Lateral root development in response to local supplies of different nitrogen forms

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1 Summary

On many agricultural plant production sites in Germany, nitrogen (N) fertilizer input exceeds plant N uptake, and this N-balance surplus represents an environmental problem. Such N-balance surplus can be reduced by placed or banded fertilizer application, in which lateral roots (LRs) grow intensively around the fertilizer band. This morphological adaptation to a localized N source relies on the observation that ammonium increases LR density while local nitrate stimulates LR elongation. Such changes in root system architecture (RSA) are considered as consequence of nutrient "sensing" processes. The aim of this Ph.D. thesis was to investigate how locally supplied ammonium increases LR formation. Therefore, ammonium or nitrate was locally supplied in vertically-splitted or horizontally-splitted agar plate systems, where just one first-order LR or a part of the PR had access to the local N source. In response to ammonium, Arabidopsis plants showed increased LR emergence and stronger branching of higher-order LRs, whereas local supply of nitrate or even mild N deficiency enhanced LR elongation. Using mutants defective in individual ammonium transporter (AMT) genes, could show that ammonium-induced higher-order LR branching is predominantly linked to AMT1;3. Since this ammonium transporter is not suppressed by local ammonium supply, it allowed uptake of ammonium, which led to auxin accumulation in the root vasculature. In contrast, auxin levels were low in nitrate-supplied roots. The quadruple *amt* mutant (*qko*), which is defective in four AMT genes, had very low ammonium uptake capacity and weak LR branching. However, auxin accumulation and LR branching in *qko* were restored by supplying elevated ammonium levels, indicating that ammonium is sensed inside root cells. Using L-methionine sulfoximine, an inhibitor of glutamine synthetase, together with ammonium led to increased auxin levels in the vasculature, suggesting that internal ammonium promotes either the de-novo synthesis or the rootward transport of auxin in the root vasculature. Using an auxin flux inhibitor to block the auxin flow from the shoot to the root suppressed ammonium-dependent LR formation. To identify the relevant genes in this process, different mutants related to auxin transport, synthesis or auxin sensing were tested for their root response to local ammonium supply. Two mutants defective in the expression of PIN-type auxin transport proteins were found with significantly altered LR formation, and the corresponding proteins showed contrasting regulation in response to ammonium. While PIN4 most likely increased the rootward auxin flow in the vasculature, ammonium-induced suppression of PIN2 led to auxin accumulation in lateral root primordia, both promoting ammonium-dependent LR formation. Moreover, ammonium uptake lowered the pH value in the root tissue and the rhizosphere. As shown by use of a fluorescing reporter line for auxin, this drop in pH increased lateral auxin diffusion in the

root tissue. In consequence, a larger amount of protonated (neutral) auxin can laterally diffuse to cortical and epidermal cells to promote cell wall loosening and LR emergence. Taken together, the present Ph.D. work elucidated the molecular mechanism how ammonium interferes with auxin transport to enhance LR formation. This work contributes to a better understanding of the adaptation of roots to local nitrogen supplies, which is an important issue for efficient use of placed fertilizers in agricultural plant production.

2 Introduction

Plants need 14 mineral elements to maintain growth, fulfil their life cycle and to produce fertile seeds. Plant availability of these nutrients depends on the chemical properties of the nutrients themselves and of the soil matrix (Giehl and von Wirén, 2014). Since mineral elements interact differently with altered soil constituents or are dislocated by water out of the rooted soil volume, their plant availability may decrease or become spatially restricted, leading to nutrient deficiency. In natural and agricultural soils, the ability of plants to acquire nutrients depends mostly on their ability to modify the root system architecture (RSA) or the chemical conditions in the root-influenced soil, i.e. the rhizosphere (Giehl et al., 2014). These foraging responses, which encompass morphological and physiological processes, such as the release of nutrient-mobilizing root exudates or the expression of nutrient transporters (Hinsinger et al., 2009; Gojon et al., 2009), determine the competitive success and productivity of plants and most likely evolved due to natural selection pressure or man-made selection in breeding processes. The different responses of plants to the environment are often spatially coupled in order to increase the root-soil interaction zone and improve the ability to mobilize precipitated or fixed mineral elements (Neumann and Martinoia, 2002; Schünmann et al., 2004). This study focuses on the molecular mechanisms leading to changes in RSA in response to the most abundant inorganic nitrogen sources for plants, nitrate and ammonium.

2.1 Nitrogen as plant nutrient

In plant nutrition, N is classified as macronutrient and builds 1 - 5 % of the total plant dry matter. N is besides all other nutrients the quantitatively most abundant in the plant tissue, and N limitations repress plant growth and lower the yield in agricultural plant production (Forde, 2002). N is present in proteins, nucleic acids and many other important cellular compounds including chlorophyll or phytohormons (Hawkesford et al., 2012). Regarding the high N content of the tetrapyrrole ring in the chlorophyll molecule, N is responsible for the establishment of the photosynthetic apparatus and maintenance of photosynthetic efficiency (Gammelvind et al., 1996). In consequence, N deficiency leads to gradual chlorosis of old leaves, the accumulation of anthocyanins and reduced plant growth as well as lower yield (Williams et al., 2001; Lawlor, 2002).

To prevent yield losses due to N deficiency, 85 - 90 million metric tons of N fertilizers are applied every year to agricultural soils worldwide (Frink et al., 1999), but since N fertilizers

are often associated with adverse environmental impact and are one of the most expensive expenses in crop production (Masclaux-Daubresse et al., 2010), the amount of applied N fertilizers has to be reduced as much as possible.

Worldwide, urea is the predominantly fertilized N form in agricultural systems. In soils, urea is degraded by ureases to ammonium (NH_4^+) and further nitrified to nitrate (NO_3^-) by microorganisms (Merigout et al., 2008). Other sources of plant-available N forms in the soil originate from the degradation of organic matter. Those N forms originate from N-containing macromolecules, which are degraded by extracellular enzymes to oligopeptides, amino acids or nucleic acids. These low molecular-weight compounds are further mineralized by microorganisms to the inorganic N form ammonium, before being nitrified by microbes to nitrite and nitrate (Miller et al., 2008). The conversion of the N forms highly depends on the chemical conditions in the soil and the microbial activity (Schimel and Bennett, 2004) and may be disturbed by different factors. For instance, urease activity decreases under cold (Moyo et al., 1989) or anaerobic conditions (Pulford and Tabatabai, 1988). Nitrification decreases especially under cold, low pH, accumulation of phenolic-based allelopathic compounds, or low oxygen availability in the soil. As consequence of lower nitrification and continuing or even increased ammonification, soil ammonium levels are increasing (Britto and Kronzucker, 2002). However, millimolar concentrations of ammonium in the soil may cause problems, as plants are responding with growth depression due to leaf chlorosis, ionic imbalances, disturbance of pH gradients across plant membranes, or oxidative stress (Gerendás et al., 1997; Britto and Kronzucker, 2002; Li et al., 2014; Bittsánszky et al., 2015; Esteban et al., 2016). On the other hand, ammonium-based fertilizers can be an environmentally friendly alternative to commonly used urea- or nitrate-based nitrogen fertilizers Therefore, it is important to futher investigate, how ammonium is affecting the root system architecture and N uptake efficiency. Such knowledge will help designing new ammonium-based fertilizer strategies.

2.2 Mobility of N forms and associated problems

The inorganic N forms nitrate and ammonium behave in opposite ways in the soil matrix when it comes to their mobility. The anion nitrate is highly mobile due to its transport via the mass flow of water and can therefore easily be washed out from the soil matrix. In contrast, the cation ammonium is relatively immobile in the soil and mainly bound to negatively charged soil colloids like clay or organic matter, which tremendously lowers the risk of N losses due to leaching (Jensen, 2006). An often criticised disadvantage of mineral

N fertilizers is the N loss directly after fertilizer application or during the growth period of the crop plants, when sometimes even more than 50% of the fertilized N is not incorporated by the crop plants. Residual N then remains either in the soil or leaches in form of NH_3, NO or N_2O into the atmosphere, or in form of nitrate to the ground water (Galloway and Cowling 2002). Since such N losses cause a broad spectrum of environmental problems, the European Union released the “Nitrates Directive” (Council Directive 91/676/EEC of 12 Dec. 1991), with the goal to reduce N surpluses in agricultural crop production. From 1996 there is a restriction for N fertilization in Germany, and from 2018 a novel fertilizer ordinance (Düngeverordnung) has entered into force, which further increases the pressure to reduce N fertilizer input (BMEL, 2014).

In most natural and agricultural ecosystems, nitrate is the major inorganic nitrogen source for plants (Miller et al., 2007) but in the micromolar concentration range, most plant species prefer uptake of ammonium over nitrate (Gazzarrini et al., 1999), which makes ammonium a suitable alternative to nitrate as fertilizer N form. However, there are also disadvantages related to ammonium-based fertilizers. If the concentration of ammonium in the soil solution reaches millimolar concentrations, ammonium often causes toxicity (Britto and Kronzucker, 2002), which results in the inhibition of root elongation and shoot growth. An elevated accumulation of ammonium in agricultural soils is often a result of urea-based fertilization, when urea is applied in large amounts. An established strategy to fertilize ammonium in a more economic and environmentally friendly way, is the so-called CULTAN fertilization practice, in which mostly liquid ammonium fertilizer is placed as a band at a certain soil depth. At the border of the fertilizer band non-toxic ammonium concentrations allow plant roots to grow close to or later also into the N depot, after ammonium has partially been nitrified. In association with localized ammonium fertilization plant roots show increased higher-order LR branching close to the ammonium source (Sommer, 1992), which has been deemed beneficial for nutrient and water uptake by the plant.

To improve ammonium-based fertilization practices it is of great importance to understand especially the sensing and signalling mechanisms related to ammonium uptake and their effect on root system architecture.

2.3 Ammonium and nitrate uptake and assimilation

Plant roots have evolved specific transport systems for nitrate and ammonium, which are needed to regulate nitrogen uptake by roots tightly and to ensure a well-balanced nitrogen homeostasis. For the uptake of nitrate, plants evolved a high-affinity transport system (HATS) and a low-affinity transport system (LATS), which mediate nitrate uptake across the plasma membrane of epidermal and cortical root cells by using the electrochemical gradient across the plasma membrane (Crawford, 1995). The HATS transports nitrate at concentrations below 200 µM, and at higher concentrations nitrate is taken up by the LATS. Both transport systems consist of constitutively expressed and inducible nitrate transporters. While low-affinity uptake is facilitated by transporters of the NRT1 family, high-affinity uptake is driven by transporters of the NRT2 family (von Wirén et al., 1997).
In Arabidopsis roots, high-affinity nitrate transport is mediated by NRT1;1 (CHL1) and NRT2;1, which are the major components of the HATS (Cerezo et al., 2001) and up-regulated under nitrogen limitation or after re-supply of small amounts of nitrate during the so-called primary nitrate response (Tsay et al., 2007). Under high nitrate supply, NRT1;1 switches upon de-phosphorylation to a low-affinity nitrate transporter and has therefore been described as a dual-affinity nitrate transporter (Liu and Tsay, 2003). In contrast, elevated nitrate supply represses *NRT2;1*, mainly via reduced N metabolites resulting from nitrate assimilation, such as ammonium or amino acids (Krouk et al., 2006). The transcriptional regulation of *NRT2;1* depends on the nitrate sensing function of NRT1;1, which was shown by strongly repressed *NRT2;1* transcript levels in the *nrt1;1* mutant compared to wild-type plants, when cultivated under high N (Krouk et al., 2006). The calcineurin B-like (CBL)-interacting protein kinase (CIPK) CIPK23 was found to play a role in nitrate signaling. *CIPK23* was rapidly induced in the presence of nitrate (Ho et al., 2009) and the characterization of a *cipk23* mutant showed that the CIPK23 protein acts a negative regulator of high-affinity transport by phosphorylating the threonine at position 101 in the C-terminus of NRT1;1 (Ho et al., 2009). In contrast, CIPK8 positively regulates the low-affinity transport of NRT1;1 (Hu et al., 2009). With this mechanism nitrate uptake is fine-tuned to meet a specific nitrate demand of the plant. Additionally, a transcription factor, *nodule inception-like protein 7* (*NLP7*) was found to be involved in nitrate signalling and is modulating nitrate assimilation and root architecture in response to nitrate signals (Castaings et al., 2009). *NLP7* regulates the expression of the nitrate transporters *NRT2;1*, *NRT2;2* and the nitrate reductase gene *NIA* (Castaings et al., 2009). Taken together, CIPK23, CIPK8 and NLP7 are required for the regulation of nitrate transport and assimilation in plant roots. In general, nitrate uptake involves complex gene regulatory networks and nitrate-dependent signaling pathways (O´Brian et al., 2016; Bellegarde et

al., 2017). So far, several transcription factors have been identified to regulate nitrate uptake, including the MADS-box-type transcription factors *ANR1, LBD37, LBD38, NLP6, NLP7* or the GARP-type transcription factors *HRS1, TGA1, TGA4, SPL9* and *AFB3*. These transcription factors regulate N-dependent transport, signaling and developmental responses locally or systemically (Remans et al., 2006; Vidal et al., 2013; O´Brian et al. 2016). Interestingly, *Nitrate Regulatory Gene 2* (*NRG2*) and *Teosinte Branched1/Cycloidea/Proliferating Cell Factor1-20* (*TCP20*), which physically interact with *NLP7*, are shown to regulate the expression of *NRT1;1* and are involved in different nitrate signaling pathways (Xu et al., 2016; Guan et al., 2017). This large number of involved transcription factors may indicate that the transcriptional regulation of nitrate uptake is modulated at several levels and subject to nitrate sensing mechanisms.

