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Research area: Genes, Development and Evolution
Cell-type specific gene expression analyses by RNA-Seq reveal local high nitrate triggered lateral root initiation in shoot-borne roots of maize by modulating auxin-related cell cycle-regulation

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Summary:

Financial source:
This research was supported by funds of the State Key Basic Research and Development Plan of China (No. 2013CB127402), the National Natural Science Foundation of China (No. 31272232) and the Innovative Group Grant of National Natural Science Foundation of China (No. 31421092) to CL, the Chinese Universities Scientific Fund (No. 2012YJ039) and the Post-graduate Study Abroad Program of China Scholarship Council (No. 201306350120) to PY. Root research in F.H.’s laboratory is supported by grants of the Deutsche Forschungsgemeinschaft (DFG).

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Plants have evolved a unique plasticity of their root system architecture to flexibly exploit heterogeneously distributed mineral elements from soil. Local high concentrations of nitrate trigger lateral root initiation in adult shoot-borne roots of maize by increasing the frequency of early divisions of phloem-pole pericycle cells. Gene expression profiling revealed that within 12 h of local high nitrate-induction, cell cycle activators (CDKs and CYCBs) were upregulated while repressors (KRP s) were down regulated in the pericycle of shoot-borne roots. In parallel, an ubiquitin protein ligase SCF$^{SKP2B}$-related proteasome pathway participated in cell cycle control. The division of pericycle cells was preceded by increased levels of free IAA in the stele resulting in DR5-RFP-marked auxin response maxima at the phloem poles. Moreover, laser capture microdissection-based gene expression analyses indicated that at the same time a significant local high-nitrate induction of the monocot-specific $PIN9$ gene in phloem pole cells modulated auxin efflux to pericycle cells. Time-dependent gene expression analysis further indicated that local high-nitrate availability resulted in PIN9-mediated auxin efflux and subsequent cell-cycle activation which culminated in the initiation of lateral root primordia. The present study provides novel insights into how adult maize roots translate information on heterogeneous nutrient availability into targeted root developmental responses.
Introduction

Roots have developed adaptive strategies to reprogram their gene expression and metabolic activity in response to heterogeneous soil environments (Osmont et al., 2007). By this way, local environmental stimuli can be integrated into the developmental program of roots (Forde, 2014; Giehl and von Wirén, 2014). In resource-depleted environments, an important heterogeneously distributed soil factor is nutrient availability, which then directs lateral root growth preferentially into nutrient-rich patches (Zhang and Forde, 1998; Lima et al., 2010; Giehl et al., 2012). Such directed lateral root development depends on regulatory networks that integrate both local and systemic signals to coordinate them with the overall plant nutritional status (Ruffel et al., 2011; Guan et al., 2014). As demonstrated by the impact of the N status-dependent regulatory module CLE3-CLV1, economizing the costs for root development is pivotal for a resource-efficient strategy in nutrient acquisition (Araya et al., 2014). In recent years, strategies on yield and efficiency improvement have been developed that are primarily based on the manipulation of root system architecture (Gregory et al., 2013; Lynch, 2014; Meister et al., 2014). A common imperative of these strategies is to develop crops that use water and nutrients more efficiently allowing the reduction of fertilizer input and potentially hazardous environmental contamination.

Maize (Zea mays L.) plays an eminent role in global food, feed and fuel production, which finally relies on a particular root system (Rogers and Benfey, 2015). The genetic analysis of maize root architecture revealed a complex molecular network coordinating root development during the whole life cycle (reviewed in: Hochholdinger et al., 2004a, 2004b). Identification of root type-specific lateral root mutants in maize emphasized the existence of regulatory mechanisms involved in the branching of embryonic roots which are distinct from those in post-embryonic roots (Hochholdinger and Feix, 1998; Woll et al., 2005). Under heterogeneous nutrient supplies, nitrate-rich patches increased only the
length of lateral roots in primary and seminal roots, while they increased both, length and
density of lateral roots from shoot-borne roots of adult maize plants (Yu et al., 2014a).
Remarkably, modulation of the extensive post-embryonic shoot-borne root stock has a
great potential to improve grain yield and nutrient use efficiency (Hochholdinger and
Tuberosa, 2010).

Lateral root branching is critical to secure anchorage and ensure adequate uptake of water
and nutrients. In maize, these roots originate from concentric single-file layers of
pericycle and endodermis cells (Fahn, 1990; Jansen et al., 2012). Lateral root initiation is
the result of auxin-dependent cell cycle progression (Beeckman et al., 2001; Jansen et al.,
2013a). Most of the molecular changes during the cell cycle, as for instance the induction
of positive regulators such as cyclins (CYCs) and cyclin-dependent kinases (CDKs) and
the repression of Kip-related proteins (KRPs) thus account for a reactivation of the cell
cycle (Beeckman et al., 2001; Himanen et al., 2002, 2004). In eukaryotes,
ubiquitin-mediated degradation of cell-cycle proteins plays a critical role in the regulation
of cell division (Hershko, 2005; Jakoby et al., 2006). Conjugation of ubiquitin to a
substrate requires the sequential action of three enzymes: ubiquitin-activating enzyme
(E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). The E3
enzymes are responsible for the specificity of the pathway, and several classes of E3
enzymes have been implicated in cell cycle regulation including the SCF
(SKP1-cullin-F-box protein) and RING-domain ubiquitin ligases (del Pozo and Manzano,
2013). The F-box protein SKP2B (for S-Phase Kinase-Associated Protein2B) encodes an
F-box ubiquitin ligase which plays an important role in the cell cycle by regulating the
stability of KRP1 and pericycle founder cell division during lateral root initiation (Ren et
al., 2008; Manzano et al., 2012).

It has been demonstrated that auxin is involved in long-distance signaling to adjust root
growth in response to local nutrient availability (Giehl et al., 2012), and it is likely to
serve in long-distance signaling for local nutrient responses as well (reviewed in: Rubio et al., 2009; Krouk et al., 2011; Saini et al., 2013; Forde, 2014). Polar auxin transport is instrumental for the generation of local auxin maxima, which guide these cells to switch their developmental program (Vanneste and Friml, 2009; Lavenus et al., 2013). In *Arabidopsis*, the PIN-FORMED (PIN) family of auxin efflux carrier proteins controls the directionality of auxin flows to maximum formation at the tip or pericycle cells (Benková et al., 2003; Laskowski et al., 2008; Marhavý et al., 2013). Auxin responses in proto-xylem or proto-phloem cells of the basal meristem coincide with the site of lateral root initiation (De Smet et al., 2007; Jansen et al., 2012). In these defined pericycle cells, the phloem pole pericycle founder cells are primed before auxin accumulation occurs (De Smet et al., 2007; Jansen et al., 2012, 2013a). In contrast to dicots, the larger PIN family in monocots has a more divergent phylogenetic structure (Paponov et al., 2005). It is likely that monocot-specific *PIN* genes regulate monocot-specific morphogenetic processes such as the development of a complex root system (Wang et al., 2009; Forestan et al., 2012).

The molecular control of lateral root initiation of the root system to heterogeneous nitrate availabilities is not yet understood in maize. In the present study, the plasticity of lateral root induction in adult shoot-borne roots of maize in response to local high concentration of nitrate was surveyed in an experimental set-up that simulated patchy nitrate distribution. RNA-sequencing experiments and cell type-specific gene expression analyses demonstrated that local nitrate triggers progressive cell cycle control during pericycle cell division. In addition, tissue specific determination of IAA and its metabolites combined with auxin maxima determination by DR5 supported a role of basipetal auxin transport during lateral root initiation in shoot-borne roots. Thereby, this study provides novel insights in how auxin orchestrates cell cycle control under local nitrate stimulation in the shoot-borne root system of maize.
Results

Local high nitrate promotes early pericycle cell divisions and lateral root formation

Heterogeneous nitrate environments were simulated in a split-root system in which different nitrate levels were supplied. To determine how local high nitrate contributes to lateral root formation, we examined emerging lateral roots from 2 days on after local high nitrate stimulation. Striking differences in length and density of lateral roots were observed 6 days after treatment (Figures 1A to 1C). The local effect on the promotion of lateral root density (136% increase) was more significant than that of lateral root length (55% increase; Figures 1B and 1C). Subsequently, early stages of lateral root initiation were monitored in pericycle cells to determine the distinct developmental stages of lateral root primordium formation at different time points and at increasing distances from the root tip. Transverse sections of paraffin-embedded root fragments were analyzed and early pericycle cell divisions were considered as anticlinal or periclinal by Safranin O and Fast Green staining (Supplemental Figure 1A). At 24 hours after treatment (Figure 1D), peak differences in pericycle cell divisions were detected between homogeneous low nitrate and local high nitrate treatments in the region between 5 and 25 mm from the root tip compared to 12 (Supplemental Figure 1B) and 36 hours (Supplemental Figure 1C) after treatment.

To obtain a comprehensive view of lateral root primordium development, microscopic analyses were conducted to determine the developmental stages and number of emerged lateral roots in the region of 5 to 25 mm region from the tip of shoot-borne roots (Figure 1E). Stages I to VIII are defined in Supplemental Figure 2A to D. Consistent with the observation of more emerged lateral roots (Figure 1C) early divisions at stages I-III were significantly induced by local high nitrate stimulation (Figure 1E).

RNA-Seq analyses of the stele of maize shoot-borne roots in response to local high nitrate stimulation
The transcriptome of stele tissue extracted from the region between 5 to 25 mm of shoot-borne roots of the maize inbred line B73 (Figure 2A) was subjected to RNA-Seq to identify genes associated with lateral root initiation in response to the previously determined 24 h local high nitrate stimulation (Figure 1D). Each treatment was analyzed in four biological replicates with 20-24 individual roots per replicate (Supplemental Dataset 1). On average, RNA-Seq experiments yielded ~24 million 90 bp paired-end reads per sample (Supplemental Dataset 1). After quality trimming and removal of duplicate reads, ~87% of all sequences were mapped to the maize reference genome (ZmB73_RefGen_v2; Supplemental Dataset 1). After removal of redundant reads, 66% of the remaining reads mapped uniquely to the filtered gene set of maize (FGSv2, release 5b.60; Supplemental Dataset 1), which comprises 39,656 high-confidence gene models. A gene was declared as expressed, if ≥5 reads mapped in all four replicates of a sample. In total, 22,796 genes were expressed in at least one nitrate treatment (Supplemental Dataset 2). A complete list of expressed genes with normalized expression values is provided in Supplemental Dataset 2.

