisation of ZmUPB1 antisense probe at different developmental stages in maize seedlings grown in the absence of nitrogen sources.

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Fig. 7. QPCR analyses of genes involved in ROS generation (Rboh, panel A), homeostasis (CATs, SOD, panel B) and signalling (MAPKs, panel C; 6PGDH and G6PD, panel D). Their relative abundances in the four root portions (zones 1, 2, 3 and 4) of seedlings grown for 24 h in a nitrogen-depleted solution then transferred to a nitrate-supplied solution (+KNO₃ 1mM, black bars) or a nitrogen-depleted solution (-N, grey bar) for 2 h are shown in the histograms. Error bars represent SEs from triplicate experiments.

157x149mm (300 x 300 DPI)

FIGURE 8. Ascorbic acid (Asc), Glutathione (GSH), cysteine (cys), cysteine-glycine and γ -glutamylcysteine contents in four root portions (zones 1, 2, 3 and 4) of maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrate-supplied solution (+ KNO_3 1mM, black bars) or a nitrogen-depleted solution (-N, grey bar) for 2 h. Data (means $\pm$ SE) are the combined results from four independent replicates with 15–20 seedlings per data point. Asterisks indicate statistically significant differences from the -N control (ANOVA, $p < 0.05$).

152x139mm (300 x 300 DPI)

FIGURE 9. Content analysis of oxidised ascorbate (DHA), oxidised glutathione (GSSG) and CGox in four root portions (zones 1, 2, 3 and 4) of maize seedlings grown for 24 h in a nitrogen-depleted solution and then transferred to a nitrate-supplied solution (+KNO₃ 1mM, black bars) or a nitrogen-deprived solution (-N, grey bar) for 2 h. Bars indicate the means ± standard errors of four replicates (n = 15-20 seedlings). Asterisks indicate statistically significant differences from the -N control (ANOVA, p < 0.05). The ratio of reduced to oxidised thiols in portions of maize seedlings are reported in panel B. Panel C shows the enzyme histochemical detection of GGT activity in maize root tips. Red staining indicates active sites of glutathione degradation.

166x125mm (300 x 300 DPI)

Fig. 10 Proposed model for ROS modulation by UPBEAT1 in the primary root apex of maize. Short-term (2 h) nitrate provision (+KNO₃, 1mM) is sufficient to inhibit the transcription of a putative orthologue of UPBEAT1 in nitrogen-deprived maize seedlings (24 h, -N). In nitrogen-deprived plants, ZmUPB1 TF down-regulates a member of the class III peroxidases, which results in regulation of the production of H₂O₂ in the elongation zone (zone 3). At the same time, O₂⁻ is overproduced in the meristematic zone (zone 1) of roots exposed to nitrate compared with nitrogen-deprived plants. These cellular processes meet in the transition zone (zone 2). In our work, we have demonstrated that the gradient of O₂⁻ and H₂O₂ was controlled early on by the presence of nitrate in the nutrient solution. The meristematic cells divide in the proliferation zone (zone 1), which has a high level of O₂⁻. After reaching the transition zone (zone 2), in the face of increased H₂O₂ accumulation they stop dividing and start to elongate and differentiate, ultimately reaching the differentiation zone. In this scenario, by regulating the expression level of the bHLH transcriptional factor UPB1, nitrate also regulates the size of the primary root, thereby balancing the O₂⁻ / H₂O₂ ratio.

159x60mm (300 x 300 DPI)