For the uptake of ammonium, plant roots developed a HATS and LATS as well. The HATS is taking up ammonium at very low external ammonium concentrations below 0.5 mM, in contrast to the LATS, which facilitates ammonium uptake at external concentrations above 0.5 mM. In Arabidopsis and other crop plants, ammonium is taken up as a preferential N-source when nitrate and ammonium are supplied at equimolar concentrations (Gazzarrini et al., 1999a; von Wirén et al., 2000). In plant roots, high-affinity ammonium uptake is mediated by ammonium transporters of the AMT/Rh/MEP family. In Arabidopsis, five high-affinity ammonium transporters are expressed in roots, namely AMT1;1, AMT1,2, AMT1;3, AMT1;5 and AMT2,1. However, just the ammonium transporters AMT1;1, AMT1;3 and AMT1;5 are localized in the rhizodermis, where they transport ammonium from the soil solution into root cells. AMT1;2 is expressed predominantly in the endodermis where it facilitates the transport of ammonium from the apoplast into endodermis cells. In more mature root zones, when endodermal cells suberize, AMT1;2 is no longer expressed in the endodermis but in the cortex (Duan et al., 2018). The localization of AMT2;1 in pericycle cells contributes to the loading of ammonium into the xylem (Giehl et al., 2017; **Figure *1***). An *amt1;1 amt1;2 amt1;3 amt2;1* quadruple mutant (*qko*) shows severe growth depression under ammonium supply and maintains only 5 to 10% of the wild-type high-affinity ammonium uptake capacity, emphasizing the exclusive role of AMTs in the HATS (Yuan et al. 2007a). The ammonium transporters AMT1;1 and AMT1;3 show an overlapping cell type-specific expression pattern in the epidermis and comparable biochemical transport properties (Loqué et al., 2006; Yuan et al., 2007a). These two AMTs are able to form hetero-trimers consisting of both AMT1;1 and AMT1;3. It has been speculated that in the case of ammonium, membrane transport and ammonium sensing in roots are coupled events. In the trimer, AMTs are regulated by the external ammonium concentration as under rising external ammonium the ammonium transport of the trimer is down-regulated by phosphorylation of the cytosolic C-terminal domain of AMT1;1. When

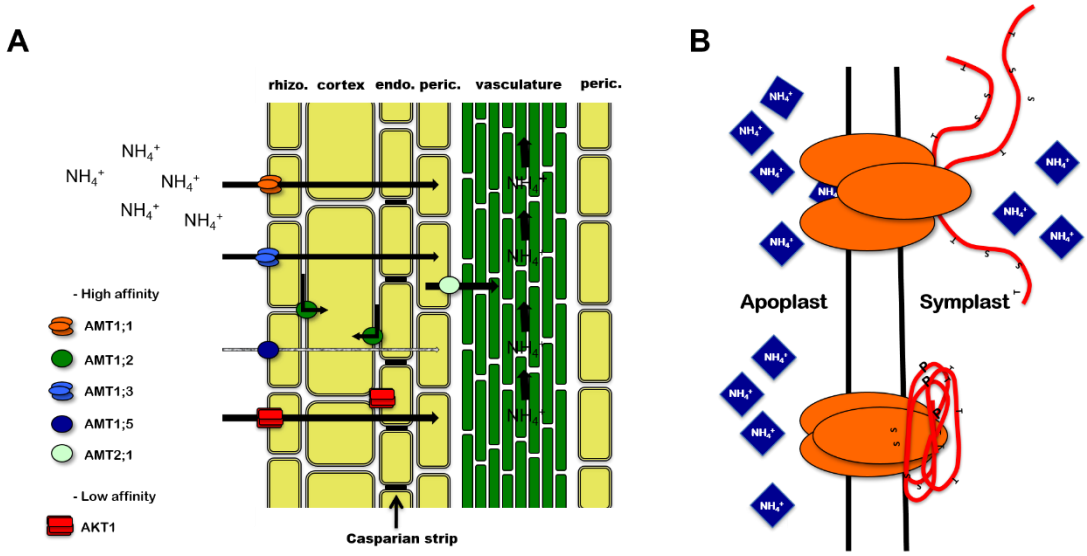

Figure 1. Organisation of ammonium transporters in roots of *Arabidopsis thaliana* and posttranscriptional regulation of AMT1;1 by phosphorylation
A) If ammonium is present in low concentrations, it is taken up from the soil into the root tissue by AMT1;1, AMT1;3 and AMT1;5, which are localized in the plasma membrane of rhizodermal cells and involved in ammonium uptake directly from the soil. Besides the radial ammonium transport throughout the symplastic route, there is also apoplastic ammonium transport. The apoplastic ammonium is transported into cortical and endodermal cells by AMT1;2. Ammonium which is entering the pericicle cells is loaded into the vasculature by AMT2;1, localized mainly in the plasma membrane of pericycle cells in the presents of ammonium. Low affinity ammonium uptake is entering the symplastic route most likely by the potassium channel AKT1, which is localized predominantly in the rhizodermis and endodermis. **B)** At low ammonium concentration, a receptor kinase (CIPK23) senses the extracellular ammonium availability and the phosphorylation site T460 in the C-terminus of AMT1;1 is not phosphorylated. If ammonium concentrations are increased, the CIPK23 senses the high external ammonium concentrations and triggers phosphorylation of T460, which leads to a conformational change in the C-terminus of AMT1;1. This will inactivate the whole trimer and prevents further ammonium uptake. rhizo: rhizodermis; endo: endodermis; peri: pericycle (Yuan *et al.*, 2007 and Straub *et al.*, 2017; modified)

high external ammonium concentration are present, the cytosolic C-terminus of AMT1;1 interacts with other subunits in the trimer leading to an trans-inactivation of the whole trimeric complex (**Figure 1**) (Loqué et al., 2007). The allosteric regulation of ammonim uptake is mediated by the specific phosphorylation of the threonine residue T460 in AMT1;1 and comes into play at ammonium concentrations as low as 50 µM (Loque et al., 2007; Lanquar et al., 2009). At that time, it was hypothesized that either the AMT1;1 transporter itself or a neighbouring receptor-like kinase mediates the sensing of external ammonium concentrations (Lanquar et al., 2009). Recent findings showed that the inactivation of AMT1:1 by phosphorylation is facilitated by a CALCINEURIN B-LIKE INTERACTING PROTEIN KINASE23 (CIPK23) (Straub et al. 2017), although it remains unclear whether this protein is directly linked to the sensing of external or internal ammonium.

Besides posttranscriptional regulation by phosphorylation, AMTs are also transcriptionally regulated. Ammonium uptake is controlled by the diurnal rhythm, as AMTs are up-regulated during the day and repressed in the dark (Gazzarrini et al. 1999; Lejay et al., 2003). Since, sucrose supply to roots relieved AMT repression and ammonium uptake in the dark, it was suggested that AMTs are regulated by photoassimilates (Lejay et al. 2003). All five root-expressed AMTs are up-regulated under N starvation (Sohlenkamp et al. 2002; Kaiser et al. 2002; Loque et al. 2006; Yuan et al. 2007a), and after N resupply ammonium uptake and mRNA levels of *AMT1;1* are strongly repressed. Interestingly, when ammonium assimilation was blocked by methionine sulfoximine (MSX), mRNA levels of *AMT1;1* remained high, while supply with external glutamine repressed *AMT1;1*, leading to the conclusion that AMT1;1 is transcriptionally regulated by ammonium assimilation products rather than by internal ammonium (Rawat et al., 1999). Early split-root experiments suggested that ammonium uptake depends mainly on local sensing and signalling and less on systemic signals reflecting the N status of the whole plant, because ammonium uptake and *AMT1;1* expression were only induced in those root parts growing under N starvation (Gansel et al., 2001).

The large majority of N taken up by plant roots is assimilated into C skeletons. Before that, inorganic N has to be reduced to ammonium, which is then further assimilated into glutamine (Gln) and glutamate (Glu). The reduction of nitrate to ammonium is catalyzed in two steps via cytosolic nitrate reductase and plastidic nitrite reductase (Lam et al., 1996; Krapp, 2015). The whole reduction process requires a lot of energy in the form of reducing equivalents, which is seen as reason for the preferential uptake of ammonium over nitrate (Bloom et al., 1992). At low nitrate availability, nitrate is mainly reduced in roots, whereas the assimilation of nitrate in leaves becomes more prominent at higher external nitrate concentrations (Andrews, 1986). In this case, nitrate is translocated to the shoot via the xylem (Liptay and Arevalo, 2000).

In the first step of ammonium assimilation, glutamine synthetase (GS) incorporates ammonium into glutamine. In the second step, the resulting amino group is transferred to 2-oxoglutarate by glutamate synthase (GOGAT) to finally form two molecules of glutamate. Glutamine and glutamate are the precursors for the production of further amino acids (Masclaux-Daubresse et al., 2006). The plastid-localized isoforms GS2 and ferredoxin-dependent GOGAT are catalysing the primary assimilation of ammonium and the re-assimilation of photorespiratory ammonium in leaves. In contrast, the cytosolic isoforms GS1 and NADH-dependent GOGAT mediate N assimilation predominantly in roots (Lam et al., 1996). In Arabidopsis, GLN1;2 is the major isoenzyme contributing to GS1 activity in roots (Lothier et al., 2011; Guan et al., 2016). In roots, *GLN1;2* is strongly up-regulated by external ammonium supply (Ishiyama et al., 2004; Guan et al., 2016), and

the corresponding mutant *gln1;2* shows low GS activity, high ammonium accumulation, and impaired plant growth, suggesting that GLN1;2 is not only essential for ammonium assimilation but also for its detoxification in roots (Lothier et al., 2011; Guan et al., 2016). Moreover, NADH-GOGAT, the major isoform in roots, is strongly induced by ammonium and the *nadh-gogat* mutant shows weaker glutamate formation and biomass accumulation even if ammonium is present (Konishi et al., 2014).

Most of the ammonium that is taken up or produced in the root is assimilated directly in the root and transported to the shoot via the xylem mainly in the form of amino acids, especially of glutamine, asparagine, glutamate and aspartate (Fischer et al., 1998). Non-assimilated ammonium is also translocated to the shoot, with xylem sap concentrations reaching up to 1 mM in rapeseed (Finnemann and Schjoerring, 1999).

2.4 The response to local nitrogen requires a complex sensing and signalling network

Primary root and LR growth is stimulated in a nutrient-specific manner. Especially in nutrient-enriched soil patches LRs respond with drastic changes of their root architecture. In barley local supplies of nitrate, ammonium or phosphate promoted LR formation, while LR formation was suppressed in nutrient-depleted zones (Drew, 1975; Drew and Saker, 1975; 1978). This specific response of the root system was observed in different plant species (Robinson, 1994). It has then been assumed that locally stimulated root growth requires a nutrient-sensing event by the root and a translation into a metabolic or developmental response that involves a nutrient-specific signaling cascade (Zhang et al., 1999). When plants are growing under particularly challenging conditions, the whole root system architecture (RSA) may change. Depending on the nutrient and water availability the length and number of LRs vary (Narang et al., 2000; Gruber et al., 2013), enabling plants to deal with nutrient deficiency or drought stress. A deep and highly branched root system has been shown to correlate with drought tolerance in rice and maize (Toorchi et al., 2002; Trachsel et al., 2009). Thus, it appears that an increased root surface enhances water uptake. Under nitrogen deficiency, the elongation of the PR and LRs is strongly enhanced, allowing plants roots to reach nitrogen depots in the deeper soil layers (Ma et al., 2014). Under phosphorus (P) deficiency, roots explore soil layers near to the soil surface and respond to P deficiency with repressed PR elongation and enhanced LR formation (López-Bucio et al., 2002). The LR growth increases in density and length,

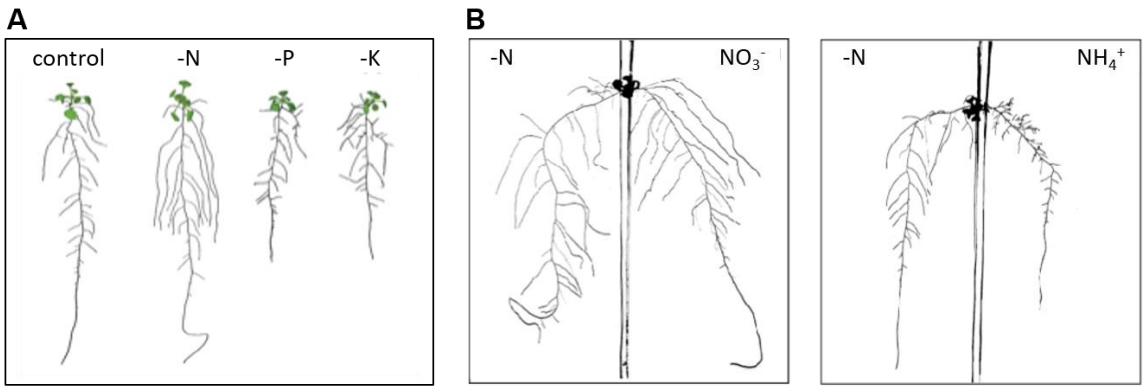

Figure 2. Response of the root system architecture of *Arabidopsis thaliana* to depletion of the macronutrients nitrogen, phosphorus or potassium, or to a local supply of nitrate or ammonium.
A) Root system architecture of Arabidopsis under nitrogen (N), phosphorus (P) or potassium (K) deficiency. To induce N deficiency, seedlings were pre-cultivated for 4 days on 165 µM N and then grown on 550 µM N for 14 days. To induce P deficiency, seedlings were pre-cultivated for 5 days with no P added to the medium and then grown on 50 µM P for 13 days. In case of K deficiency, seedlings were pre-cultivated for 7 days on 60 µM K and then grown in the absence of K for 12 days. **B)** Lateral roots respond to nitrate and ammonium in an opposite way. Plants were grown on vertically-splitted agar plates, in which local nitrate supply to the first lateral root in the right compartment led to an elongation of second-order LRs, whereas local ammonium repressed LR elongation but enhanced LR branching (modified from Gruber et al., 2013 and Lima et al., 2010).

forming a shallow and highly branched root system (López-Bucio et al., 2003) which can exploit the phosphate accumulating in the topsoil layer.