Pairwise comparisons were performed to determine genes differentially expressed between local high nitrate and homogeneous low nitrate treatment (control). To specifically focus on genes with a strong response to local high nitrate stimulation, only genes with a false discovery rate (FDR) ≤5% and a fold changes (Fc) ≥2 were considered in subsequent analyses. In total, 582 genes were differentially expressed in response to local high nitrate stimulation, among which 508 genes (87%) were upregulated and 74 genes (13%) were downregulated (Figure 2B).

**Overrepresentation of differentially expressed genes involved in cell cycle control and ubiquitin-dependent protein degradation**

Differentially expressed genes were functionally classified according to Gene Ontology (GO) terms using agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php). To identify
significantly overrepresented (FDR ≤1%) functional categories, a singular enrichment anal-
alysis (SEA) was performed. SEA compares each annotated gene to all annotated expressed genes in a given nitrate treatment. Among the 582 differentially expressed genes (Figure 2B), 513 genes were annotated and 26 GO terms displayed overrepresentation (Supplemental Table 1). Among those, several GO terms related to cell cycle regulation were overrepresented (GO: 0006950, 0007050, 0022402, 0004861, 0016538).

Differentially expressed genes were further assigned to MapMan functional categories to compare the distribution of over- and under-represented functional classes between nitrate treatments (Supplemental Table 3). The expected number of genes for each functional group was calculated based on all expressed genes and subsequently compared with the detected number of genes in this group. In the over-represented group “Protein”, 24 genes related to protein degradation (proteolysis) were enriched in bin 29 of MapMan outputs (Supplemental Figure 3, simplified illustration according to MapMan; Supplemental Table 4, complete gene list extracted from MapMan). Protein degradation is partially dependent on proteasomes in cooperation with SKP, Cullin, the F-box-containing complex (SCF) and RING-E3 activities. In total 9 genes that encode F-BOX proteins that belong to the SCF E3 ligase complex were identified in this study (Supplemental Figure 3; Supplemental Table 4). In detail, we identified a SKP2B subunit (GRMZM2G138176) and a subunit of Cullin (GRMZM2G551108) which belongs to an ubiquitin-protein ligase. Moreover, 13 subunits (Supplemental Table 4) of the RING E3 ligase complex and 1 subunit (GRMZM2G428119) of BTB/POZ Cullin E3 ligase complex were found (Supplemental Figure 3). Finally, a subunit (GRMZM2G038126) related to a 26S proteasome regulatory protein was found. The detailed features of ubiquitin-dependent degradation are summarized in Supplemental Table 4.
Expression dynamics profiling on cell cycle progression

RNA-Seq results suggested differential expression of numerous cell cycle genes in the overrepresented group “Cell” and enriched GO term GO:0007050 that control cell cycle arrest upon induction with local high nitrate. Among those, transcript levels of six cell-cycle repressors of the KRP family (GRMZM2G037926, GRMZM2G084570, GRMZM2G101613, GRMZM2G116885, GRMZM2G157510, GRMZM5G854731) that arrest progressive cell cycle activity were downregulated. In contrast, three cell-cycle activators of the CDKB class (CDKB1;1: GRMZM2G495626, CDKB2;1: GRMZM2G068193 and CDKB2;2: GRMZM2G070115) and eight cell-cycle activators of the CYCB family (GRMZM2G005619, GRMZM2G034647, GRMZM2G062453, GRMZM2G073003, GRMZM2G073671, GRMZM2G138886, GRMZM2G310115) were upregulated (Figure 2C; Supplemental Table 2).

To further validate the effects of local high nitrate on cell cycle regulation, expression patterns of selected maize cell cycle genes were examined by qRT-PCR in the stele tissue. After clustering, two diametrically opposed expression patterns were observed compared to the homogeneous low nitrate treatment: CYCB genes were upregulated while KRP genes were downregulated under local high nitrate conditions (Figure 2C). Repression of KRP genes and activation of CYCB genes was observed as early as 12 h after local high nitrate induction (Figure 2D). These results are in line with early pericycle cell divisions after 24 h of high-nitrate stimulation (Figures 1D; Supplemental Figure 1A). Pericycle cells at the phloem poles were isolated by laser capture microdissection (LCM) to determine cell specific expression of cell cycle genes by qRT-PCR. The results of these experiments showed that local high nitrate induced the expression of CYCB, CDKB genes, while the expression of KRP genes was inhibited (Figure 2E).

Auxin redistribution and basipetal auxin transport
RNA-Seq results suggested progressive cell cycle activation and a higher demand for SCF ubiquitin-ligase-mediated protein degradation. Both processes are typical for auxin responses in lateral root initiation and emergence. A maize marker line expressing RFP (red fluorescent protein) under the control of a DR5 auxin-responsive promoter was used to analyze the spatial characteristics of the auxin response in shoot-borne roots. To study the effect of deficient auxin transport on the formation of lateral roots and the localization of the auxin response in maize, shoot-borne roots were split incubated in the presence of 1-N-naphthylphthalamic acid (NPA) once they were initiated (Supplemental Figure 4). In shoot-borne roots treated for 4 days with NPA, no lateral roots emerged compared to either nitrate treatment (Supplemental Figure 4A). Moreover, auxin maxima were observed in differentiated meta-xylem vessels of roots subjected to local high nitrate (Supplemental Figures 4B), while no maxima were found in NPA-treated roots (Supplemental Figures 4C). Instead, at a distance of 6 mm to 8 mm from the root tip, an auxin maximum was established in pericycle cells of local high nitrate treated roots (Supplemental Figure 4D), whereas a diffuse signal was detected and no auxin response maximum was formed in the stele tissue of NPA-treated shoot-borne roots (Supplemental Figure 4E).

To survey the effects of local high nitrate on polar auxin transport, shoot-borne roots grown for 4 days in NPA were transferred to high or low nitrate. After 24 hours, local high nitrate-treated shoot-borne root tissues expressed the RFP signal in both, columella and lateral root cap cells (Figure 3A). In contrast, incubation in homogeneous low nitrate enhanced the RFP signal exclusively within root apical meristem cells (Figure 3B). Typically, a strong RFP signal was observed at the phloem poles of roots grown under local high nitrate in the lateral root initiation zone (Figures 3C). In contrast, weak signals were monitored after homogeneous low nitrate treatment (Figures 3D). Moreover, IAA and its metabolites concentrations were determined to address the effect of nitrate on IAA distribution and its biosynthesis and degradation within root tissues (root tip, stele and...
cortex) in shoot-borne roots (Figure 3E and 3F; Supplemental Figure 5). Significantly higher IAA levels in the root tip and relatively uniform levels across tissues were detected when roots were grown in homogeneous low nitrate condition (Figures 3E and 3F). IAA was distributed asymmetrically across three distinct tissues when induced by local high nitrate, with the highest level in the stele followed by a significant decrease in cortex and root tip (Figures 3E and 3F). In contrast, methyl-IAA (MeIAA), an inactive form of IAA, was not induced by local high nitrate (Supplemental Figure 5A and 5B). This observation was supported by spatio-temporal IAA activity measurements. These experiments demonstrated that auxin was transported from root tip and cortex to the stele as soon as 12 hours after stimulation. Hence, measured auxin was not generated by local biosynthesis or degradation because the concentrations of indole-3-acetonitrile (IAN), a putative IAA precursor and 2-oxoindole-3-acetic acid (OxIAA), a degradation product of IAA remained unchanged when induced by local high nitrate (Supplemental Figure 5C to 5F). In addition, the other two conjugated forms IAA-Ala and IAA-Glu were also not induced by local high nitrate application (Supplemental Figure 5G to 5J).

**Tissue- and cell-specific expression dynamics of ZmPINs**

RNA-Seq revealed that ZmPIN1a, ZmPIN1c and ZmPIN9 were significantly induced in the shoot-borne root stele by local high nitrate treatment (Figure 4A). To further study potential roles of ZmPIN genes in auxin transport between different tissues, tissue-specific expression of the three ZmPIN genes was further monitored by qRT-PCR. These experiments independently confirmed and extended the RNA-Seq results (Figures 4B). ZmPIN1a and ZmPIN1c transcript levels were induced in the root tip (Figure 4C). In cortex tissue only ZmPIN1a was upregulated (Figures 4D). ZmPIN9 was the only of these genes which was exclusively expressed in the stele and subjected to a linear time-dependent induction by local high nitrate (Figures 4B to 4D). Transcript levels of ZmPIN9 closely correlated with those of CYCB, CDKB (Figures 4E) but displayed
reciprocal correlation to those of the KRP genes (Figures 4F). Taken together, tissue-specific expression of ZmPIN genes and the local distribution of DR5 reporter activity indicated that auxin transport might directly or indirectly affect cell cycle processes via regulation of the SCF ubiquitin-ligase complex.

Pericycle founder cells related to lateral root initiation were captured by LCM from shoot-borne roots to further explore the effects of cell-specific auxin efflux on auxin maxima formation. Epi-fluorescence microscopy of unstained transverse sections revealed smaller xylem-pole pericycle cells with thick, auto-fluorescent cell walls, while the large pericycle cells at the phloem poles had thinner cell walls and lacked auto-fluorescence (Supplemental Figures 6A). In maize, pericycle cells and endodermis cells at the phloem poles contribute to lateral root formation (Supplemental Figures 6B and 6C). These cell types were isolated by LCM from transverse root sections at a distance of 5 and 10 mm from the root tip (Supplemental Figures 6D and 6E). Excellent RNA quality was obtained from these samples (RIN values > 6.7; Supplemental Figure 7).

Cell-type specific expression analyses of ZmPIN1a, ZmPIN1c and ZmPIN9 revealed that ZmPIN9 (Figure 5A, Supplemental Figure 8) transcripts were much more abundant under local high-nitrate treatment in phloem pole cells compared to pericycle and endodermis cells, while ZmPIN1a and ZmPIN1c were not (Figure 5B, Supplemental Figure 8). The cell type-specific upregulation of ZmPIN9 under local nitrate indicated that this transporter contributes to auxin provision of adjacent pericycle cells. Taken together, net auxin efflux direction of local high nitrate stimulation navigated by ZmPIN9 is illustrated in Figure 6A.
Discussion

Lateral root branching is a pivotal developmental process of root systems to extract nutrients from heterogeneous soil environments. Local nitrate remolds lateral root patterning by local signaling and in dependence of systemic regulation (Ruffel et al., 2011; Forde, 2014; Guan et al., 2014). Impressive progress has been made in understanding the mechanisms regulating lateral root development in roots of *Arabidopsis* (Giehl and von Wirén, 2014) and crops (von Behrens et al., 2011; Smith and De Smet, 2012; Orman-Ligeza et al., 2013). However, little is known about the mechanisms controlling lateral root branching in adult post-embryonic shoot-borne roots of cereals. The extensive shoot-borne root system of maize substantially contributes to grain yield and nutrient use efficiency (reviewed in: Hochholdinger et al., 2004a, b; Hochholdinger and Tuberosa, 2009). Through our studies of nitrate on lateral root patterning in maize, we identified a dramatic and highly cell type-specific response of post-embryonic shoot-borne roots to local nitrate. With the nitrate-dependent regulation of auxin maxima in phloem-pole cells and downstream cell cycle control, this study unravels a novel link between the availability of heterogeneous nitrate and the developmental programs of lateral root formation.

**Local high nitrate promotes auxin-mediated cell cycle activation during lateral root initiation**

A foraging response of post-embryonic shoot-borne roots and subsequently altered lateral root morphology in adult maize roots was simulated in a “split-root” system (Yu et al., 2014a, b). There, the local availability of nitrate exerted a higher number of lateral root primordia in stages I to III in shoot-borne roots relative to those grown under low nitrate (Figure 1E). This observation suggested that local high nitrate supply significantly affects the first anticlinal and periclinal divisions of pericycle founder cells. In *Arabidopsis*, regulation of CDKs and a class of CDK-inhibitory proteins have been proposed as
regulatory links between the control of cell division and morphological plasticity (De Veylder et al., 2001; Himanen et al., 2002, 2004). The increased frequency of dividing pericycle cells and emerged lateral roots suggested that local high nitrate activates competent but previously inactive pericycle cells (Figures 1C and 1E). This notion is supported by the observation that the gene expression levels of six KRP inhibitors (GRMZM2G037926, GRMZM2G084570, GRMZM2G101613, GRMZM2G116885, GRMZM2G157510, GRMZM5G854731), that suppress the division of competent pericycle cells, were downregulated (Figure 2). Cell cycle genes are differentially expressed in activated and non-activated pericycle cells in Arabidopsis (de Almeida Engler et al., 2009). KRP1, KRP2 and KRP4 have been proposed as primary candidates for controlling the activation of divisions in non-dividing cells and to specifically prevent cell cycle induction for formative divisions of lateral roots in the pericycle (Himanen et al., 2002, 2004; Ren et al., 2008; de Almeida Engler et al., 2009). Accordingly, KRP1 overexpression inhibited auxin-mediated cell division of pericycle cells, resulting in a dramatic decrease in the number of lateral roots (Ren et al., 2008). KRP2 regulates early lateral root initiation by blocking the G1-to-S transition and was downregulated transcriptionally after 4 h of auxin induction (Himanen et al., 2002). KRP4 responded less strongly and showed a weak negative response upon auxin induction (Himanen et al., 2002; de Almeida Engler et al., 2009). The response of KRP3 deviated significantly from that of the other KRP genes and was induced upon transfer to auxin, when it was highly expressed in actively dividing cells in Arabidopsis (De Veylder et al., 2001; Himanen et al., 2002). In contrast, in the stele of maize the orthologous KRP3 gene showed a similar transcript pattern as the other KRP genes in response to auxin or nitrate (Jansen et al., 2013a; Figure 2D). This suggests divergent cell cycle control mechanism between dicots and monocots. Thus, these results show that the homeostasis of KRP proteins, which actively regulate the G1-to-S transition in the pericycle cells, is auxin-dependent. During spontaneous lateral root initiation, competent pericycle cells proceed to the G2 phase of
the cell cycle while the remaining pericycle cells stay in the G1 phase (Beeckman et al., 2001). In the present study, a set of CYCB genes (GRMZM2G005619, GRMZM2G034647, GRMZM2G062453, GRMZM2G073003, GRMZM2G073671, GRMZM2G138886, GRMZM2G310115) and CDKB1;1 (GRMZM2G495626), CDKB2;1 (GRMZM2G068193), CDKB2;2 (GRMZM2G070115) were upregulated and likely act in G2-to-M phase regulation (Figure 2; Supplemental Table 2; Himanen et al., 2002, 2004). In Arabidopsis, CYCB proteins stimulate cell division and tissue growth (Lee et al., 2003). Moreover, B1-type and B2-type CDK proteins display a maximum of kinase activity at the G2-to-M transition and during mitosis (Inzé and De Veylder, 2006). In addition, CYCB2;2 promotes mitotic cell cycle and cell divisions probably through association with CDKB2 in the root meristem (Lee et al., 2003; Sabelli et al., 2014). In the present study, qRT-PCR based time-course experiments on stele-specific gene expression have been implemented to survey the effects of local high nitrate stimulation on the cell cycle. The expression patterns of selected CDKB, CYCB and KRP genes are distinctively clustering in line with their phase-specific control of the cell cycle (Figures 2D and 2E). These nitrate-inducible patterns are thoroughly analogous to the expression profiles observed in Arabidopsis during lateral root initiation induced by auxin (Himanen et al., 2002; Jansen et al., 2013a; Figure 2D). Stability of KRP2 is controlled by CDKB1;1 phosphorylation (Verkest et al., 2005a), although KRP1 does not interact with CDKB1;1 and CYCB proteins (De Veylder et al., 2001). In this model, the CDKB1;1 kinase inhibits KRP2 activity, resulting in a higher activity at the G2-to-M transition and consequently more mitotic cell divisions (Verkest et al., 2005a). In view of these results, we conclude that in maize local high nitrate supply promotes early pericycle cell divisions by modulating mitosis-specific cell cycle progression.

A role of the ubiquitin-proteasome system in nitrate-dependent cell cycle control.
It has been demonstrated that ubiquitin-dependent degradation of key regulatory proteins of the cell cycle plays a crucial role in a sequence of events leading to cell division (reviewed in: del Pozo and Manzano, 2013). In addition to CDK protein phosphorylation, KRP1 and KRP2 are also known to be degraded by the 26S proteasome during the G1-to-S-phase transition of the cell cycle (Verkest et al., 2005a, b; Jakoby et al., 2006). S-phase kinase-associated protein 2B (SKP2B), an F-box protein, that controls cell cycle by targeting KRP1 protein turnover in *Arabidopsis* (Ren et al., 2008). In the stele-specific RNA-Seq analysis presented here, genes contributing to the ubiquitin-proteasome system SCF^{SKP2B} complex were strikingly enriched as illustrated by MapMan visualization (Supplemental Figure 3; Supplemental Table 4). In *Arabidopsis*, KRP1 degradation is dependent on the SCF^{SKP2B} complex that consists of CULLIN (GRMZM2G428119), SKP2B (GRMZM2G138176) and the 26S proteasome (GRMZM2G038126) (Ren et al., 2008; Supplemental Figure 3). Recently, we have also demonstrated that the *rum1* mutant, which is defective in lateral root formation, displays significant inhibition of ubiquitin-dependent protein degradation and cell cycle/division processes (Zhang et al., 2014). Thus, our RNA-Seq results, together with the finding that *SKP2B* overexpression promotes KRP1 degradation in *Arabidopsis* (Ren et al., 2008), suggest that ubiquitin-dependent SCF^{SKP2B} complex might target KRP protein degradation for reactivation of the mitotic cell cycle in maize (Figure 6). This does, however, not exclude the possibility that the cell cycle is directly regulated by auxin at the transcriptional level (Himanen et al., 2002, 2004; Figure 6).

### Basipetal auxin transport modulated by local high nitrate

Auxin is a key regulator of lateral root formation (De Smet et al., 2007; Dubrovsky et al., 2008; Rubio et al., 2009; Krouk et al., 2011; Saini et al., 2013) but also involved in lateral root development in response to local nutrient availability (Krouk et al., 2011; Giehl et al., 2012). Induction of genes specific for G1/S cell cycle transition and activation of protein
degradation is controlled by auxin signal perception and transduction (Himanen et al., 2002, 2004). As early as 3 h or 6 h but not later than 12 h after stimulation with local high nitrate, the genes CYCB1;1 and CYCB2;1 involved in the G2-to-M transition showed simultaneous induction by auxin (Himanen et al., 2002; Jansen et al., 2013a; Figure 2D). This observation supports the notion that phloem pole pericycle cells gain mitotic activity which synchronizes with the induction of G2/M phase-specific genes at the same time (Figures 1D and 2E; Supplemental Figure 1A). In contrast, early changes of cell division induced by nitrate are at least 12 h delayed compared to auxin induction (Figure 2D; Himanen et al., 2002). Based on this observation, we hypothesize that local high nitrate modulates auxin homeostasis required for pericycle cell divisions rather than directly act on the cell cycle genes. In fact, transcriptome and reverse genetic studies have shown that the auxin status and auxin transporters are strongly modulated by nitrogen availability (reviewed in: Krouk et al., 2011).