When plants grow under mild N deficiency, primary and LRs elongate (Gruber et al., 2013). However, this growth response is halted when N deficiency becomes more severe. Then, a CLE-CLV1 signaling module comes into play that restricts the expansion of the lateral root system into N deficient environments (Araya et al., 2014). Localized supply of the inorganic nitrogen forms nitrate and ammonium modulate RSA in a completely opposite manner. Localized nitrtae leads to elongation of LRs, while localized ammonium represses root elongation but enhances LR branching (**Figure 2**; Lima et al., 2010). Since nitrate depots are mostly found in deeper soil layers, an elongated root system may increase the change to reach those N sources. In contrast, ammonium may be distributed in the soil in a patchy manner, following hot spots of organic matter mineralization (Giehl and von Wirén, 2014). Taken together, these responses show that RSA is highly plastic and under control of local and systemic nitrogen-dependent signalling (Giehl et al., 2014).

In Arabidopsis the dual-affinity nitrate transporter NRT1.1 is the most important protein shaping the root system architecture in response to nitrate and is required for LR elongation into nitrate-containing patches (Remans et al., 2006). Furthermore, NRT1.1 functions as sensor for the transcriptional regulation of nitrate -inducible genes (Ho et al., 2009). Under local nitrate supply LR elongation is strongly promoted and this increased

LR elongation correlates with enhanced cell proliferation in the meristem of the LR reaching into the nitrogen source (Zhang et al., 1999). However, when nitrate was supplied locally in high concentrations, LR elongation was inhibited (Zhang et al., 1999; Zhang and Forde, 2000). The *nia1 nia2* double mutant, defective in nitrate assimilation, reacts to a local nitrate supply like wild-type plants, suggesting that the nitrate-ion itself acts as signal triggering LR elongation (Zhang and Forde, 2000). Transcript levels of the MADS-box transcription factor *ANR1* increased in response to nitrate, and this increased expression was dependent on the nitrate transporter NRT1;1, indicating that *ANR1* is downstream of NRT1;1 in the nitrate signalling cascade. In split-root experiments, LR elongation was exclusively observed in response to nitrate and could not be induced by other nitrogen forms (Remans et al., 2006). The expression of *ANR1* was strongly reduced in the *nrt1;1* (*chl1*) mutant, which exhibited a much weaker LR elongation. The co-localization of NRT1;1 and *ANR1* in root tips supports the sensor function of NRT1;1, which is required for nitrate dependent LR elongation (Remans et al., 2006). This conclusion was supported by experiments in which elevated L-glutamate was supplied to roots. High L-glutamate concentrations led to growth inhibition of PRs and enhanced LR branching, which could be reversed by external nitrate supply (Walch-Liu and Forde, 2008). The inhibitory effect of L-glutamate on PR growth appears to be related to the sensing function of NRT1;1, because the *nrt1;1* (*chl1*) mutant was insensitive to the antagonistic effect of L-glutamate on PR growth. The inhibitory effect of L-glutamate on PR growth and the enhanced LR branching were restored, when *NRT1;1* was reintroduced in the *nrt1;1* mutant. However, the de-phosphorylated form of NRT1;1 (*NRT1;1T101A*) did not rescue L-glutamate-induced PR inhibition, even in the presence of nitrate concentrations up to 5 mM, suggesting that NRT1;1 acts as a sensor just in its phosphorylated form (Walch-Liu and Forde, 2008). When Gifford et al. (2008) conducted a systems biology approach together with fluorescence-assisted cell sorting of GFP-tagged cell lines to investigate the responses of the transcriptome to nitrate, they identified a broad spectrum of genes expressed in different cell layers. They found the *microRNA167a/b*, which is expressed in the pericycle and the LR cap. This microRNA, together with *ARF8* (*AUXIN RESPONSE FACTOR8*), is involved in a regulatory pathway mediating LR outgrowth (Gifford et al., 2008). Interestingly, *ARF8* acts as repressor of LR emergence and is induced by nitrate but blocked by the glutamine synthetase inhibitor MSX. Taking together, organic N rather than nitrate seems to be the predominant signal regulating the repression of LR emergence (Gifford et al., 2008).

Since primary and LR elongation is repressed while LR branching is enhanced under ammonium supply (Britto and Kronzucker, 2002; Li et al., 2010; Lima et al., 2010; Liu et al., 2013; Araya et al., 2016), and ammonium nutrition increases the internal concentration

of organic N (Liu and von Wirén, 2017), further, so far undiscovered mechanisms must be involved in the inhibition of root elongation or the induction of LR initiation or emergence in response to ammonium. The elongation of roots is determined by two linked cell physiological processes, which are cell division and cell expansion (Beemster and Baskin, 1998). In the root apical meristem, ammonium represses cell proliferation and reduces longitudinal cell expansion, while the structure and activity of the stem-cell niche remains unaffected (Liu et al., 2013). Several mechanisms have been proposed to underlie the inhibition of root elongation by ammonium, including extra- and intra-cellular pH changes (Britto and Kronzucker, 2002), elevated ROS formation (Patterson et al., 2010; Xie et al., 2015), enhanced ammonium efflux in the root elongation zone that reinforces inhibition of longitudinal cell expansion (Li et al., 2010), or a decrease in protein glycosylation (Qin et al., 2008; Barth et al., 2010; Tanaka et al., 2015). However, besides inhibiting root elongation, ammonium triggers the formation of LRs, which is beneficial for nutrient and water acquisition. Several studies have revealed that ammonium triggers multiple physiological and morphological responses, such as specific changes in gene expression, metabolism, redox-status or root system architecture (Patterson et al., 2010; Li et al., 2010; Lima et al., 2010; Fernández-Crespo et al., 2015). The ammonium transporters AMT1;1 and AMT1;3, which show an overlapping cell type-specific expression pattern in roots (Loqué et al., 2006; Yuan et al., 2007a) were tested for their impact on higher-order LR formation in an ammonium patch. Growing the *amt1;1 amt1;2 amt1;3 amt2;1* quadruple mutant (*qko*) with reconstituted expression of AMT1;3 (*qko+13*) or AMT1;1 (*qko+11*) on vertically-splitted agar plates Lima et al. 2010 investigated the response of the root system to local ammonium supply. Third-order LR density of *qko+13* plants achieved wild-type levels, whereas *qko+11* plants did not restore third-order LR formation and remained at a similar level as *qko* plants. This result suggested that only AMT1;3 plays a crucial role in ammonium-dependent higher-order LR formation and that AMT1;3 may has an ammonium sensor function to regulate LR formation. In yeast, the high-affinity ammonium transporter Mep2p acts as a transceptor crucial for N-dependent pseudohyphal growth, which increases the surface of N-absorbing cells (Lorenz and Heitman, 1998; Marini and André, 2000). This pseudohyphal growth relies on an intact intracellular C-terminus that is required for the signal perception induced by N starvation (Rutherford et al., 2008). Additionally, filamentous growth is dependent on the cyclic AMP-protein kinase A (cAMP-PKA) (Van Zeebroeck et al., 2008). Surprisingly, methylammonium (MeA) can also activate the Mep2p-PKA signalling cascade, but only ammonium leads to conformational changes in the C-terminus of Mep2p as required for pseudohyphal growth (Van Nuland et al., 2006). Ammonium binding to the carrier triggers a conformational change that initiates

signaling (Van Nuland et al., 2006). This transceptor mechanism may be conserved among Meps and AMTs and responsible for higher-order LR branching in plants.

In Arabidopsis, ammonium is transported into the vacuole to prevent high cytosolic ammonium concentrations, and this compartmentation has recently been described as a target of cytosolic ammonium sensing by the receptor-like kinase CAP1 (Bai et al., 2014a). Root hair elongation is highly sensitive to external ammonium, which is related to a disturbed cytosolic calcium gradient in root hairs. Interestingly, higher ammonium concentrations in the cytosol could be induced in isolated vacuoles of the mutant *cap1-1,* in which ammonium fluxes across the tonoplast are lower. The presence of ammonium led to autophosphorylation of CAP1, as shown *in vitro* (Bai et al., 2014a). This result was confirmed by an independent phosphoproteomics approach, which detected phosphorylated CAP1 in Arabidopsis seedlings after exposure to ammonium (Engelsberger and Schulze, 2012). CAP1 is expressed in almost all cell types of roots, leaves, and flowers (Bai et al., 2014a), and has been proposed to function in those tissues as intracellular ammonium sensor and in cytosolic ammonium detoxification in by vacuolar compartmentation.

Interestingly, the application of local nitrate and ammonium together shows a complementary effect on the LR growth leading to an increase of both LR length and number (Lima et al., 2010). However, it is still unclear whether enhanced LR branching is primarily a consequence of the ammonium-triggered inhibition of the parental root meristem (Liu et al., 2013) and altered phytohormone flows or rather an adaptive response to ammonium availability independent of the fate of the parental root. To investigate the molecular mechanisms, which are crucial for higher-order LR branching in an ammonium-dependent manner the processes of LR development have to be linked to ammonium sensing and signalling

2.5 Lateral root development

The research on LR formation in the previous years discovered important regulatory processes involved in the morphological changes of the root system. In Arabidopsis, LRs originate from pericycle founder cells located on the opposite side of the xylem poles. LRs develop in a chronological fashion (Malamy and Benfey, 1997), which can be divided in three major steps defined as LR initiation, emergence and elongation (Péret et al., 2009). Auxin plays a critical role in almost every aspect of root development (Overvoorde et al, 2010). In the past decade, many studies reveal that auxin acts as an important mediator

for the integration of external nutritional signals into plant root developmental programs (Giehl and von Wirén, 2014). Auxin perception, biosynthesis or distribution have been shown to be modulated by heterogeneous nutrient availabilities to alter root system architecture. For example, phosphate deficiency enhances auxin sensitivity by upregulating the expression of the auxin receptor *TRANSPORT INHIBITOR RESPONSE1 (TIR1)* in pericycle cells, and therefore stimulating LR formation (Pérez-Torres et al., 2008). Mild Nitrogen deficiency induces the expression of *TRYPTOPHAN AMINOTRANSFERASE RELATED2 (TAR2)*, a member of the TAA family that catalyzes tryptophan-dependent auxin biosynthesis in roots (Stepanova et al., 2008), leading to an elevated auxin biosynthesis in roots, stimulating LR growth in response to low nitrogen availability (Ma et al., 2014). Localized iron enhances an AUX1-mediated shoot-to-root transport of auxin, which triggers LR elongation into an iron-rich patch (Giehl et al., 2012). The first steps in LR formation is the process of LR initiation, which happens at the proto-xylem in the basal meristem. Pericycle cells in the root apex de-differentiate and re-enter mitotic cell division. While the pericycle cells of the phloem poles remain in the G1 phase, the xylem pole cell changes into the G2 phase (Casimiro et al., 2003). Thereby, xylem-pole pericycle cells maintain their ability to divide after leaving the root apical meristem (Dubrovsky et al., 2000). In the region right behind the root apical meristem, the xylem pole pericycle cells further divide asymmetrically and generate pairs of founder cells. In this process two short cells are generated that are flanked by two longer cells. This stage of LR initiation is defined as stage I (Malamy and Benfey, 1997; Casimiro et al., 2001). After a radial expansion, the central short daughter cells divide periclinally to form a primordium composed of an inner and an outer cell layer, which is specified as stage II. In stage III, the outer cells undergo another round of periclinal cell division to form three cell layers in the young LR primordium. The inner cells also divide periclinally to create a fourth cell layer, defined as stage IV. In stage V, the LR primordium breaks through the parental cortex and epidermal cells and finally emerges to become a LR (Malamy and Benfey, 1997).

2.5.1 Initiation

The initiation of LRs is induced upon auxin-dependent signalling processes, where LR initiation depends on both locally synthesised auxin and shoot-derived auxin transported to the root apex through the phloem (Ljung et al., 2005; De Rybel et al., 2012). The regular distribution of LRs around the parental root is controlled by an endogenous “clock-like” mechanism that generates oscillations of auxin pulses in the protoxylem cells within the

oscillation zone every 15 hours (De Smet et al., 2007; Moreno-Risueno et al., 2010). Interestingly, each peak of auxin correlates with the initiation of a new LR primordium along the parental root (De Smet et al., 2007). Thus, auxin pulses close to the neighbouring xylem pole cells regulate the spacing of LR initiation. Nevertheless, the exact mechanisms underlying auxin-mediated differentiation of xylem pole pericycle cells into founder cells are not yet completely understood, but most likely is auxin the plant hormone responsible for competence acquisition of de-differentiating cells (Sena et al., 2009).

The existence of an auxin signal in the basal meristem supports the importance of the root tip for the regulation of root branching and supports the idea that the auxin pool in the root tip drives the initial stages of LR primordia formation (De Smet et al., 2007; De Rybel et al., 2012). The auxin efflux carriers of the PIN family have a big impact on LR initiation and are localized in the vascular parenchyma and phloem. There are 8 members of those plant-specific plasma membrane-localized proteins and the *pin1 pin3 pin4 pin7* quadruple mutant shows reduced LR formation, indicating that rootward auxin transport is needed for the formation of LR primordia as well (Benková et al., 2003). After the induction of new primordia by anticlinal cell division, PIN1 is required for cell plate formation (Geldner et al., 2004). The auxin transporter AUX1 (AUXIN RESISTANT1) is essential for gravitropic root responses and plays a central role in the processes leading to LR formation (De Smet et al., 2007; Bennett et al., 1996). AUX1 controls overall auxin levels in the root tip and is involved in the unloading of auxin from the phloem. Therefore, AUX1 ensures auxin availability at the site of LR initiation. AUX1-mediated auxin transport strongly depends on the shootward auxin transport from the root tip to the LR initiation zone (Marchant et al., 2002). Furthermore, auxin can be transported by MULTI-DRUG-RESISTENT/P-GLYCOPROTEINS (ABCB/PGPs). The ABCB/PGP P-glycoproteins are members of the ATP-binding cassette (ABC) protein subfamily. Those transporters are able to transport amphipathic and anionic molecules upon ATP-hydrolysis (Titapiwatanakun and Murphy, 2009). In Arabidopsis only a few ABCBs were characterized as auxin transporters, among them PGP1 (ABCB1) and PGP19 (ABCB19) are related to rootward auxin transport (Lewis et al., 2007; Wu et al., 2007; Yang and Murphy, 2009). PGP4 contributes to auxin transport in the root as well and is supporting AUX1 and PIN2 (Wu et al., 2007). The complementary and additive transport function of AUX1, PIN and PGP proteins are needed for the fine-tuning of polar auxin flows and are essential for LR primordia development (Titapiwatanakun and Murphy, 2009).