The basipetal flow of auxin in the lateral root cap and epidermis and its subsequent accumulation in pericycle founder cells are thought to drive the first formative divisions in pericycle cells (Dubrovsky et al., 2001; De Smet et al., 2006). Disruption of this process inhibits lateral root outgrowth in Arabidopsis (Casimiro et al., 2001; Himanen et al., 2002). To address the effects of local high nitrate stimulation on basipetal auxin transport in shoot-borne roots, lateral root initiation was synchronized experimentally by NPA before induction by different nitrate levels (Supplemental Figure 4). NPA treatment keeps pericycle cells in an undifferentiated or cell division-competent state (Jansen et al., 2012, 2013b). Indeed, early vascular differentiation was inhibited at a distance of 6 to 8 mm from the root tip (Supplemental Figures 4B and 4C). Visualization of the activity of the auxin-responsive DR5 reporter indicated that the auxin signal was elevated in those lateral founder cells which were subjected to local high nitrate (Figure 3A) but not in roots grown in homogeneous low nitrate (Figure 3B). Accordingly, higher auxin maxima were formed in the phloem poles and their neighboring cells in the lateral root initiation.
zone in response to nitrate (Figures 3C). In contrast, addition of NPA caused accumulation of auxin in root apical meristem cells (Figure 3B), which decreased auxin levels to a suboptimal level for the induction of founder cell division in the lateral root initiation zone (Casimiro et al., 2001; Figure 3D). These results indicated that local high nitrate may partly restore basipetal auxin transport and release the inhibition from NPA. Auxin maxima in the phloem poles could be a major trigger for the priming of neighboring pericycle cells and for subsequent anticlinal divisions (Jansen et al., 2012; Supplemental Figure 5C). Tissue-specific determination of active IAA and its metabolites validated possible auxin transport from root tip and cortex to root stele tissue as soon as 12 hours after treatment in response to local nitrate supply (Figures 3E, 3F, 4C and 4D; Supplemental Figure 5), which is in agreement with the release of KRP proteins at the same time (Figure 2D). The concentration of MeIAA was not induced by local high nitrate although it has a stronger capacity to induce lateral roots than IAA (Supplemental Figures 5A and 5B). In addition, both the precursor (IAN) (Supplemental Figures 5C and 5D) and the degradation product (OxIAA) (Supplemental Figures 5E and 5F) of IAA were not induced by local high nitrate. Furthermore, conjugated forms of IAA (IAA-Ala and IAA-Glu) presented here might not contribute to the auxin homeostasis (Supplemental Figures 5G to 5J). Based on these results, local nitrate-enhanced basipetal auxin transport may contribute to early auxin gradients in pericycle cells in the lateral root initiation zone.

Cell-type specific expression dynamics of PIN genes navigate auxin efflux for pericycle priming

Polar auxin transport, partially mediated by PIN auxin efflux carriers, is crucial for generating auxin gradients between cells (Benková et al., 2003; Friml et al., 2003; Blilou et al., 2005). Despite their functional redundancy, each PIN protein has been implicated in particular developmental processes in Arabidopsis (Friml et al., 2002a, b, 2003; Blilou
et al., 2005; Marhavý et al., 2013). In contrast to dicots, the phylogenetic structure of the larger PIN families in monocots is more divergent (Paponov et al., 2005; Wang et al., 2009; Forestan et al., 2012). ZmPIN1a, ZmPIN1b, and ZmPIN1c, which are orthologs of AtPIN1, may perform the functions of AtPIN3, AtPIN4, and AtPIN7, for which no orthologs have been identified in maize (Forestan et al., 2012; Villiers and Kwak, 2012). ZmPIN1-mediated transport of auxin and the related auxin fluxes during maize axillary meristem, kernel and ligule development have been studied in detail (Carraro et al., 2006; Gallavotti et al., 2008; Forestan et al., 2010; Johnston et al., 2014). These studies have significantly extended the understanding of auxin transport on the initiation of shoot organs in maize. On top of this, the results of the present study add that ZmPIN1 is likely involved in shootward auxin flows to be channeled through the lateral root cap (Band et al., 2014; Figures 3A and 4C). It is important to note that basipetal auxin flow results in a considerable increase in auxin levels in phloem pole cells at approximately the site where pericycle cells start to divide. This observation supports the notion that local nitrate-mediated basipetal auxin transport plays a role in the activation of pericycle cells.

PIN-mediated auxin efflux further promotes periclinal auxin transport, enabling auxin to move between epidermal, cortical, and endodermal cells (Band et al., 2014). Auxin movement into founder cells has been reinforced by PIN3 localized laterally in the inner-membrane in endodermis cells. This provides a local auxin reflux pathway important for further lateral root initiation (Marhavý et al., 2013). Auxin treatment induces PIN3-GFP in endodermal and cortical cell files of the Arabidopsis root (Marhavý et al., 2013). In maize, ZmPIN1a is exclusively induced by local high nitrate stimulation in the cortex. Hence, ZmPIN1a may contribute to auxin export from the cortex to the stele (Figure 4D). This conclusion is further supported by the expression profile of ZmPIN1a, which displayed significantly higher transcript levels in the endodermis compared to the pericycle (Supplemental Figure 8). The upregulation of ZmPIN1a supports a regulatory function for the endodermis during auxin maximum formation. Moreover, this indicates
that the interaction between pericycle cells and the adjacent endodermis cells defines a
development-specific auxin reflux pathway, which supports pericycle founder cells to
reach auxin threshold levels required to initiating lateral roots. Monocot root-specific
*PIN9* genes have been identified in maize, rice and wheat (Paponov et al., 2005; Wang et
al., 2009; Forestan et al., 2012). In rice, a higher expression level of *OsPIN9* was found in
the root and the stem base compared to other tissues (Wang et al., 2009). Likewise,
*ZmPIN9* is exclusively expressed in shoot nodes and roots of maize (Forestan et al., 2012).
Moreover, *PIN9* was highly expressed in lateral root primordia, pericycle cells and
vascular tissues in rice (Wang et al., 2009), as well as in the endodermis, pericycle and
phloem of maize (Forestan et al., 2012). Taken together, the divergent structure and
phylogeny and the expression of the monocot *PIN9* genes indicate a sub-functionalization
associated with monocot-specific morphogenetic processes (Paponov et al., 2005;
Forestan et al., 2012). In the present study we reveal that *ZmPIN9* expression in the stele
is induced in parallel with the transcriptional upregulation of CYCB proteins and
repression of KRP proteins after local supply of high nitrate (Figure 2D; Figures 4B, 4E
and 4F). Taken together, these results suggest a major role of *ZmPIN9* in auxin efflux
from phloem to pericycle cells (Figures 5 and 6A), and that in maize the dividing
potential of phloem pericycle cells rather than that of xylem pericycle cells relies on
auxin efflux by the monocot-specific *ZmPIN9* protein.

In summary, the present study supports a model for PIN-dependent auxin efflux and cell
cycle activation under local nitrate availability (Figure 6B). Lateral roots are initiated
from the pericycle a process mediated by auxin-related cell cycle progression. Prior to
cell cycle activation, pericycle cells need to be specified as the founder cells by auxin.
*ZmPIN1*-mediated basipetal auxin transport to the lateral root initiation zone and
*ZmPIN9*-dependent redistribution of auxin to pericycle cells are orchestrated by local
high nitrate supply. Auxin relieves the inhibition from KRP proteins for progressive cell
cycle either directly or via induction of the SCF<sup>SKP2B</sup>-regulated ubiquitin-dependent
proteasome. Thus, the present study uncovered a direct regulatory pathway between heterogeneous nitrate stimulation and auxin transport dynamics that underpins cell cycle decisions prior to lateral root initiation.
Material and Methods

Plant material and growth condition

Seeds of the maize inbred line B73 were surface sterilized and germinated in paper rolls in distilled water as previously described (Hetz et al., 1996). Seven days after germination, the endosperm of each seedling was excised and uniform seedlings with two visible leaves and four embryonic roots were transferred to low nitrate (0.5 mM NO$_3^-$, Ca(NO$_3$)$_2$ was used) nutrient solution and grown as previously described (Yu et al., 2014a; “long-term experiment”). Initiation of shoot-borne roots was recorded in a time-course experiment until the emergence of the 7th whorl of shoot-borne roots which were split-treated by high (4 mM NO$_3^-$, Ca(NO$_3$)$_2$ was used) and low nitrate solution as control. The experiment was conducted in a growth chamber in a 16 h light, 25°C and 8 h dark, 21°C cycle for two months.

Synchronization of lateral root initiation

Synchronization of lateral root initiation was performed according to a modified protocol of Himanen et al. (2002) and Jansen et al. (2013b). Plants were grown in low nitrate solution until the 7th whorl of shoot-borne roots started to emerge (~60 days after germination). Then plants were transferred into a 25 μM solution of the polar auxin transport inhibitor NPA (N-1-naphthylphthalamic acid) (Sigma-Aldrich) in 0.5 mM NO$_3^-$ for 4 days. Lateral roots were then synchronously induced by transferring the plants to low (0.5 mM NO$_3^-$) or local high (4 mM NO$_3^-$) nitrate solution without NPA (Supplemental Figure 4).

Morphological and anatomical analysis of lateral roots initiation

To determine lateral root length and density, nitrate-treated shoot-borne roots were harvested, scanned using ScanMaker 9800XL (Microtek, Shanghai, PRC) and subsequently analyzed by WinRHIZO (www.regent.qc.ca). Lateral root density was
calculated by the number of emerged lateral roots divided by the length of the region where lateral roots emerged. Microscopic analysis was conducted to determine the number of lateral root primordia and emerged lateral roots. For this purpose, the total number of lateral root initiation events was counted, and lateral root primordia were classified according to their developmental stages I-III, IV-V, V-VII, and VIII (Malamy and Benfey, 1997; Supplemental Figure 2).

Histochemical, histological and microscopic analysis

Visualization of cell divisions by Safranin O and counter staining by Fast Green Maize shoot-borne roots were fixed in 4% (w/v) paraformaldehyde for 24 h at 4°C as described by Lim et al. (2000). 10 μm sections were prepared by a RM2125RTS-microtome (Leica; www.leicabiosystems.com). Staining of deparaffinized specimens with Safranin O (Roth) and Fast Green FCF (Sigma-Aldrich) was performed according to standard histological procedures (Johansen, 1940). Stained specimens were analyzed under an AxioCam MRC microscope (Carl Zeiss Microimaging, Göttingen, Germany) and documented with Axio-Imager software (Carl Zeiss Microimaging).

Localization of auxin signal response

Transgenic lines carrying a DR5::RFP auxin response reporter with a synthetic auxin promoter were subjected to histological analysis (Gallavotti et al., 2008). Roots were fixed in 4% (w/v) para-formaldehyde (1 × PBS buffer, pH 7.4) under vacuum (500 Mpa) for 10 min at 4°C. The solution was replaced with fresh fixative following two vacuum infiltration steps. Subsequently, the infiltrated samples were incubated at 4°C for > 1 h. Tissues were then rinsed at 4°C several times with 1 × PBS buffer. Root samples were then embedded in 8% low melting agarose (peqGOLD Universal Agarose, peQLab Biotechnologie, Erlangen, Germany) with 0.5% gelatin (AppliChem). Trimmed agarose blocks with properly oriented samples were sectioned in 100 μm increments by a VT1200
vibratome (Leica, Nussloch, Germany), mounted with distilled water and immediately observed under an Axio-Imager epifluorescence microscope (Carl Zeiss) and a confocal laser scanning microscope (Carl Zeiss LSM 780). Auto-fluorescence was detected with an AxioCam MRc microscope (Carl Zeiss) and images were taken with an Axio-Imager (Carl Zeiss). A filter set (Carl Zeiss Filter Set 43) for rhodamine was used for visualization of the RFP signal. Another two filter sets for the CFP signal (Carl Zeiss Filter Set 55) were used and nucleus staining with DAPI (Carl Zeiss Filter Set 38) was performed.

**Indole acetic acid detection by UPLC-ESI-MS/MS**

Based on the histological and DR5 fluorescent results, the most apical 5 mm of the shoot-borne root were excised and defined as root tip. Subsequently, the stele was separated from the cortical parenchyma and epidermis by peeling off the outer tissue layers (Saleem et al., 2009). For each biological replicate, ten individual shoot-borne roots were pooled for root tip, cortical parenchyma and stele tissues and immediately frozen in liquid nitrogen before storage at –80°C and freeze drying.

**SPE extraction of auxins**

The phytohormone extraction procedure was adapted and modified from Kojima et al., (2009) and Seo et al., (2011). Frozen tissues were crushed to fine powder using a TissueLyser (Qiagen, Hilden, Germany) with two steel balls in a 2 ml Eppendorf tube, and then mixed with 180 µl extraction solution in H₂O (Milli-Q® Reference System, Merck, Germany)/methanol (Th. Geyer, Germany) 50/50 containing 0.5% formic acid (Biosolve Chimie, France) for 30 s. The mixture was sonicated for 5 min, then extracted with an overhead shaker for 15 min. Next, 720 µl H₂O were added and the complete extraction solution was again mixed in the overhead shaker for 1h. After centrifugation at 14,000 rpm for 10 min, the supernatant was transferred to a new Eppendorf tube. All
steps were performed at 4°C. Afterwards, the procedure was repeated and the supernatants were combined.

Internal standards were dissolved in methanol and added to each combined supernatant (Supplemental Table 5). The methanol of the extracted solution was then evaporated in a vacuum centrifuge for 20 min and the residue was then used for SPE clean up. The supernatant was then added to a 1cc/30 mg HLB cartridge (Waters, Milford, MC, USA) which was conditioned before with 1 ml methanol containing 0.1% formic acid and equilibrated with 2 × 1 ml H₂O containing 0.1% formic acid. The sample was then washed twice with 1 ml H₂O containing 0.1% formic acid and eluted with 2 × 600 µl 90% methanol with 0.1% formic acid. Now the methanol was evaporated with a vacuum centrifuge and the water residue was filled up to 1 ml with H₂O containing 0.1% formic acid. Then all was mixed for 30 s and sonicated for 2 min at 4°C. In the second SPE step a 1cc/30 mg MCX cartridge (Waters) was used. The MCX cartridge was conditioned with 1 ml methanol containing 0.1% formic acid and equilibrated with 2 × 1 ml H₂O containing 0.1% formic acid. Samples were then added to the column and washed twice with 1 ml H₂O containing 0.1% formic acid. Auxin was then eluted with 2 × 600 µl 100% methanol. Samples were then evaporated in a vacuum centrifuge to dryness, resolved in 10 µl 50% methanol containing 0.5% formic acid and mixed for 30 s and sonicated for 2 min, filled up to 50 µl with 40 µl H₂O and transferred to a UPLC vial for analysis.

LC-MS-MS analysis

10 µl extracted samples were then injected into an ACQUITY Ultra-performance LC system (Waters) coupled with a Xevo TQ MS mass spectrometer (Waters). The sample analytes were separated on an ACQUITY UPLC® BEH C18 (Waters) coupled with a VanGuard pre-column BEH C18 (Waters). For quantification of the analytes, one fragment ion was used for quantification, two for qualification, and two as internal standards (Supplemental Table 5). MS data was processed by using TargetLynx V4.1
SCN 904 (Waters) (Supplemental Text 1). The peak area of the diagnostic product ion was used for quantification.

Quantitative RT-PCR

qRT-PCR was performed in a CFX 384TM Real-Time System (Bio-Rad, Munich, Germany). RNA was isolated from the three root tissues described above using the RNeasy® Mini Kit (Qiagen). All RNA samples were quantified and qualified using a NanoDrop spectrophotometer (Thermo Scientific, PeqLab, Germany) and agarose gel electrophoresis, respectively. cDNA for qRT-PCR analysis was synthesized from 1 μg total RNA with the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Each PCR reaction contained 4 μl MESA Blue qPCR™ Mastermix Plus for SYBR Assay no ROX (Eurogentec, Cologne, Germany), 1 μl cDNA sample and 100 nM gene-specific oligonucleotide primers to a final volume of 8 μl. The primer efficiency of each oligonucleotide was calculated using the following dilution series: 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128. The relative expression levels of the transcripts were calculated with reference to the housekeeping gene *myosin* (Genbank AC: 486090G09.x1) previously used in the laboratory to quantify gene expression in maize roots (Dembinsky et al., 2007). Significant differences in gene expression levels were determined by paired Student’s *t*-test or one-way ANOVA analysis. Cell cycle and *PIN* gene expression analyses were performed with primer pairs as previously described (Rymen et al., 2007; Forestan et al., 2010, 2012).

Root stele-specific RNA-Seq experiment

RNA isolation and cDNA library preparation for RNA-sequencing

The steles of shoot-borne roots were separated from cortical parenchyma between 5 and 25 mm from the root tip. Dissected stele tissues were pooled and ground in liquid nitrogen. RNA was extracted by the RNeasy® Plus Universal Mini Kit (Qiagen). RNA
quality was assessed via agarose gel electrophoresis and an Agilent RNA 6000 Nano
LabChip® on Agilent 2010 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA).
All samples had an excellent quality as documented by RIN (RNA integrity number)
values >9.7. During the quality control steps, an Agilent DNA 1000 LabChip (Agilent
Technologies) and ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems,
Foster City, CA, USA) were used for quantification and qualification of the sample
libraries. The cDNA libraries for RNA sequencing were constructed according to the
TruSeq™ RNA Sample Prep Kit (Illumina, San Diego CA, USA). For sequencing, eight
libraries were pooled in one lane of a flow cell. Cluster preparation and paired-end read
sequencing were performed according to the HiSeq™ 2000 guidelines (Illumina).

Processing, mapping of RNA-sequencing reads and statistical procedures for analyzing
differentially expressed genes

Raw sequencing reads generated by the Illumina HiSeq 2000 system were initially
processed and quality trimmed with SHORE
(http://1001genomes.org/software/shore.html). At least 20 million clean reads were
obtained by removing reads containing adapters, reads containing >10% unknown bases,
and low quality reads containing >50% low quality bases. Read mapping was performed
with CLC Genomics Workbench
(http://www.clebio.com/products/clc-genomics-workbench/). All high quality reads were
mapped to the maize B73 reference genome (RefGen_v2;
ftp://ftp.gramene.org/pub/gramene/maizesequence.org/) allowing large gaps of up to 50
kb to span introns. In addition, redundant reads mapping at the same starting coordinate
and mapping orientation (stacked reads) were removed from the set of uniquely mapping
RNA-Seq reads. The remaining reads of all samples were projected to the filtered gene
set (FGSv2, release 5b.60; http://ftp.maizesequence.org/release-5b/filtered-set/) of the
B73 reference genome derived from maize genome sequencing project. Only those reads
uniquely mapping to the reference data set were subsequently used for analyses. Differential gene expression was determined using the normalized FPKM (fragments per kilobase of transcript per million reads) values in conjunction with empirical analysis of differential gene expression (Robinson et al., 2010) implemented in the CLC Genomics Workbench using a False Discovery Rate (FDR) ≤5% cutoff (Benjamini and Hochberg, 1995) and a log₂ fold change of ≥1.

Gene Ontology (GO) and metabolic pathway analysis

Gene Ontology (GO) functional categories were assigned to differentially expressed genes using the web-based agriGO software (http://bioinfo.cau.edu.cn/agriGO/analysis.php). Singular enrichment analysis (SEA) was used to compute enriched categories by comparing a list of differentially expressed genes to all expressed genes. According to Benjamini and Yekutieli (2001), multiple comparison correction was performed and FDR was controlled at ≤1%.

The MapMan bin classification system (Thimm et al., 2004) was performed for functional annotation of differentially expressed genes and subsequently visualized based on the functional annotated file ZmB73_5b_FGS_cds_2012 (http://mapman.gabipd.org). To determine if specific functional groups were overrepresented among the differentially expressed genes with reference to all genes represented in the whole genome, the expected number of genes for each functional category was calculated based on the distribution of functional categories among all genes on the genome. To determine if significantly more or fewer genes than expected were detected for each individual category, a Chi-Square Test for independence with Yates’ continuity correction (Yates, 1934) was performed.

Cell-specific gene expression analysis by laser capture microdissection

Fixation, protection and embedding of samples
Target cell types were isolated from frozen sections according to improved modified protocol of Nakazono et al. (2003). Root segments from 5 to 10 mm from the root tip were dissected and fixed immediately in a freshly prepared mixture with 75% (v/v) ethanol and 25% (v/v) acetic acid by vacuum infiltration (300 Mpa) for 15 min on ice and afterwards swirled gently for 1 h on ice at 4°C. The solution was replaced with fresh fixative and vacuum infiltration/swirl steps were repeated twice. To minimize the formation of ice crystals, the fixative was replaced by fresh 15% sucrose (w/v) (Sigma-Aldrich) in 0.01 M diethyl pyrocarbonate (Sigma-Aldrich)-treated PBS buffer (137 mM NaCl, 8.01 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄, pH 7.3) and infiltrated as described before. The 15% sucrose (w/v) vacuum infiltration/swirl step was repeated again. Subsequently, fresh 40% (w/v) sucrose (in 0.01 M diethyl pyrocarbonate-treated PBS buffer) was used to replace 15% sucrose, then the vacuum infiltration/swirl step was conducted as described above and repeated with fresh 40% sucrose solution for three times. The root segments were embedded in 7 × 7 × 5 mm disposable molds (Polysciences Europe GmbH, Heidelberg, Germany) with PolyFreeze-clear tissue freezing medium (Polysciences Europe GmbH). Then molds with oriented segments were held close to the surface of liquid nitrogen until the surface freezing medium of molds froze. Samples were then immediately immersed into liquid nitrogen and stored at –80°C until cryosectioning. The procedure of fixation/protection/embedding was performed within one day.