2.5.2 Emergence

Once a LR primordium has been initiated, it must form an autonomous meristem and emerge from the parental root tissue (Malamy and Benfey, 1997). Since the pericycle, where newly formed primordia are originating, is located deep within the root tissue, new primordia have to break through three overlying outer cell layers. *Arabidopsis thaliana* has a very simple root anatomy and the root consists of a diarchy vascular cylinder surrounded by a layer of pericycle, a layer of endodermis, a layer of cortex and a layer of epidermis cells (Dolan et al., 1993). Penetration of these three cell layers is defined as LR emergence (Péret et al., 2009). Microarray experiments of auxin-treated roots discovered an increased expression of cell wall-remodeling genes during LR development. Especially, pectin methylesterase and pectate lyase genes were up-regulated, which are involved in pectin catabolism. These results indicated that cell layers overlying primordia need to undergo cell separation, allowing primordia to emerge (Laskowski et al., 2006). For this process the expression of *expansin, xyloglucan endotransglucolyase/hydrolase* and *polygalacturonase* in the epidermal cells overlaying the LR primordia is required (Vissenberg et al., 2000; Van Sandt et al., 2007; Ogawa et al., 2009). However, outgrowth of the primordium from the parental root requires cell wall loosening to support LR emergence (Laskowski et al., 2006; Swarup et al., 2008). The whole LR emergence process also relies on auxin, and it seems that the auxin, crucial for this process, mainly derives from shoot apex, since the removal of leaves and cotyledons blocks LR emergence (Swarup et al., 2008). At later stages of LR development, LRs become independent of the parental root and synthesize auxin on their own, which has a big impact on the overall auxin pool in the root (Ljung et al., 2005). LR initiation and emergence compete for auxin and it has been shown by stochastic modelling and different mutants with disturbed auxin transport, that in the initiation zone of the parental root auxin reflux creates an auxin accumulation up to a certain threshold. The freshly formed primordium depletes the auxin source in the initiation zone by consuming auxin derived from the shoot. Finally, the primordium will emerge if its auxin content exceeds the threshold for emergence and stops the consumption of auxin from the parental root. LR branching is fine-tuned by the competition for auxin between LR initiation and emergence (Lucas et al., 2008). Specific auxin transporters are necessary for the LR emergence process. In the parental root, LAX3 (LIKE AUX1-3) is localized in cortical and epidermal cells overlaying LR initials. LR emergence is facilitated by auxin originating from the dividing pericycle founder cells and is coupled with the induction of cell wall remodelling genes in the endodermal cells. Followed by this step, auxin induces the expression of *LAX3*. This auxin influx carrier is located in the cortical cells overlaying the endodermis and increases the

cellular permeability for auxin. Then, cortical cell softening leads the LR primordium penetrating the overlaying cell layers to emerge. Supporting this hypothesis, the *lax3* mutant is defective in LR emergence, and exhibits a severe reduction in LR number (Swarup et al., 2008). PIN2 is expressed in epidermis cells mainly in the root elongation zone of the LR and is described as auxin transporter (Benková et al., 2003). PIN2 transports auxin from the root tip in a shootwart direction (Rashotte et al., 2000) and its regulation plays a crucial role during LR emergence. Auxin accumulation, in the primordia of the mutant *pin2* was significantly higher compared with the wild-type control as monitored by the auxin reporters DR5::GFP, which coincides with enhanced LR emergence in the *pin2* mutant. These results indicate that auxin accumulation in the LR primordium facilitates LR emergence through the LAX3-dependent auxin induction of cell wall remodelling genes (Swarup et al., 2008).

2.5.3 Elongation

After the breakthrough of the LR primordium through the overlaying cell layers of the parental root the activation of its meristem is required for an independent development of the freshly emerged LR. The further development of the young LR depends on auxin, produced in the LR meristem (Ljung et al., 2005). LR elongation relies on auxin transport from the shoot or PR tip, which was demonstrated by decreased LR elongation in the *mdr1* (multi drug resistence1) mutant. This mutant exhibits disrupted rootward auxin transport. The MDR1-like ABC transporter facilitates the rootward auxin transport and an auxin maximum in the LR tip, which is required for LR elongation (Wu et al., 2007). Since auxin is involved in promoting LR elongation in response to nitrate and NRT1.1 can facilitate cell-to-cell auxin transport when it functions as auxin transporter, NRT1;1 has to be considered as a crucial factor for nutrient-dependent auxin responses. The *nrt1.1* (*chl1*) mutant accumulated auxin in LR primordia and showed increased LR growth, which led to the hypothesis that under low nitrate availability, auxin accumulation in the root tip is low and LR elongation is reduced (Krouk et al. 2010b). A crucial role of the auxin transporter AUX1 in LR elongation in response to Fe was investigated by Giehl et al. (2012), where *AUX1* was expressed in LR apices. Interestingly, the mutant *aux-1* was not able to elongate LRs into Fe-enriched agar patches. The localized supply of Fe markedly increased AUX1-dependent fluorescence in the apex of already emerged LRs in the reporter line *proAUX1-AUX1:YFP* (Giehl et al., 2012). Furthermore, the reduction of PR elongation due to ammonium treatment appeared to be connected to the expression of *PIN2* and *AUX1*. In the presence of ammonium *PIN2* and *AUX1* were strongly repressed,

whereas the expression of *PIN3* and *PIN7* was just slightly downregulated. Therefore, AUX1 and PIN2 are likely to be involved in the regulation of auxin redistribution needed for root elongation (Liu et al., 2013).

2.6 Auxin signalling is obligatory for all stages of lateral root formation

Besides the auxin transport also auxin signaling pathways play a very important role in LR formation (Fukaki et al., 2007). Analysis with cell-cycle and cell-differentiation markers revealed that the gene *SOLITARY ROOT1* (*SLR1*) is involved in cell divisions of pericycle cells during LR initiation. The *slr-1* mutant completely lacks LRs, is defective in root hair formation, and in the gravitropic responses of the root. Moreover, its phenotype cannot be rescued by the application of exogenous auxin, suggesting that SLR1 is involved in auxin reception and signalling. *SLR1* encodes IAA14, a member of a large family of AUX/IAA proteins, indicating that SLR/IAA14 acts as a transcriptional repressor, and therefore is a key regulator in auxin-regulated growth and development, and especially in LR formation (Fukaki et al., 2002). Further AUX/IAA proteins regulate LR development, e.g. IAA1, IAA3, IAA18, IAA19 or IAA28 reduce the numbers of emerged LRs, but still allow LR initiation (Tian and Reed, 1999; Rogg et al., 2001; Tatematsu et al., 2004; De Rybel et al., 2010; Arase et al., 2012). Interestingly, these AUX/IAA proteins control LR development at different stages of the LR formation (Péret et al., 2009). In roots, auxin is sensed by TIR1 (TRANSPORT INHIBITOR RESPONSE 1) or AFB1-3 (AUXIN SIGNALING F-BOX PROTEIN1-3)-dependent receptor mechanism. These proteins are components of the cellular protein complex known as the SCF TIR1/AFB E3 ubiquitin ligase complex (Skp1-Cullin-F-box complex) (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b). The SCF TIR1/AFB E3 ubiquitin ligase complex is needed for the recruitment of AUX/IAA proteins in an auxin-dependent manner (Quint and Gray, 2006) and stabilizes the interaction between TIR1 and AUX/IAA. This interaction leads to AUX/IAA ubiquitination and subsequent degradation of AUX/IAA by proteasomes, which results in a de-repression of auxin-responsive genes involved in LR formation (Dharmasiri et al., 2005b; Tan et al., 2007; Vanneste and Friml, 2009). To activate the transcription of auxin-regulated genes, AUXIN RESPONSE FACTORs (ARFs) need to be released from AUX/IAA interaction. Once released from the AUX/IAA protein complex, ARFs bind to auxin-responsive promoter elements to activate auxin-induced genes. In Arabidopsis, 23 genes are encoding for ARFs, whereas *ARF7* and *ARF19* are described to be directly involved in LR initiation. LR emergence depends on the degradation of the SHY2/IAA3 or SLR1/IAA14

repressor, which is almost exclusively expressed in the endodermis (Swarup et al., 2008; Fukaki et al., 2002; Vanneste et al., 2005), and degraded in the presence of auxin, which induces the expression of *LAX3* in cortical cells by ARF7 and ARF19. However, this creates a positive feedback loop reinforcing auxin influx into cortex cells overlaying the emerging LR primordia (Swarup et al., 2008). Interestingly, the *arf7 arf19* double mutant is defective in LR initiation, like the *slr1* mutant (Fukaki et al., 2002; Vanneste et al., 2005). Since, the *arf7 arf19* double mutant is able to form a certain number of LRs, additional ARF genes seem to contribute to lateral initiation (Fukaki et al., 2007). Besides the SLR1/IAA14-ARF7/ARF19 dependent LR initiation module, a second auxin signalling module, the BODENLOS (BDL)/IAA12-ARF5 dependent module was discovered, and both modules are required for proper organogenesis. The transcription factors *LATERAL ORGAN BOUNDARIESDOMAIN/ASYMMETRIC LEAVES-LIKE* (*LBD/ASL*) were able to rescue the *arf7 arf19* mutant phenotype when overexpressed in the mutant background and were therefore identified as downstream genes regulated by ARF7 and ARF19 (Okushima et al., 2007). The direct activation of *LBD16/ASL18* and *LBD29/ASL16* by ARFs indicates a very specialized auxin signaling cascade, which regulates a whole regulatory network to develop a LR (**Figure 3**) (Okushima et al., 2007; De Smet and Jürgens, 2007). Furthermore, ARF-type transcription factors activate *PUCHI*, a protein-class, which was classified as a member of the *AP2/EREBP* family of transcription factors (van der Graaff et al., 2000; Chandler et al., 2007, Cole et al., 2009). PUCHI is expressed in all primordia cells and affects the auxin distribution or primary transcriptional response to auxin during LR formation. The *puchi* mutant is disturbed in the process of cell division during LR development, and seems to be involved in an auxin-dependent patterning in LR primordia (Hirota et al., 2007).

Nevertheless, whether higher-order LR branching on local ammonium supply is mainly triggered by changes in the auxin reception and signalling or by altered auxin transport through the side of LR initiation or emergence is not investigated until now.

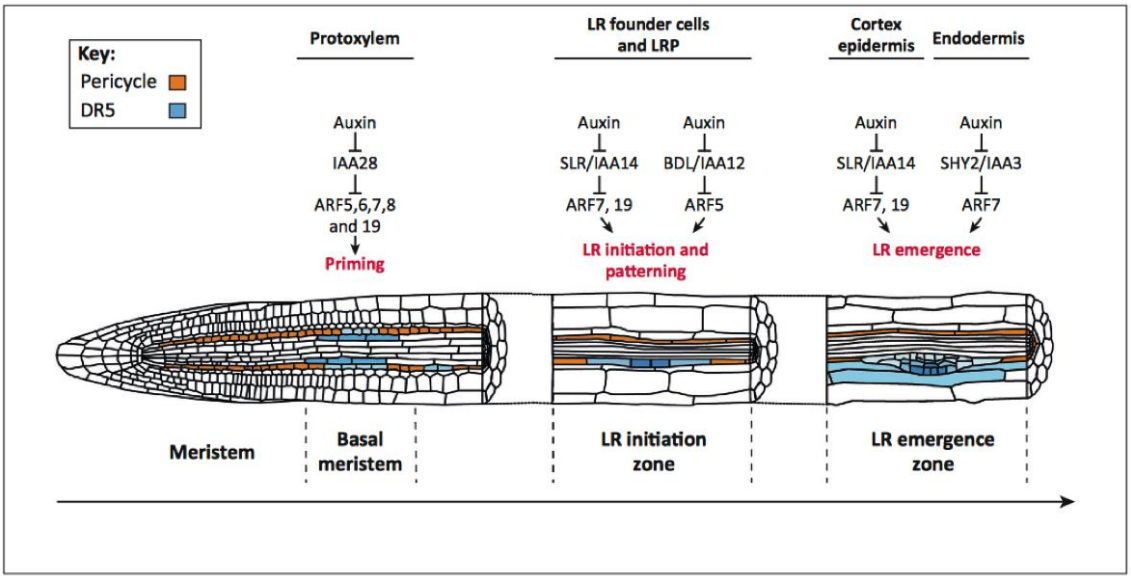

Figure 3. Auxin controls LR development through multiple auxin-signalling modules.
Lateral root (LR) founder cell priming involves the IAA28–ARF5, 6, 7, 8, 19 module in the basal meristem. The IAA14/SLR–ARF7, 19 module controls the polarisation of LR founder cell pairs, which leads to the coordinated migrations of the LR founder cell nuclei toward the common cell walls. Both the IAA14/SLR–ARF7, 19 and the IAA12/BDL–ARF5 modules are necessary for triggering LR initiation, which begins with a first round of asymmetric anticlinal divisions. These modules also regulate the morphological and histological patterning of the LR primordium (LRP). As the LRP develops, it pushes its way through the outer tissues of the parental root. This process is facilitated by local cell wall remodelling in the endodermis, cortex, and epidermis cells overlaying the primordium. This LR emergence, is controlled by the auxin diffusing from the LRP tip. In the endodermis, the auxin response during LR emergence is under the control of the IAA3/SHY2–ARF7 module, whereas it depends on the IAA14/SLR–ARF7, 19 module in the cortex and epidermis layers. The IAA14/ SLR–ARF7, 19 module regulates the auxin response during LR development, both in the LRP and in the outer tissues (Lavenus et al., 2013; modified).