Cryosectioning and isolation of pericycle founder cells by laser capture microdissection

Prior to cryosectioning, the blocks with root segments were equilibrated in a precooled Cryostat at –28°C for about 15 min, then trimmed into 20 μm thick sections using CM1850 cryotome (Leica) at –28°C. Serial frozen sections were mounted on PEN membrane slides (Carl Zeiss Microscopy, Göttingen, Germany) pre-coated with Poly-L-lysine (0.1% w/v) (Sigma-Aldrich). Subsequently sections were incubated in
ice-cold 70% (v/v) ethanol for several minutes to reduce RNase activity. Prior to LCM, the freezing medium was removed and sections were dehydrated. Sequential washes were performed in staining jars as follows: 50% (v/v) ethanol at RT for 5 min; 70% (v/v) ethanol at RT for 30 s; 95% (v/v) ethanol at RT for 30 s; absolute ethanol at RT for 1 min and dehydration of samples for 2 min in xylene twice at RT. After dehydration, slides were air dried then kept in a vacuum desiccator with silica gel (Sigma-Aldrich). Laser microdissection was performed with a PALM MicroBeam platform (Carl Zeiss Microimaging; www.zeiss.com). At least 40 pericycle founder cells at the phloem poles were isolated from individual cross section. In total ~2000 cells (40 cells × 50 sections) were collected per adhesive cap (Carl Zeiss Microscopy) within 1 h and pooled for downstream analyses.

Cell-specific RNA isolation and linear amplification for quantitative RT-PCR

RNA was extracted from microdissected cells with 10 μl lysis buffer from the Arcturus™ PicoPure® Frozen RNA Isolation Kit (Life Technologies). Reaction tubes were incubated upside down on a 42°C block for 30 min, subsequently they were briefly centrifuged at 800g for 2 min, then RNA extraction was performed according to the manufacturer’s protocol. RNA quality was evaluated using a RNA 6000 Pico LabChip® Kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer (Agilent Technologies). Then, total RNA (>3 ng) was linearly-amplified by the RiboAmp® HS PLUS RNA Amplification Kit (Applied Biosystems), according to the manufacturer’s instructions and RNA quality was determined by a RNA 6000 Nano LabChip® Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNAs were synthesized from linear-amplified RNA using qScript cDNA SuperMix (Quanta Biosciences) and quantitative RT-PCR was performed as described previously.

Accession numbers
Raw sequencing data are stored at the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP058076.
Supplemental material

Supplemental Figure 1 online. Early pericycle cell divisions in shoot-borne roots. (A) Longitudinal section of a shoot-borne root and the first anticlinal divisions of pericycle (red arrows) and endodermis cells (yellow arrows). In the region 5-25 mm proximal of the root tip, peak differences of early pericycle cell divisions (see Figure 1D) were monitored between homogeneous low nitrate (homo LN) and local high nitrate (local HN) treatments. Scale bar: 50 μm. Early pericycle cell divisions in shoot-borne roots 12 (B) and 36 (C) hours after local high nitrate treatment (HAT). Error bars represent SE for four biological replicates. Asterisks denote a significant difference according to paired Student’s t test (*: p ≤0.05; **: p ≤0.01). Homogeneous low nitrate: homo LN; local high nitrate: local HN.

Supplemental Figure 2 online. Different stages of lateral root primordium formation are illustrated by representative images: (A) I-III, anticlinal and periclinal divisions create two or three layers. (B) IV-V, further periclinal division creates a fourth layer. (C) VI-VII, periclinal and anticlinal divisions make the tip of lateral root primordium visible. (D) VIII, emerged lateral root. Scale bar: 50 μm.

Supplemental Figure 3 online. Schematic overview of the components of the ubiquitin-dependent degradation system (SCF^{SKP2B} complex) induced in response to local high nitrate. Log2-fold changes of gene expression (local HN versus homo LN) are illustrated by MapMan. DUB, deubiquitinating enzyme; E1, ubiquitin-activating enzymes; E2, Ubiquitin-conjugating enzymes; RING, C3HC4 RING-domain-containing ubiquitin E3 ligase; HECT, HECT type E3; APC, anaphase-promoting complex; E3, Ubiquitin ligases; Cullin, SKP, FBOX and RBX, subunit of the E3 ligase. Homogeneous low nitrate: homo LN; local high nitrate: local HN.

Supplemental Figure 4 online. Inhibition of lateral root formation and auxin response by NPA treatment. (A) Shoot-borne roots grown in homogeneous low nitrate, local high...
nitrate and NPA solution for 4 days. Comparison of vascular differentiation and auxin response between local high nitrate (B) and NPA (C) treatment. White arrows indicate auxin signal in the late meta-xylem. Auxin maxima signal in dividing pericycle cells (D) and those after NPA treatment (E). Yellow dotted boxes indicate the pericycle cell layer. X represents a xylem cell. Scale bars: 150 μm (B, C), 50 μm (D, E). Homogeneous low nitrate: homo LN; local high nitrate: local HN.

**Supplemental Figure 5 online.** MeIAA (A, B), IAN (C, D), OxIAA (E, F), IAA-Ala (G, H), IAA-Glu (I, J) concentrations in root tip, stele and cortex of shoot-borne roots induced by homogeneous low and local high nitrate 12 hours after treatment (HAT) and 24 HAT. Error bars represent SE for four biological replicates.

**Supplemental Figure 6 online.** Pericycle cells dividing at the phloem poles and illustration of LCM-microdissected cell types. Heterogeneous arrangements of pericycle cells alternated by phloem and xylem poles are shown by autofluorescence (A). (B) Lateral roots originating from early divisions of competent pericycle cells at the phloem poles illustrated by toluidine blue staining. (C) Auxin maxima and pericycle cell divisions are highlighted by DR5::RFP fluorescence and DAPI counterstaining. Pc: pericycle cell; asterisk: phloem, X: xylem cell. Scale bars: 150 μm (A), 20 μm (B, C). (D) Color-coded scheme of three cell types (endodermis, pericycle and phloem) isolated by LCM. X: meta-xylem vessel. (E) As an example micrographs taken before and after capturing pericycle cells are shown (red arrows).

**Supplemental Figure 7 online.** RNA quality of LCM-dissected cell types evaluated by the RNA integrity number (RIN). Color codes indicate phloem (red), pericycle (green), and endodermis (yellow) cells. Homogeneous low nitrate: homo LN; local high nitrate: local HN.

**Supplemental Figure 8 online.** Relative expression level of *ZmPIN1c* in three cell-types related to lateral root initiation. Different letters indicate significant differences among
means when given a single homogeneous low nitrate (lower case letters) or local high nitrate (capital letters) treatment ($p \leq 0.05$ by one-way ANOVA analysis). Homogeneous low nitrate: homo LN; local high nitrate: local HN.
Supplemental Dataset 1 online. Overview of sample collection, RNA-Seq output, mapping results and alignments to the B73 reference genome.

Supplemental Dataset 2 online. Complete list of 22,796 expressed genes.
Supplemental Table 1 online. Comprehensively enriched GO terms obtained by singular enrichment analyses among differentially expressed genes in pair-wise comparisons between local high nitrate and homogeneous low nitrate treatments.

Supplemental Table 2 online. Gene list of cell cycle related genes extracted from bin 31 of the MapMan analysis.

Supplemental Table 3 online. Determination of overrepresented and underrepresented functional classes among differentially expressed genes (Fc ≥2; FDR ≤5%) that were functionally annotated by MapMan. Fold changes (local HN vs homo LN) are indicated as logarithmic (log_{2}) values. Homogeneous low nitrate: homo LN; local high nitrate: local HN.

Supplemental Table 4 online. Gene list of the ubiquitin-dependent protein degradation pathway extracted from bin 29 of the MapMan analysis.

Supplemental Table 5 online. Internal standards used for the analysis of IAA and its metabolites by UPLC-ESI-MS/MS.
Supplemental methods online. Detailed procedure of MS data processing using TargetLynx V4.1 SCN 904.
Acknowledgement

We thank David Jackson (Cold Spring Harbor Laboratory) for sharing DR5::RFP transgenic maize seeds, Andreas Meyer (University of Bonn) for support in confocal laser scanning microscopy and Mikio Nakazono (Nagoya University) for stimulating discussions on the optimization of Laser Capture Microdissection.
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von Behrens I, Komatsu M, Zhang Y, Berendzen KW, Niu X, Sakai H, Taramino G, 


**Figure legends**

**Figure 1.** Effect of homogeneous low and local high nitrate supplies on lateral root development in shoot-borne roots of maize. (A) Shoot-borne roots were grown hydroponically in low nitrate (0.5 mM NO$_3$-). Subsequently, two shoot-borne roots were split-supplied with low nitrate (0.5 mM NO$_3$-) (left) or high nitrate (4 mM NO$_3$-) (right) for a period of 6 days. Representative roots are displayed. Scale bar: 1 cm. (B) Average length (in mm) and (C) density (LRs mm$^{-1}$) of lateral roots initiated from the whole shoot-borne roots. (D) Early pericycle cell divisions in shoot-borne roots 24 hours after local high nitrate stimulation. (E) Frequency of lateral root primordia (LRP) in the region between 5 and 25 mm from the root tip at 24 HAT. Stages I-III, IV-V, VI-VII and VIII were indicated according to Supplemental Figures 2A-E. Homogeneous low nitrate: homo LN; local high nitrate: local HN. (B) to (E): Error bars represent SE for four biological replicates. Asterisks denote a significant difference according to paired Student’s t test (*: $p \leq 0.05$; **: $p \leq 0.01$).