2.7 Apoplastic and cytosolic pH affect N uptake and N related RSA

Interestingly, ammonium and nitrate transport across the membrane of root epidermal cells lead to pH changes in the root tissue, the apoplast or in the rhizosphere (Zhu et al., 2009; Ortiz-Ramirez et al., 2011; Khademi et al., 2004). The altered rhizosphere pH influences N acquisition, because plants compensate for pH fluctuations by regulating plasma membrane proton-ATPase activity to maintain the electrochemical gradient across the plasma membrane during N uptake (Zhu et al., 2009). The nitrate transporters belonging to the NRT1 or NRT2 family are nitrate-proton symporters (Espen et al. 2004; Xu et al. 2012) and therefore directly regulated by apoplastic pH (Fan et al., 2016). Surprisingly, the response of the nitrate transceptor NRT1.1 to nitrate and its transport activity is high at low pH (around 5), but reduced if the pH in the rhizosphere is increased to 7 independently of the nitrate concentrations in the medium (Ho et al., 2009). Furthermore, the low-affinity transporter OsNPF2.4 is expressed in the root epidermis of

rice, and its transcript levels and transport activity are regulated by pH, suggesting OsNPF2.4 is most likely involved in N sensing triggered by the rhizosphere pH (Xia et al., 2015). Besides external pH also cytosolic pH is strongly influenced by N acquisition. The nitrate transporter, OsNRT2.3b has been identified as sensor of cytosolic pH changes and is involved in balancing nitrate and ammonium uptake according to the cytosolic pH (Fan et al. 2016). However, the buffering of the cytosolic pH is assumed to enhance plant adaption to fluctuating rhizosphere pH by regulating the activity of nitrate or ammonium transporters.

Nevertheless, besides influencing N transport soil pH directly and indirectly modulates RSA. Changes in soil pH lead to massive transcriptional responses and have a big impact on the expression of a large number of auxin-responsive genes, indicating that pH-mediated changes in root architecture are most likely mediated through manipulations in auxin signalling (Lager et al., 2010). Interestingly, under alkaline conditions PIN2 shows increased auxin transport activity, which is coupled to the activation of plasma membrane-localized proton-ATPases. Those proton-ATPases are needed for the proton secretion from the root tips, which seems to be essential for the acidification of the rhizosphere to maintain root elongation (Xu et al., 2012). However, additional studies are required to discover the effects of cytosolic and apoplastic pH on N acquisition and changes in RSA. The impact of pH on auxin signalling and transport might be a key to understand the integration of pH-mediated signaling into root architectural tails.

2.8 Aims of the study

A large number of external and internal signals influence root system architecture during plant development. The present thesis focussed on the influence of a localized supply of ammonium on root system architecture and set off to investigate which ammonium-dependent signalling processes or molecular components are involved in higher-order LR branching under ammonium nutrition.

The high affinity ammonium transporter AMT1;3 was identified as the most crucial ammonium transporter involved in the root architecural changes due to local ammonium (Lima et al., 2010). In a first step, it was clarified if AMT1;3 is directly involved in the ammonium sensing and signalling processes or if the transported ammonium itself triggers the signals leading to higher-order LR branching in an ammonium patch. For this purpose, AMT reporter lines and mutants were used, as well as experiments with alterd ammonium supplies. Additionally, AMT- or ammonium-dependent signaling was suggested to facilitate changes in the auxin distribution in roots in response to ammonium. It was investigated which developmental process of lateral root development is affected by ammonium, before auxin reporters, auxin transport mutants, as well as auxin synthesis and transport inhibitors were used to determine how ammonium interferes with auxin-dependent lateral root formation. Finally, a series of experiments was conducted that addressed the question to what extent ammonium-induced pH changes in the apoplast alter auxin transport processes and lateral root formation. For comparison, most experiments were conducted on different local N supplies, where ammonium or nitrate were used as N source. In parallel the quadrouple mutant *qko*, defective in high-affinity ammonium uptake, was used to evaluate the impact of high affinity ammonium transporters on changes in root architectural traits or auxin distribution in roots.

With these approaches and the obtained results, the present Ph.D. thesis could elucidate the crucial role of AMT1;3 in higher-order lateral root formation and show how ammonium interferes with auxin signalling to induce lateral root branching.

3 Materials and Methods

3.1 Plant material

The wild-type (*Arabidopsis thaliana*) ecotype used in this study was Col-0. The following and previously described mutants and transgenic lines (in Col-0 background) were used: *pin2/eir1-1* (Roman et al., 1995), *pin3-5* (Friml et al., 2003), *pin4* (N89779) and *pin7* (N91554; Torii et al., 1996), *aux1* (SALK_020355C; Fischer et al., 2006), *lax1* (SALK_088482C) and *lax3-1* (SALK_135975C; Peret et al., 2012), *chl1-5* (*nrt1;1*) (Tsay et al., 1993), *abcb1* (SALK_083649C; Wang et al., 2013), *tir1-T* (SALK_151603C; Men et al., 2008), *pgp4-1* (N16269; Tripathi et al., 2009), *pgp19-4* (N31389; Rojas-Pierce et al. (2007), *tar2-1* (N16404; Stepanova et al. 2011), *taa1* (SALK_022743C), *qko (amt1;1, amt1;2, amt1;3, amt2;1*) (Yuan et al., 2007a)*, AMT1;1pro-AMT1;1gene::GFP* and *AMT1;3pro-AMT1;3gene::GFP* (Loqué et. al. 2006), *Col-0-DR5::GFP* (Friml et al. 2003), while *qko-DR5::GFP* line was generated by Joni Lima introducing the *DR5rev:GFP* construct (Vanneste and Friml, 2009) into the *qko* background by Agrobacterium-mediated transformation via floral dip. Moreover, *PIN2pro-PIN2gene::GFP* (Xu and Scheres ,2005), *PIN4pro-PIN4gene::GFP* (Vieten et al., 2005), *Apo-pHusion* (Gjetting et al., 2012) and *DR5v2-ntdTomato–DR5-n3GFP* (Liao et al., 2014), *DII-VENUS* (Brunoud et al., 2012) were obtained from the respective labs.

3.2 Growth conditions

Seeds were surface-sterilized with 70% ethanol mixed with 0.05% (v/v) TritonX-100 and germinated on MGRL medium (Fujiwara et al., 1992) containing 5 mM MES (2-[morpholino] ethanesulphonic acid; Sigma, Germany; pH 5.7), 0.5% sucrose and 1% Difco agar (Becton, Dickinson and Company). The plates were incubated at 4°C for 4 days to synchronize seed germination. Plants were pre-cultured on vertically-oriented plates for 10 days in mild nitrogen deficiency with 0.5 mM KNO_3 as sole N source.

The 10-d-old seedlings were transferred to freshly prepared vertically-splitted agar plates (Remans et al., 2006) or horizontally-splitted agar plates (Zhang and Forde, 1998) containing the above described MGRL medium, with almost no nitrogen (5 µM KNO_3), supplemented locally with KCl, KNO_3 or NH_4Cl at indicated concentrations. One seedling per plate was transferred to segmented plates with 10 mm of the oldest first-order LR apex touching the top of the high N (HN) segment, oriented in a vertical position.

To mimic pH changes occurring during ammonium uptake in N-free medium (0.8 mM KCl), pH was lowered to 5.0 by adding MES buffer to the HN plate side after 7 days of cultivation on vertically-splitted agar plates. Root system architecture was assessed usually after 12-15 d of growth in growth cabinets under a 22°C/18°C and 10/14-h light/dark regime and light intensity adjusted to 120 µmol photons m^{-2} s^{-1}.

3.3 IAA and NAA treatment

To evaluate the effect of exogenous auxin supply on higher-order LR formation, IAA (Indole-3-acetic acid; Sigma, Germany) as well as NAA (1-Naphthaleneacetic acid; Sigma, Germany) were applied to the HN side of the vertically-splitted agar plate in a concentration of 0.1 µM. Wild-type Col-0 and *qko* were grown in the above-described agar system. IAA and NAA were dissolved in 1 M KOH and diluted to working solution with double-distilled H_2O before applied to agar plates. After 15 d of cultivation on 0.8 mM KCl, 0.8 mM KNO_3, 0.8 mM NH_4Cl, as control treatment or 0.8 mM KCl + 0.1 µM IAA or NAA, 0.8 mM KNO_3 + IAA or NAA, 0.8 mM NH_4Cl + IAA or NAA, the RSA of Col-0 plants as well as *qko* plants were analysed.

3.4 Inhibiting auxin efflux carrier and auxin synthesis by NPA and kynurenine treatment

To inhibit polar auxin transport, N-naphthylphtalamic acid (NPA) was dissolved in DMSO and supplied to a fourth Y-shaped agar segment in vertically-splitted agar plates, in which the shoot and a part of the first-order LR was placed (**Figure 25A**). The final concentration of NPA was 5 mM. In control plates, only DMSO was supplied to the fourth Y-shaped agar segment at a final concentration of 0.1% (v/v).

To inhibit auxin biosynthesis in roots L-kynurenine (Kyn) was dissolved in DMSO and supplied to the HN agar segment in vertically-splitted agar plates, in which the first-order LR was placed (Figure 22A). The final concentration of Kyn was 5 mM. In control plates, only DMSO was supplied to the HN plate side at a final concentration of 0.1% (v/v). 0.8 mM NH4Cl were used as sole N source on the HN plate side.

3.5 Block of ammonium assimilation using MSX

To investigate the role of internal ammonium on the auxin accumulation in the root vasculature independent of AMTs, the *qko-DR5::GFP* line was grown in vertically-splitted agar plates in the presence of a glutamine synthetase inhibitor. Therefore, the *qko-DR5::GFP* line was treated locally with 0.8 mM KCl, KNO_3 or NH_4Cl as control treatment or was additionally supplied with 20 µM MSX (L-methionine-sulfoxamine; Sigma, Germany) for 48 hours after 10 d of growth on agar plates. After 10 d of incubation roots were analysed by confocal microscopy.

3.6 Inhibition of root growth using methylammonium

To analyse the role of different phosphorylation sides in the C-terminus of AMT1:3, different phosphorylation variants of AMT1:3 were tested in the background of *qko* by growing them on agar plates in the presence of the toxic ammonium analog, metylammonium (MeA). Therefore, the with the different AMT1;3 phosphorylation variants recomplemented *qko* lines were treated with 50 µM MeA (Methylammoniumclorid; Sigma, Germany) for 8 d. After 0 - 8 d of incubation root growth rates were analysed.

3.7 Root growth measurements

After 15 d of incubation on vertically-splitted agar plates, root systems were harvested and combed (lateral roots were moved away from the main roots to fully expand the root system) on plastic slides (transparent foil for monochrome copier, DIN A4, Lyreco, Germany) to scan the root systems by an Epson Expression 10000XL scanner (Seiko) at a resolution of 400 dpi. Root growth measurements were taken from scanned images using WhinRHIZO version Pro2007d software (Regents Instruments Canada). Root length and number of first-, second- and third-order LRs were analysed and used for calculations. Since local ammonium supplies resulted in a strong reduction in first-order LR length, LR numbers were used to calculate LR density (Dubrovsky et al., 2009).

Third-order LR initials were counted on second-order LRs of the *Col-0-DR5::GFP* line (Friml et al. 2003) and the *qko-DR5::GFP* line (generated by Joni Lima, *DR5rev:GFP* construct introduced into *qko* background by Agrobacterium-mediated transformation via floral dip) after 7d, 10d and 12d of incubation on vertically-splitted agar plates on the HN-

plate side containing 0.8 mM NH_4Cl. Developmental stages of LR initials were classified according to Casimero et al. (2003). LR initials were grouped in three groups of developmental stages containing stage I-IV, stage V-VII and emerged. All the experiments were performed at least twice and yielded similar results.

3.8 Microscopy analyses

GFP images were obtained with a confocal microscope LSM 780 T-PMT (Carl Zeiss MicroImaging GmbH, Jena, Germany). Roots were stained with propidium iodide (30 µg mL-1) for 10 min. GFP-dependent fluorescence was detected by excitation at 488 nm with an argon laser and filtering the emitting light at 505 to 530 nm. The 488 nm excitation and 458-514 nm emission lines were used to image propidium iodide-derived fluorescence. All confocal sections across samples were recorded with the same microscope settings. Image superimposition and fluorescence quantification were made by means of the Zeiss LSM 510 software version 3.0. The same microscope settings were used to record all of the confocal sections across samples.

3.9 pH measurements in agar plates

Wild-type Col-0 was grown in vertically-splitted agar plates and treated locally with 0.8 mM KCl, KNO_3 or NH_4Cl and agar medium was stained with bromocresol purple after 15 days over night, to observe changes in pH. Medium pH was buffered with 5 mM MES (2-[morpholino] ethanesulphonic acid; Sigma, Germany; pH 5.7). Additionally, agar pieces with a size of 2 cm by 2 cm were cut out of the agar near the base of the first-order LR and dissolved in 80° C warm water. After cooling down to room temperature pH was measured using a pH-meter.

3.10 Mutant screening

In order to screen for mutants with altered higher-order LR formation on locally supplied ammonium, homozygous T-DNA insertion lines mentioned in chapter 3.1 were grown on vertically-splitted agar plates like described in chapter 3.2.