**Figure 2.** Stele-specific transcriptome analysis and differentially expressed cell cycle genes in response to local high nitrate stimulation. (A) Shoot-borne roots were manually dissected in three tissues: 5 mm root tip (5 mm), stele covering all central cell types covered by the pericycle (5-25 mm stele), cortex covering all radial tissues between endodermis and epidermis (5-25 mm). (B) Frequency of differentially expressed genes normalized according to log$_2$-fold changes (Fc) with FDR $\leq$5% and $|\text{Fc}| \geq 2$. (C) RNA-Seq revealed differentially expressed cell cycle genes, $CDKB$ and $CYCB$ genes (G2/M transition, left) were induced by local HN while $KRP$ genes were inhibited by local HN (G1/S transition, right). (D) Heat map of selected cell cycle genes ($CDKB1;1$, $CDKB2;1$, $CYCB1;1$, $CYCB1;3$, $CYCB2;1$, $CYCB2;2$, $KRP1$, $KRP2$, $KRP3$, $KRP4$) in the stele determined by qRT-PCR after a time course experiment of 36 h. Transcript abundance was normalized to myosin and expressed in base log$_2$ values (Genbank AC: 486090G09.x1). (E) Expression of cell cycle genes ($CDKB1;1$, $CDKB2;1$, $KRP1$, $KRP2$, $KRP3$, $KRP4$).
GCYCB1;1, CYCB2;1) in pericycle cells at the phloem poles determined by qRT-PCR 24 hours after local HN stimulation. Error bars represent SE for four biological replicates. Asterisks denote a significant difference according to paired Student’s t test (*: p ≤0.05; **: p ≤0.01). Homogeneous low nitrate: homo LN; local high nitrate: local HN.

Figure 3. Effect of local high nitrate on tissue-specific auxin distribution. The initiated shoot-borne roots were incubated in NPA solution for 4 days then followed by low or high nitrate treatment for 24 hours. DR5 reporter activity in DR5::RFP-expressing transgenic maize lines in longitudinal orientation close to the root tip (A, B) or in cross sections (C, D) of the lateral root initiation zone were detected after high and low nitrate stimuli respectively. White dotted arrows indicate the basipetal auxin transport direction and yellow asterisks mark the differences of auxin accumulation in columella. White solid arrows indicate auxin accumulation in the phloem poles. IAA concentrations in root tip, stele and cortex of shoot-borne roots induced by homogeneous low and local high nitrate 12 hours after treatment (HAT) (E) and 24 HAT (F). Error bars represent SE for four biological replicates. Asterisks denote a significant difference between homogeneous low nitrate and local high nitrate treatments according to paired Student’s t test (*: p ≤0.05; **: p ≤0.01). Different letters indicated significant differences among means when given a single homogeneous low nitrate (lower case letters) or local high nitrate (capital letters) treatment (p value ≤0.05 by one-way ANOVA analysis). Scale bars: 150 μm (A to D). Homogeneous low nitrate: homo LN; local high nitrate: local HN.

Figure 4. Tissue-specific transcript abundance of ZmPIN genes. (A) RNA-Seq revealed three ZmPIN genes significantly upregulated in the root stele when induced by local high nitrate. ** indicate FDR corrected p-value ≤1%. The expression values were normalized by FPKM (fragments per kilobase of transcript per million reads) of local HN vs homo LN. Expression patterns of ZmPIN1a, ZmPIN1c and ZmPIN9 in stele (B), root tip (C) and cortex (D) as determined by qRT-PCR after local high nitrate induction in a time-course
experiment. Different letters indicate significant levels at the $p \leq 0.05$ after the ANOVA analysis. (E) Correlation between transcript levels of $CYCB$, $CDKB$ genes and $ZmPIN9$ in the stele (open circles). (F) Correlation between transcript levels of $KRP$ genes and $ZmPIN9$ in the stele (open triangles). The data points were extracted from Figure 2D and Figure 4B at 0 h, 6 h, 12 h, 24 h local high nitrate induction. Different letters indicated significant differences among means ($p$ value $\leq 0.05$ by one-way ANOVA analysis).

Homogeneous low nitrate: homo LN; local high nitrate: local HN.

**Figure 5.** Cell-type specific localization of $ZmPIN9$ (A) and $ZmPIN1a$ (B) expression as determined in three cell-types related to lateral root initiation. Cells were isolated by laser capture microdissection (LCM) 24 h after transfer to local high nitrate. Paired Student’s $t$ test was performed for homogeneous low nitrate and local high nitrate treatments and significance levels are indicated by * at $p \leq 0.05$ or ** at $p \leq 0.01$. Different letters indicate significant differences among means when given a single homogeneous low nitrate (lower case letters) or local high nitrate (capital letters indicated) treatment ($p$ value $\leq 0.05$ by one-way ANOVA). Homogeneous low nitrate: homo LN; local high nitrate: local HN.

**Figure 6.** Model of the molecular mechanisms underlying local high nitrate-induced lateral root initiation in shoot-borne roots of maize. (A) Schematic illustration of cell-type specific $ZmPIN9$ expression pattern. Auxin maxima in phloem poles have been highlighted (red). Black solid arrows indicate suggested net auxin flux directions. (B) At low nitrate concentrations, auxin accumulates in the root apical meristem (Figure 3B and 3F). Low auxin efflux capacity fails to induce basipetal auxin transport. In the presence of local high nitrate basipetal auxin transport (thick arrows) is facilitated by $ZmPIN1a$ and $ZmPIN1c$ (Figures 3A and 4C). Increased auxin in the lateral root initiation zone is transported into competent pericycle cells by $ZmPIN9$ (Figure 3C, 4B and 5A). Lateral root initiation is mediated either by auxin directly or by the auxin-mediated ubiquitin-dependent protein degradation machinery ($SCF^{SKP2B}$ complex) (Supplemental...
Figure 3), either of which repress KRP s to facilitate for cell cycle activation (Figure 2). E: epidermis; C: cortex; En: endodermis; P: pericycle; Ph: phloem; LR: lateral root; LRP: lateral root primordium.
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Supplemental Figure 1. Early pericycle cell divisions in shoot-borne roots. (A) Longitudinal section of a shoot-borne root and the first anticlinal divisions of pericycle (red arrows) and endodermis cells (yellow arrows). In the region 5-25 mm proximal of the root tip, peak differences of early pericycle cell divisions (see Figure 1D) were monitored between homogeneous low nitrate (homo LN) and local high nitrate (local HN) treatments. Scale bar: 50 μm. Early pericycle cell divisions in shoot-borne roots 12 (B) and 36 (C) hours after local high nitrate treatment (HAT). Error bars represent SE for four biological replicates. Asterisks denote a significant difference according to paired Student’s t test (*: p ≤ 0.05; **: p ≤ 0.01). Homogeneous low nitrate: homo LN; local high nitrate: local HN.
Supplemental Figure 2. Different stages of lateral root primordium formation are illustrated by representative images: (A) I-III, (B) IV-V, (C) VI-VII, (D) VIII. Scale bar: 50 μm.
Supplemental Figure 3. Schematic overview of the components of the ubiquitin-dependent degradation system (SCF^{SKP-2B} complex) induced in response to local high nitrate. Log$_2$-fold changes of gene expression (local HN versus homo LN) are illustrated by MapMan. DUB, deubiquitinating enzyme; E1, ubiquitin-activating enzymes; E2, Ubiquitin-conjugating enzymes; RING, C3HC4 RING-domain-containing ubiquitin E3 ligase; HECT, HECT type E3; APC, anaphase-promoting complex; E3, Ubiquitin ligases; Cullin, SKP, FBOX and RBX, subunit of the E3 ligase. Homogeneous low nitrate: homo LN; local high nitrate: local HN.
Supplemental Figure 4. Inhibition of lateral root formation and auxin response by NPA treatment. (A) Shoot-borne roots grown in homogeneous low nitrate, local high nitrate and NPA solution for 4 days. Comparison of vascular differentiation and auxin response between local high nitrate (B) and NPA (C) treatment. White arrows indicate auxin signal in the late meta-xylem. Auxin maxima signal in dividing pericycle cells (D) and those after NPA treatment (E). Yellow dotted boxes indicate the pericycle cell layer. X represents a xylem cell. Scale bar = 150 μm (B, C), 50 μm (D, E). Homogeneous low nitrate: homo LN; local high nitrate: local HN.
Supplemental Figure 5. MeIAA (A, B), IAN (C, D), OxlIAA (E, F), IAA-Ala (G, H), IAA-Glu (I, J) concentrations in root tip, stele and cortex of shoot-borne roots induced by homogeneous low and local high nitrate 12 hours after treatment (HAT) and 24 HAT. Error bars represent SE for four biological replicates.
Supplemental Figure 6. Pericycle cells dividing at the phloem poles and illustration of LCM-microdissected cell types. Heterogeneous arrangements of pericycle cells alternated by phloem and xylem poles are shown by autofluorescence (A). (B) Lateral roots originating from early divisions of competent pericycle cells at the phloem poles illustrated by toluidine blue staining. (C) Auxin maxima and pericycle cell divisions are highlighted by DR5::RFP fluorescence and DAPI counterstaining. Pc: pericycle cell; asterisk: phloem, X: xylem cell. Scale bars: 150 μm (A), 20 μm (B, C). (D) Color-coded scheme of three cell types (endodermis, pericycle and phloem) isolated by LCM. X: meta-xylem vessel. (E) As an example micrographs taken before and after capturing pericycle cells are shown (red arrows).
Supplemental Figure 7. RNA quality of LCM-dissected cell types evaluated by the RNA integrity number (RIN). Color codes indicate phloem (red), pericycle (green), and endodermis (yellow) cells. Homogeneous low nitrate: homo LN; local high nitrate: local HN.
**Supplemental Figure 8.** Relative expression level of ZmPIN1c in three cell-types related to lateral root initiation. Different letters indicate significant differences among means when given a single homogeneous low nitrate (lower case letters) or local high nitrate (capital letters) treatment ($p \leq 0.05$ by one-way ANOVA analysis). Homogeneous low nitrate: homo LN; local high nitrate: local HN.
Supplemental Text 1 Detailed procedure of MS data processing using TargetLynx V4.1 SCN 904.

The column temperature was set to 40 °C and the autosampler was set to 4 °C. The mobile phases were H2O containing 0.1 % FA (A), MeOH containing 0.1 % FA. The mobile phase flow was 0.4 ml min⁻¹. The gradient conditions were as follows: A, 90 % in 0 – 0.3 min; 90 – 80 % in 0.3 – 0.7 min; 80 – 40 % in 0.7 – 8 min; 40 – 1 % in 8 – 8.5 min; 1 % in 8.5 – 8.9 min; 1 – 90 % in 8.9 – 9.0 min; 90 % in 9.0 – 10.0 min.