3.11 Expression analysis

Table 1. Gene-specific primers used for qRT-PCR.

	oligonucleotides in 5'-3' orientation	
	Forward	Reverse
LAX3	CACCGAGAGTGGTAGGAGGA	CTCCAAACCCGAACCCAACT
PIN1	AACGCTCCGGTGGTTGG	CGGCGTGGTGGTTTCCT
PIN2	CAGAGGTTCTTCCACCGATGT	CTTCCCGGTGACATGTTCTCT
PIN3	GAGCTTCACATGTTCGTCTGG	CTTGATCGTTGTCAGGTGCTC
PIN4	ACAACGTGGCAACGGAACAA	CCTCTAGCATTACTCTTTCGAGGT
PIN7	ATCGTTCAAAGTTGAGTCCGATGT	TCTCCGAGAAGCGTTTGATTTTCT
TAA1	TGGCTAGGGACGAAGGAAGA	GCTCTCCAGCTCTGCTCATT
TAR2	TGCATTCTTGTTTCTTGGTTTTGTG	GCCAACGAGAGGACCAACAT
YUC1	GTCTCTCATCAGATCCGGCA	CACCTCCCTGAAGCCAAGTA
YUC2	ACCCATGTGGCTAAAGGGAGTGA	AATCCAAGCTTTGTGAAACCGACTG
YUC3	GGTGGTGGGATGTGGAAACT	CATGAACAGCGCTACGAACG
YUC4	ACGACCGTCTCAAACTCCAT	CGAACTCTGCTTTCTCGACG
YUC5	ATGATGTTGATGAAGTGGTTTCCTCTG	ATCAGCCATGCAAGAATCAGTAGAATC
YUC6	GAGACGCTGTGCACGTCCTA	AGTATCCCCGAGGATGAACC
YUC7	AGTTTGGTCCGGGAAAGGTC	GGTGAATCCCACCGCATACA
YUC8	ATCAACCCTAAGTTCAACGAGTG	CTCCCGTAGCCACCACAAG
YUC9	TGGGCTTAAAAGGCCTGACAT	GTCAAGAACCGGCGTCTTTC
YUC10	AGCTCCGTTTGCAATTGGTT	AGCTCCGTTTGCAATTGGTT
YUC11	TCAACTCCCTCACATGCCAT	TTCACCACCTTCACGATCCA
AUX1	TGGGCTTAAAAGGCCTGACAT	GTCAAGAACCGGCGTCTTTC
UBQ10	CTTCGTCAAGACTTTGACCG	CTTCTTAAGCATAACAGAGACGAG
ACT2	GACCAGCTCTTCCATCGAGAA	CAAACGAGGGCTGGAACAAG

Extraction of RNA was conducted with the TRIzol® reagent (Invitrogen) which is based on a single-step method (Chomczynski and Sacchi, 1987). After treatment with RQ1 RNase-Free DNase (Promega), 1 µg total RNA was reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) and oligo-dT-primers. Gene expression was measured by quantitative real-time PCR (qRT-PCR) with a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) and iQ SYBR

Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The expression levels of the genes analyzed in this study were assessed using the specific primers listed in table 1. Relative expression was calculated according to Pfaffl (2001) and normalized to *UBQUITIN10* (*UBQ10*) and *ACTIN2* (*ACT2*) as constitutively expressed housekeeping gene.

3.12 Hormone measurements by LC-MS-MS

The concentration of phytohormones was determined by a method developed by Kai Eggert (Molecular Plant Nutrition, IPK Gatersleben, Germany) for the sequential determination of various phytohormones as described in Eggert and von Wirén (2017). Auxins were extracted twice from 3 mg – 10 mg freeze-dried, grinded plant material with a cold 0.1% formic acid (FA) solution. Internal standards were added to the crude extract. The crude extract was added to an 1cc/30 mg HLB cartridge (Waters, USA), which was pre-conditioned with 1 ml MeOH containing 0.1% FA and equilibrated with 2 x 1 ml 0.1% FA. The HLB cartridge was washed twice with 1 ml 0.1% FA and eluted with 2 x 600 µl 90% MeOH containing 0.1% FA. Then, MeOH was evaporated using a vacuum centrifuge, and the residue was resuspended in 1 ml 0.1% FA and sonicated at 4°C for 2 min. In the second step, acidic and neutral compounds (ABA, auxins, salicylic acid) were separated from the basic cytokinins using a 1cc/30 mg MCX cartridge (Waters, USA). The MCX cartridge was pre-conditioned with 1 ml MeOH containing 0.1% FA and equilibrated with 2 x 1 ml 0.1% FA. Samples were then added to the column and washed twice with 1 ml 0.1% FA. Acidic and neutral compounds were eluted with 2 x 600 µl 100% MeOH.
10 µl of the purified extract was injected into an Ultra-Performance LC system (Acquity) coupled with a Xevo TQ mass spectrometer (Waters, Milford, MA, USA). The sample analytes were separated on an Acquity UPLC® BEH C18 1.7-µm, 2.1 x 100 mm column coupled with a VanGuard pre-column BEH C18 1.7 µm, 2.1 x 5 mm. The column and autosampler temperature were set to 40°C and 4°C, respectively.

3.13 Accession Numbers

Sequence data from this thesis can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:
At5g57090 (PIN2), At1g70940 (PIN3), At2g01420 (PIN4), AT1G23080 (PIN7), At2g38120 (AUX1), AT5G01240 (LAX1), AT1G77690 (LAX3), AT2G36910 (ABCB1), AT2G47000 (PGP4), AT3G28860 (PGP19), AT4G24670 (TAR2), AT1G70560 (TAA1).

4 Results

4.1 AMT1;3-mediated ammonium transport is crucial for higher-order lateral root branching

4.1.1 Higher-order lateral root branching on local ammonium supply depends on AMT-mediated ammonium uptake

To investigate the effect of different local N supplies on the root system architecture (RSA), a vertically-splitted agar plate system (**Figure 4A, B, C**) was established by Lima et al. (2010). Arabidopsis plants were placed in a way that just one first-order LR was grown on N-supplemented agar, while the remaining root system continued to grow under N-deficient conditions. Like shown in the study by Lima et al. 2010, localized nitrate supply stimulated the growth of first- and second-order LRs as indicated by their increase in length on the high nitrogen (HN) plate side. In comparison to N-deficient and ammonium-treated roots, nitrate-supplied first-order LRs were much more elongated (**Figure 4A, B, C, D**). Additionally, under local ammonium supply or N-deficiency the second-order LR length remained short in comparison to those growing under local nitrate supply and were in case of –N-treated roots 7-fold shorter and in case of ammonium nearly 2-fold shorter (**Figure 4F**). Like described by Lima et al. (2010), under local ammonium supply second- and third-order LR length and density were strongly stimulated. Ammonium enhanced the second- and third-order LR density in comparison to nitrate by > 75% (**Figure 4G, H, I**). This observation confirmed that ammonium provoked a local stimulation of higher-order LR branching, while nitrate and the absence of nitrogen were not.

Based on this root morphological response, third-order LR traits were selected as the morphological readout to characterize higher-order LR branching in the present work. To charactarize the mechanisms underlying the stimulation of ammonium on LR branching, the quadruple AMT knock-out mutant *qko (amt1;1 amt1;2 amt1;3 amt2;1)*, which lost 90% of the high-affinity ammonium uptake capacity relative to wild-type plants (Yuan et al., 2007a), was used. In the *qko* mutant, ammonium-induced LR branching was almost absent (**Figure 4E, I**; Lima et al., 2010). The application of *qko* mutant provides a contrast genetic material to investigate the role of localized ammonium supply on LR development.

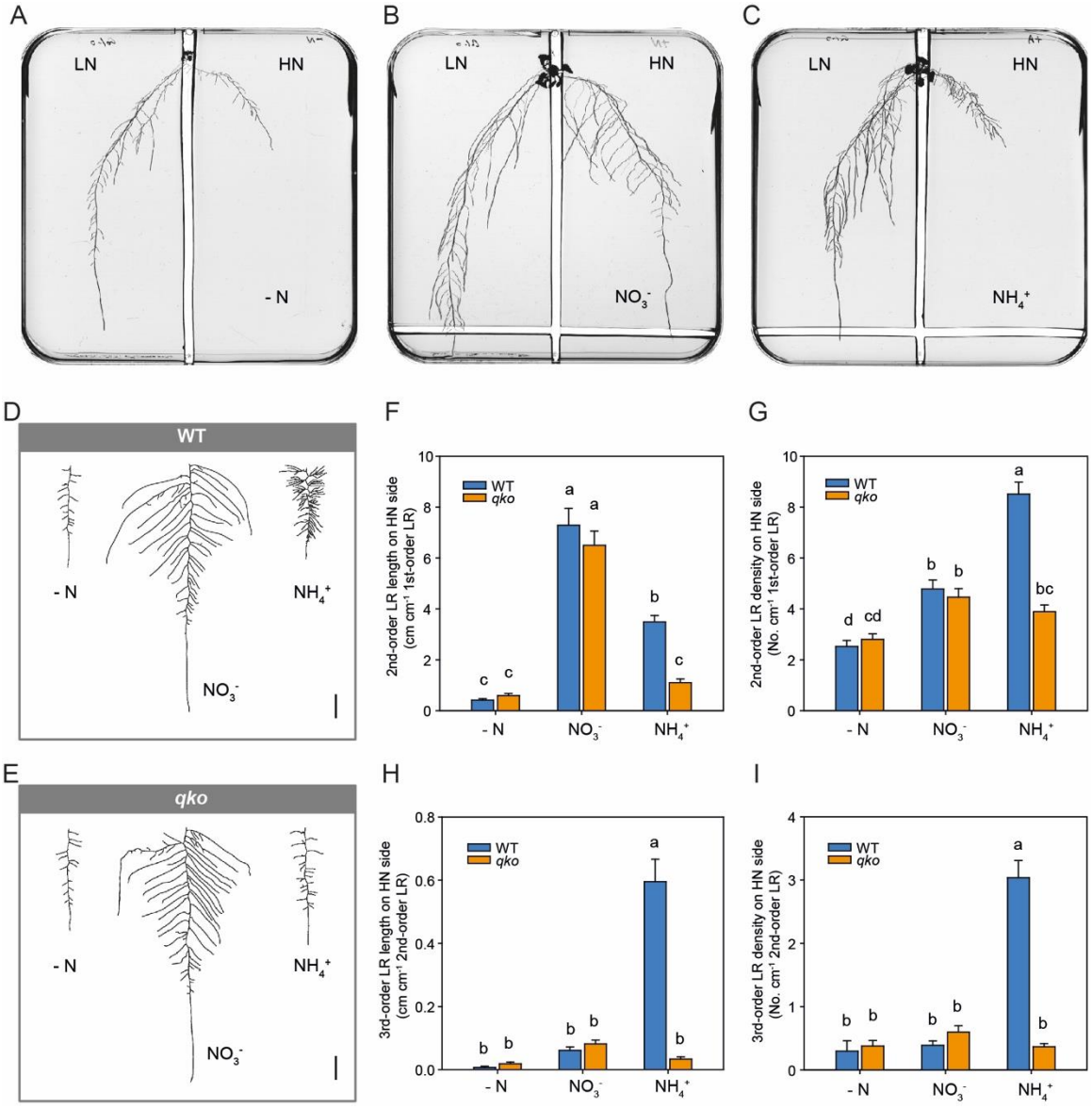

Figure 4. Higher order LR branching is induced by local ammonium supply.
Phenotype of 25 days-old *Arabidopsis thaliana* wild-type (Col-0) growing on vertically-splitted agar plates for 15 days with the first lateral root (LR) growing into a high nitrogen (HN) compartment containing **(A)** 0.8 mM KCl, **(B)** 0.8 mM KNO_3 or **(C)** 0.8 mM NH_4Cl. Primary root (PR) were grown in 5 µM KNO_3 on low nitrogen compartment (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days. First^t-order LR of wild type Col-0 **(D)** and *qko* **(E)** after 15 days of growth on the three different N treatments. Representative images from 12-16 plants per treatment were shown **(F)** Second-order LR length on the HN plate side, **(G)** second-order LR density on the HN plate side, **(H)** third-order LR length on the HN plate side, **(I)** third-order LR density on the HN plate side. Bars represent mean values (± SE) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 12 – 16 individual plants per nitrogen treatment. Bars represent 1 cm.

Additionally, to the root morphological parameters nitrogen uptake was monitored by supplementing ^{15}N-labeled ammonium to the high N (HN) side of vertically-splitted agar plates to observe the ammonium uptake ability of Col-0, *qko, qko+1;1* and *qko+1;3*. Shoot and root concentrations of ^{15}N tended to be slightly higher in Col-0 and in *qko* plants recomplemented with AMT1;1 or AMT1;3 than in *qko* plants. The PRs growing on the LN side showed no significant difference regarding the ^{15}N concentrations in the root (**Figure 5**). In agreement with similar ammonium uptake capacities as determined in long-term transport studies and same biomass production as in wild-type plants when grown on ammonium-based medium (Yuan et al., 2007a), *qko+11* and *qko+13* both accumulated ammonium-derived N to the same extent, clearly exceeding the ^{15}N accumulation by *qko* plants.

Nevertheless, the disadvantage of this long-term ^{15}N uptake study was that small differences between *qko+11* and *qko+13* were not detectable, most likely because small differences in ^{15}N concentration was coverd by the high ^{15}N content in the plant material accumulating in the plant tissue over the 15 days culture period.

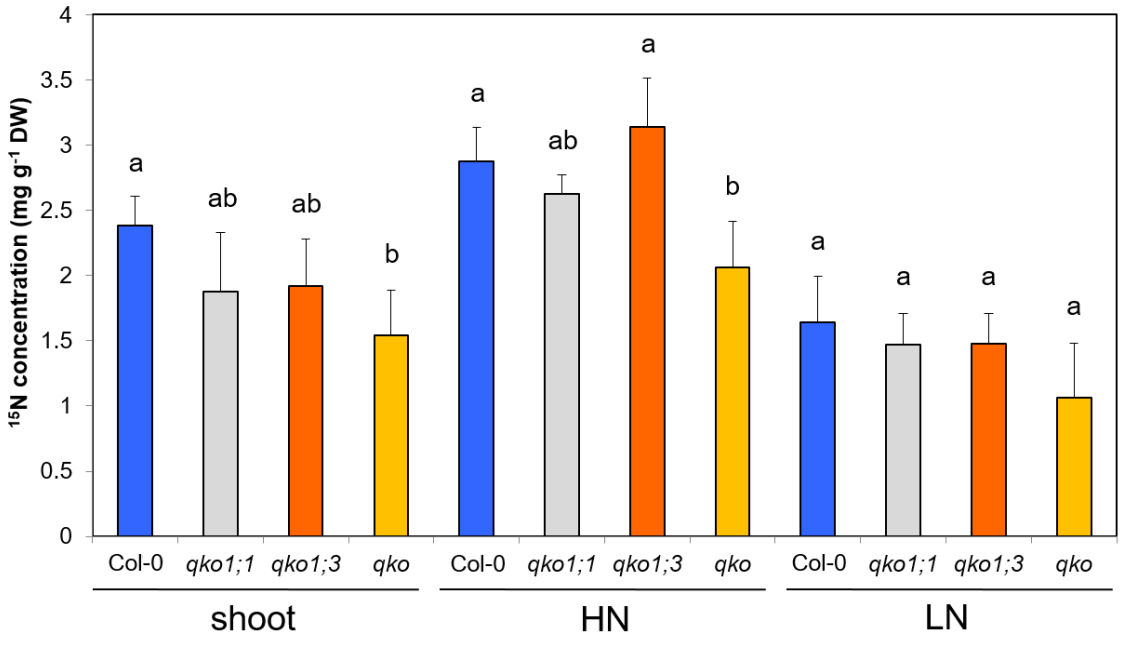

Figure 5. Ammonium uptake is strongly reduced in *qko* when grown on local ammonium supply.

Concentration of ^{15}N-labeled ammonium in wild-type Col-0, *qko* and *qko* lines with reconstructed expression of *AMT1;1* and *AMT1;3*. 25 days-old plants were grown for 15 days on vertically-splitted agar plates with the first LR growing into an high N (HN) compartment containing 0.8 mM ^{15}N-labeled NH_4Cl. Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days. ^{15}N concentrations in shoots, in roots grown on the HN plate side and in remaining roots grown on the low N (LN) plate side. Bars represent mean values (± SD) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 25 individual plants.

4.1.2 AMT1;3 is constitutively expressed in developing lateral roots under local ammonium supply

The intracellular localization of AMT1;1 and AMT1;3 was already investigated by Loqué et al. (2006) in transgenic Arabidopsis plants expressing C-terminal AMT gene–GFP fusions under the control of the native promoter. In this study plants were grown on homogeneous ammonium supply. Here, these two reporter lines were grown on vertically-splitted agar plates to observe AMT expression patterns on local N supply. GFP-dependent fluorescence of both fusion proteins increased in nitrogen-deficient and nitrate-treated second-order LRs (**Figure 6A, B, D, E**). These observations indicated that the ammonium transporters AMT1;1 and AMT1;3 reside in the plasma membrane.

AMT1;1 promoter-dependent fluorescence was mainly localized in rhizodermal cells in the elongation zone of the root. In the root cap, fluorescence was absent, being almost completely complementary in location to that of AMT1;3 promoter-dependent fluorescence (**Figure 6A, D**). Meanwhile, AMT1;3 promoter-dependent fluorescence was also found in rhizodermal cells in the elongation zone, indicating that co-localization of AMT1;1 and AMT1;3 just occurs in the elongation zone near the tip of second-order LRs. A common feature of both promoters was that their activities increased under nitrogen deficiency, in particular in rhizodermal cells near the root tip, like shown by Loqué et. al. (2006). The localized expression of AMT1;1 and AMT1;3 differed between the applied N-forms. Compared to AMT1;1, AMT1;3-dependent fluorescence was always seen independent of the supplied N-form in the split agar system (**Figure 6D, E, F**). While nitrate did not decrease the signal intensity of the GFP-dependent fluorescence derived from AMT1;1, ammonium strongly reduced this fluorescence (**Figure 6C**), indicating that AMT1;1 is repressed by ammonium like shown by Lima et. al. (2010). Summing up, on local ammonium supply AMT1;3 is induced in second-order LRs in contrast to AMT1;1, which is almost completely repressed.

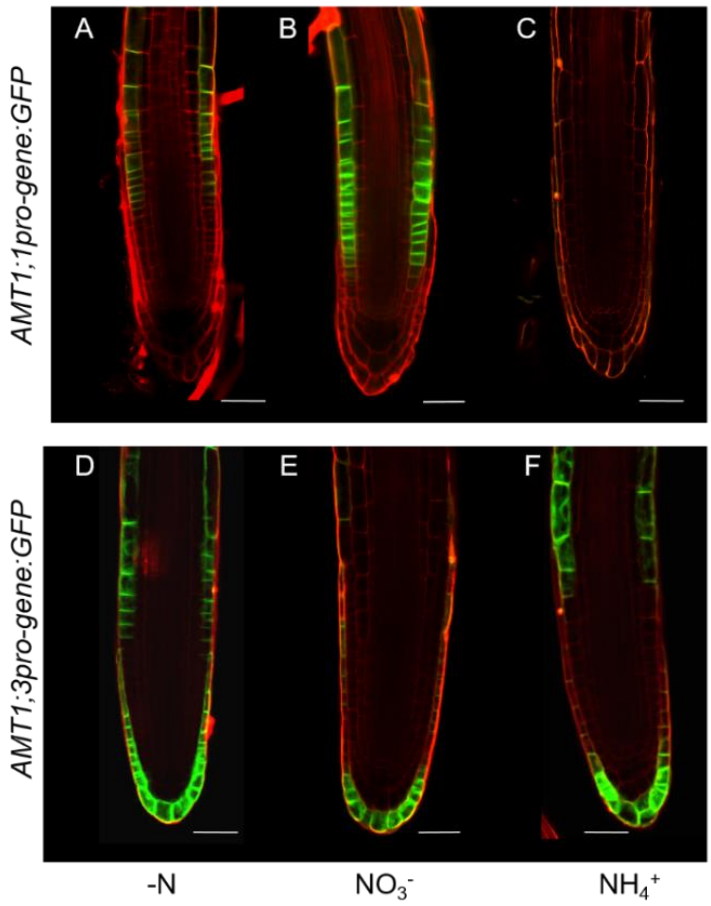

Figure 6. AMT1;3 is not repressed under localized ammonium supply.
Transgenic plants expressing *AMT1;1-promoter-ORF-GFP* **(A, B, C)** or *AMT1;3-promoter-ORF-GFP* **(D, E, F)** were grown on vertically-splitted agar plates with the first LR growing into an HN compartment containing 0.8 mM KCl **(A and D)**, 0.8 mM KNO_3 **(B and E)** or 0.8 mM NH_4Cl **(C and F)**. PR were grown in 5 µM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days. Whole-mount images from second-order LR tips were taken by confocal laser scanning microscopy 12 days after transplanting to detect AMT localization. Cell wall was stained by propidium iodide (PI). Representative images from 15-20 plants per treatment were shown. Bars represent 50 µm.

Not just the celltype-specific localization of AMT1;1 and AMT1;3 was different in transgenic Arabidopsis plants growing on the split-agar plates, there were also differences in tissue-dependent expression on the HN plate side. While AMT1;1 was strongly repressed in first- and second-order LRs under local ammonium supply (**Figure 7B, C**), AMT1;3 was expressed in the rhizodermis of freshly developing second-order LRs, which represented the highest order LRs at this developmental stage. AMT1;3 remained only weakly expressed in older, already matured first-order LRs (**Figure 7E, D**). However, under nitrogen-deficient conditions like on the pre-culture plates, AMT1;1 and AMT1;3 promoter-dependent fluorescence was clearly visible (**Figure 7A, D**), which has also been shown before (Loqué et al., 2006).

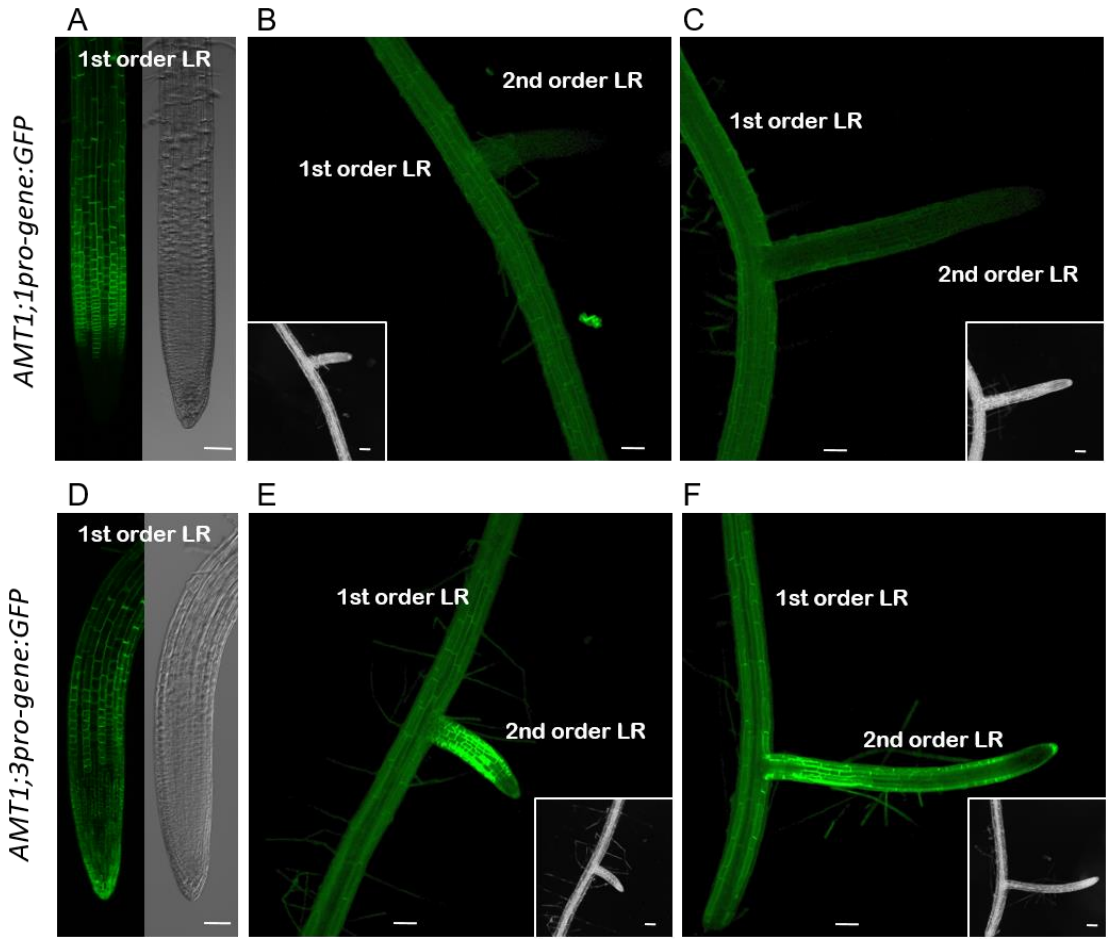

Figure 7. AMT1;3 is induced on local ammonium supply and localized in the root zone of developing LRs.
Transgenic plants expressing *AMT1;1-promoter-ORF-GFP* or *AMT1;3-promoter-ORF-GFP* grown on local ammonium. **(A)** First-order LR tip of *AMT1;1-promoter-ORF-GFP* in pre-culture after 10 days on 0.5 mM KNO_3. **(B and C)** Second-order LR growing out of the parental root after 10 days **(B)** or 12 days **(C)** on local ammonium. **(D)** First-order LR tip of *AMT1;3-promoter-ORF-GFP* in pre-culture after 10 days on 0.5 mM KNO_3. **(E and F)** Second-order LR growing out of the parental root after 10 days **(E)** or 12 days **(F)** on local ammonium. Plants were grown on vertically-splitted agar plates with the first LR growing into an HN compartment containing 0.8 mM NH_4Cl. PR were grown in 5 µM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days. Images from first- and second-order LRs were taken by confocal laser scanning microscopy to detect AMT localization. Representative images from 20-25 plants per treatment were shown. Bars represent 50 µm.

The same reporter lines were also pre-cultured on 0.5 mM nitrate as sole nitrogen source before transfer with 3 cm of the apical part of the PR to horizontally-splitted agar plates (**Figure 8A**). When the PR grew from the upper plate segment containing 0,25 mM nitrate to the lower plate segment containing 1 mM ammonium or 1 mM nitrate, the intensity of GFP-dependent fluorescence was affected. AMT1;1 got strongly repressed in the PR tip when the root was growing into local ammonium supply (**Figure 8C**). In contrast, nitrate was not leading to a repression of AMT1;1 (**Figure 8B**). As in the LRs, AMT1;3 was constantly expressed in the PR, even on local ammonium (**Figure 8D, E**).

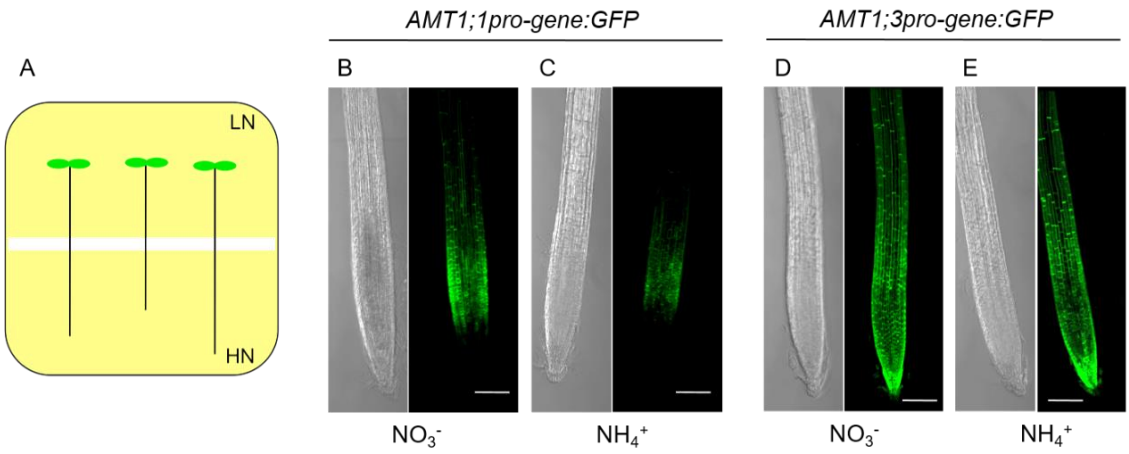

Figure 8. Expression of AMT1;1 and AMT1;3 in the PR in response to local ammonium supply.
(A) Schematic representation of the horizontally-splitted agar plate setup for the local ammonium treatment. Upper agar segment containing low nitrate concentration and lower agar segment containing ammonium. Transgenic plants expressing *AMT1;1-promoter-ORF-GFP* **(B and C)** or *AMT1;3-promoter-ORF-GFP* **(D and E)** in response to a local ammonium supply. Plants were grown on horizontally-splitted agar plates with the PR growing into a HN compartment containing 1 mM NH_4Cl or 1 mM KNO_3. The upper low nitrogen compartment contained 0.25 mM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source until PRs reached a length of 3 cm. Whole-mount images from PR tips were taken by confocal laser scanning microscopy 7 days after transplanting. First-order LR tip of *AMT1;1-promoter-ORF-GFP* grown into HN compartment containing 1 mM KNO_3 **(B)** or 1 mM NH_4Cl **(C)**. First-order LR tip of *AMT1;3-promoter-ORF-GFP* grown into HN compartment containing 1 mM KNO_3 **(D)** or 1 mM NH_4Cl **(E)**. Representative images from 18-22 plants per treatment were shown. Bars represent 50 µm.