The Xevo TQ MS operated in both ESI+ and ESI- ion mode. The electrospray capillary voltage was 2.45 kV with a cone voltage of 20 V. The cone and desolvation gas flows were set to 20 and 1000 L h⁻¹. The cone and desolvation gas flows were set to 50 and 1000 L h⁻¹. The source and desolvation temperature were 150 and 650 °C. The collision energy of the MS-MS was between 10 and 50 eV. For the quantification of the analytes we used three fragment ions one for quantification two for qualification and for the internal standards two fragment ions were used (Tab. S1). MS data processing was done by using TargetLynx V4.1 SCN 904.
**Supplemental Table 1.** Comprehensively enriched GO terms obtained by singular enrichment analyses among differentially expressed genes in pair-wise comparisons between local high nitrate and homogeneous low nitrate treatments. 

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*a* SEA was performed with agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) comparing the differentially expressed genes to all expressed genes. GO terms were declared enriched when FDR ≤0.01. Differentially expressed genes were compared between local high nitrate and homogeneous low nitrate at 24 h treatments (513 of 582 differentially expressed genes were annotated).
**Supplemental Table 2.** Gene list of cell cycle related genes extracted from bin31 of the MapMan analysis.

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</tr>
<tr>
<td>31.3</td>
<td>cell.cycle</td>
<td>GRMZM5G854731</td>
<td>KRP1; cyclin-dependent protein kinase inhibitor</td>
<td>-2.2</td>
</tr>
</tbody>
</table>
Supplemental Table 3. Determination of overrepresented and underrepresented functional classes among differentially expressed genes (Fc ≥2; FDR ≤5%) that were functionally annotated by MapMan. Fold changes (local HN vs homo LN) are given as logarithmic (log2) values. Homogeneous low nitrate: homo LN; local high nitrate: local HN.

<table>
<thead>
<tr>
<th>Bin</th>
<th>Functional group</th>
<th>Expressed genes</th>
<th>Differentially expressed genes</th>
<th>Representation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Photosynthesis</td>
<td>157</td>
<td>5</td>
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</tr>
<tr>
<td>2</td>
<td>Major CHO metabolism</td>
<td>119</td>
<td>3</td>
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<tr>
<td>3</td>
<td>Minor CHO metabolism</td>
<td>139</td>
<td>5</td>
<td></td>
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<td>4</td>
<td>Glycolysis</td>
<td>90</td>
<td>1</td>
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</tr>
<tr>
<td>5</td>
<td>Fermentation</td>
<td>20</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>Gluconeogenesis/glyoxylate cycle</td>
<td>12</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>OPP</td>
<td>33</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>8</td>
<td>TCA / org. transformation</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Mitochondrial e-transport / ATP synthesis</td>
<td>126</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>Cell wall</td>
<td>327</td>
<td>21</td>
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<tr>
<td>11</td>
<td>Lipid metabolism</td>
<td>376</td>
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<td>N-metabolism</td>
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<td>Amino acid metabolism</td>
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<td>0.0192</td>
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<tr>
<td>14</td>
<td>S-assimilation</td>
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<td>5</td>
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<td>Metal handling</td>
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<td>Secondary metabolism</td>
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<td>Hormone metabolism</td>
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<td>21</td>
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<td>18</td>
<td>Co-factor and vitamin met.</td>
<td>73</td>
<td>1</td>
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<tr>
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<td>Tetapyrrole synthesis</td>
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<tr>
<td>20</td>
<td>Stress</td>
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<td>32</td>
<td>+</td>
<td>0.0082</td>
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<tr>
<td>21</td>
<td>Redox regulation</td>
<td>214</td>
<td>10</td>
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<tr>
<td>22</td>
<td>Polyamine metabolism</td>
<td>15</td>
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<td>Nucleotide metabolism</td>
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<td>Bin</td>
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<td>Expressed genes</td>
<td>Differentially expressed genes</td>
<td>Representation</td>
<td>p-value</td>
</tr>
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<tr>
<td>24</td>
<td>Biodegradation of Xenobiotics</td>
<td>30</td>
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<tr>
<td>25</td>
<td>C1-metabolism</td>
<td>25</td>
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<td>26</td>
<td>Miscellaneous</td>
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<td>60</td>
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<td>RNA</td>
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<td>50</td>
<td>-</td>
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<td>DNA</td>
<td>526</td>
<td>2</td>
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<td>29</td>
<td>Protein</td>
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<td>93</td>
<td>+</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>Signalling</td>
<td>1235</td>
<td>20</td>
<td>-</td>
<td>&lt;0.0001</td>
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<tr>
<td>31</td>
<td>Cell</td>
<td>870</td>
<td>31</td>
<td>+</td>
<td>0.0001</td>
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<tr>
<td>32</td>
<td>MicroRNA, natural antisense</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Development</td>
<td>526</td>
<td>5</td>
<td></td>
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</tr>
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<td>34</td>
<td>Transport</td>
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<tr>
<td>35</td>
<td>Not assigned</td>
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<td>151</td>
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<tr>
<td></td>
<td>MapMan Sum</td>
<td>25064</td>
<td>622</td>
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<td>Expressed DEG Sum</td>
<td>22796</td>
<td>582</td>
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</tr>
</tbody>
</table>

a Each Bin consists of genes of similar biological function.
b All genes expressed in shoot-borne root treated by local high nitrate or homogeneous low nitrate.
c Subset of expressed genes that are differentially expressed between local high nitrate and homogeneous low nitrate treatment (Fc ≥2; FDR ≤5%).
d Over (+) or underrepresentation (-) of differentially expressed genes in functional groups relative to the distribution of all expressed genes.
**Supplemental Table 4.** Gene list of the ubiquitin-dependent protein degradation pathway extracted from bin29 of the MapMan analysis.

<table>
<thead>
<tr>
<th>BinCode</th>
<th>BinName</th>
<th>Gene ID</th>
<th>Description</th>
<th>log$_2$Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G021796</td>
<td>E3 ubiquitin-protein ligase EL5</td>
<td>1.75</td>
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<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G025255</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
<td>2.84</td>
</tr>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G027375</td>
<td>armadillo/beta-catenin repeat family protein / U-box domain-containing protein</td>
<td>1.12</td>
</tr>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G045286</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
<td>1.84</td>
</tr>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G052344</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
<td>1.64</td>
</tr>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G075782</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
<td>2.26</td>
</tr>
<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G100090</td>
<td>armadillo/beta-catenin repeat family protein / U-box domain-containing protein</td>
<td>1.1</td>
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<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G115988</td>
<td>protein protein binding protein</td>
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<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G137440</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
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<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G144782</td>
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<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G152461</td>
<td>zinc finger (C3HC4-type RING finger) family protein</td>
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<tr>
<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G324111</td>
<td>protein RING-H2 finger protein ATL5H precursor</td>
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<td>29.5.11.4.2</td>
<td>protein.degradation.ubiquitin.E3.RING</td>
<td>GRMZM2G417089</td>
<td>protein zinc finger, RING-type</td>
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<tr>
<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SC.FBOX</td>
<td>AC204641.5_FG001</td>
<td>kelch repeat-containing F-box family protein</td>
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<td>kelch repeat-containing F-box family protein</td>
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<td>GRMZM2G014022</td>
<td>protein F-box domain containing protein</td>
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<tr>
<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SC.FBOX</td>
<td>GRMZM2G025783</td>
<td>kelch repeat-containing F-box family protein</td>
<td>1.51</td>
</tr>
<tr>
<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SC.FBOX</td>
<td>GRMZM2G100121</td>
<td>FBL17; ubiquitin-protein ligase</td>
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<tr>
<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SC.FBOX</td>
<td>GRMZM2G110057</td>
<td>protein F-box protein interaction domain containing protein</td>
<td>3.07</td>
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<tr>
<td>BinCode</td>
<td>BinName</td>
<td>Gene ID</td>
<td>Description</td>
<td>log$_2$Fc</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------------------</td>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SCF.FBOX</td>
<td>GRMZM2G138176</td>
<td>SKP2B; F-box domain, cyclin-like; protein F-box/ Leucine-rich repeat protein</td>
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<td>protein.degradation.ubiquitin.E3.SCF.FBOX</td>
<td>GRMZM2G171616</td>
<td>FBL6</td>
<td>EBF1 (EIN3-BINDING FBOX PROTEIN 1); protein binding / ubiquitin-protein ligase</td>
</tr>
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<td>29.5.11.4.3.2</td>
<td>protein.degradation.ubiquitin.E3.SCF.FBOX</td>
<td>GRMZM2G325650</td>
<td>kelch repeat-containing F-box family protein</td>
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<td>29.5.11.4.3.3</td>
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<td>ubiquitin-protein ligase</td>
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<td>29.5.11.4.5.2</td>
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<td>ATBPM4 (BTB-POZ and MATH domain 4); protein binding</td>
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<tr>
<td>29.5.11.5</td>
<td>protein.degradation.ubiquitin.ubiquitin.protease</td>
<td>GRMZM2G038126</td>
<td>protein 26S protease regulatory subunit 6B</td>
<td>1.62</td>
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</table>
**Supplemental Table 5.** Internal standards used for IAA detection by UPLC-ESI-MS/MS.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Molecular mass</th>
<th>Ionization mode</th>
<th>Quantification ion</th>
<th>First target ion</th>
<th>Second target ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole-3-acetonitrile</td>
<td>IAN</td>
<td>156.2</td>
<td>ESI+</td>
<td>89.9</td>
<td>117.0</td>
<td>130.0</td>
</tr>
<tr>
<td>Indole-3-acetamide</td>
<td>IAM</td>
<td>174.2</td>
<td>ESI+</td>
<td>77.0</td>
<td>103.0</td>
<td>130.0</td>
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<tr>
<td>Indole-3-acetic acid</td>
<td>IAA</td>
<td>175.2</td>
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<td>$[^2\text{H}_5]$ Indole-3-acetic acid</td>
<td>D-IAA</td>
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<td>180.2</td>
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<td>Indole-3-acetic acid methylester</td>
<td>IAAMe</td>
<td>189.2</td>
<td>ESI+</td>
<td>76.9</td>
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<td>IAAEt</td>
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<td>Indole-3-L-isoleucin</td>
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<td>ESI+</td>
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and pericycle promotes lateral root initiation. EMBO J 32: 149-158