4.1.3 The repression of primary root elongation by decreased meristem length depends on the presence of AMT1:3

To investigate the impact of AMTs on PR growth in respone to external ammonium, *Arabidopsis thaliana* (Col-0), *amt1;1* and *amt1;3* were pre-cultured on basal medium with 0.5 mM nitrate as sole nitrogen source, and then seedlings with 3 cm PRs were transferred to a horizontally-splitted agar plate system. When the PR grew from the upper plate segment containing 0,25 mM nitrate to the lower plate segment containing 1 mM ammonium, root growth in wild type and amt1;1 was significantly inhibited four days after roots were growing in to the local ammonium patch. As measure for the inhibition of PR elongation the approximate length of the root meristem, defined as the distance between the quiescent center and the first elongated cortical cell, of which cell length is two times higher than the cell width. Cell production depends on the number of dividing cells and on the rate of cell division. On day 5, the apparent length of the meristem was reduced in wild-type and *amt1;1* plants on local ammonium supply (**Figure 9A, B**). Cortical cell number in the meristem of wild-type and *amt1;1* plants was about 20% lower under ammonium- than nitrate supply (**Figure 9D**). These results are consistent with the reduced

cell production rate under ammonium found by Liu et al. (2013), who showed that under homogenous ammonium treatment PR elongation is inhibited. In contrast to *amt1;1*, *amt1;3* showed no difference in cortical cell number in the PR meristem between ammonium or nitrate treated roots (**Figure 9C, D**). This indicates, a deletion of *AMT1:3* reduces the susceptibility of the PR root to local ammonium, where a deletion of *AMT1;1* does not.

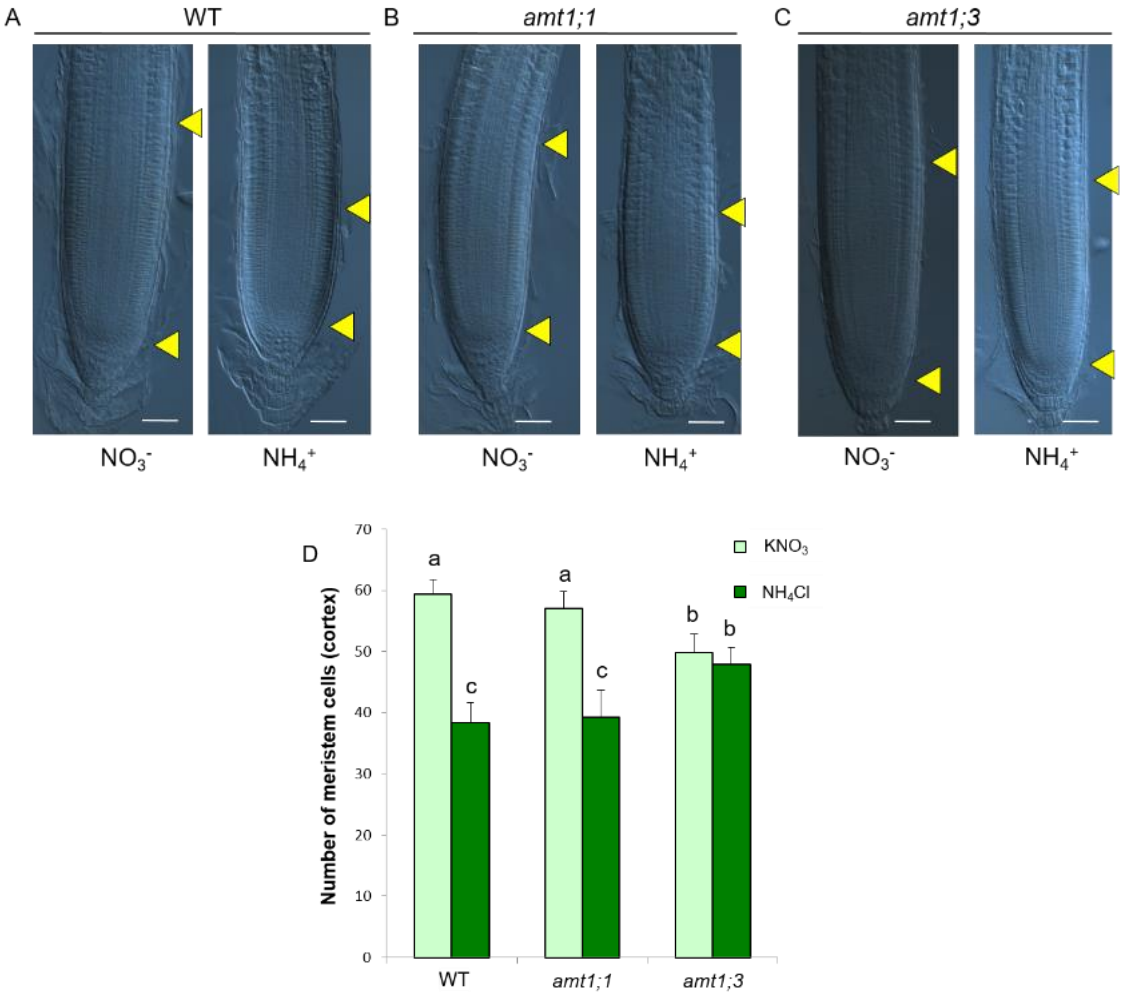

Figure 9. The amt1;3-1 mutant is tolerant to ammonium-induced repression of PR elongation.

Comparison of cortex cell number in PR meristem of wild type, *amt1;1-1* and *amt1;3*-1 grown into a local ammonium patch **(A)** PR tip of wild type growing on local nitrate or ammonium, **(B)** amt1;1 growing on local nitrate or ammonium and **(C)** amt1;3 growing on local nitrate or ammonium. Representative images from 25 - 30 plants per treatment were shown. **(D)** Number of cortex cells of PR meristem of wild type, amt1;1 and amt1;3 growing on nitrate or ammonium. Plants were grown on horizontally-splitted agar plates with the PR growing into a HN compartment containing 1 mM NH_4Cl or 1 mM KNO_3. The upper low-nitrogen compartment contained 0.25 mM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source until PR length reached 3 cm. Whole-mount images from PR tips were taken by light microscopy 4 days after PR grew on local N. Bars represent 50 µm. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 25 – 30 individual plants per nitrogen treatment and genotype.

Unexpectedly, the PR meristem of *amt1;3* was already smaler than that of wild-type and amt1:1 on local nitrate supply. In contrast, in the local ammonium treatment the number of cortex cells in the meristem of the PR of *amt1;3* was significantly higher than in the other two lines (**Figure 9D**). This observation indicated that AMT1;3 confers ammonium toxicity in the PR under ammonium supply, probably by increasing ammonium uptake into the meristem and stem cell niche. However, AMT1;3 appears also important under nitrate supply, maybe by retrieving ammonium leaking out of cells into the apoplast and thereby maintaining a higher N level in the meristem.

The C-terminus of AMT1;1 was shown to play a crucial role in allosteric regulation and functionality of a trimeric AMT complex (Loqué et al., 2007). A similar role of the C-terminus has been presumed for AMT1;3 here. The cooperation partner Prof. Lixing Yuan, Chinese Agricultural University, Beijing, had prepared transgenic Arabidopsis lines containing certain amino acid substitutions at different positions in the C-terminus of AMT1;3 to mimic phosphorylation or de-phosphorylation, which altered the activation state of the AMT1;3 protein. Corresponding to a T464D substitution in the AMT1;1 (Loqué et al., 2007), the amino acids T or S to D were substituted at several positions to mimic phosphorylation, which shuts off the protein activity, when referring to corresponding positions in AMT1;1 (Loqué et al., 2007 and Lima et al. 2010). In total ten AMT1;3 variants were analyzed, namely T464A, T464D, T471A, T471D, S480A, S480D, S487A, S487D, T494A and T494D. These AMT1;3 variants were ectopically expressed in the quadrouple knock-out line *qko*. To verify which phosphorylation site affects high-affinity ammonium uptake, the length of the PR was analyed as proxy for the ammonium uptake capacity, when plants were cultivated on metylammonium (MeA). Thereby, the question was addressed, whether ammonium transport and signalling by AMT1;3 depends on its phosphorylation state and is coupled to PR repression. Col-0, *qko* and *qko* expressing wild-type AMT1;3 under the control of an UBQ promoter or under the control of a 35S promotor were used as control. When the root growth rate was determined 0 - 8 days after transfer to MeA, phosphorylation mimicry in the C-terminus at T464 or T471 restored root growth, indicating that the protein was inactive and did not confer MeA uptake (**Figure 10**). However, phosphorylation mimicry by S480D, S487D or T494D did not rescue PR growth. Thus, phosphorylation downstream of position 471 had no effect on the deactivation of AMT1;3 and thereby did not allow de-repression of PR growth under MeA (**Figure 10**).

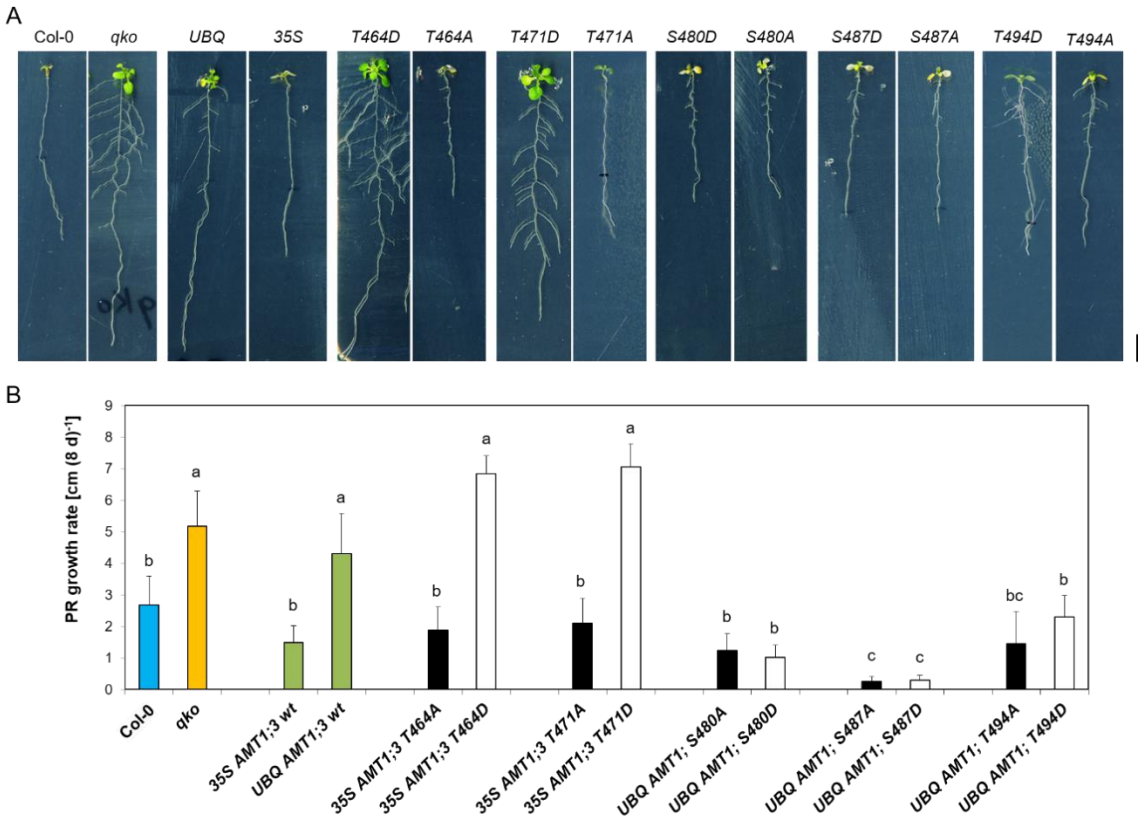

Figure 10. Metylammonium sensitivity in *qko* lines expressing AMT1;3 variants with modified C-termini.

Ten AMT1;3 phosphorylation variants (*qko+35S-AMT1;3-T464A*, *qko+35S-AMT1;3-T464D*, *qko+35S-AMT1;3-T471A*, *qko+35S-AMT1;3-T471D*, *qko+UBQ-AMT1;3-S480A*, *qko+UBQ-AMT1;3-S480D*, *qko+UBQ-AMT1;3-S487A*, *qko+UBQ-AMT1;3-S487D*, *qko+UBQ-AMT1;3-T494A*, *qko+UBQ-AMT1;3-T494D*) were grown on agar plates containing methylammonium (MeA) to verify critical phosphorylation sides in the C-terminus crucial to confer MeA uptake. **(A)** Phenotype of the ten tested AMT1;3 phosphorylation variants and control plants 8 days after growing on MeA containing agar medium. Representative images from 10 - 12 plants per treatment were shown. Scale bar is 1 cm **(B)** Root growth rate of the ten tested AMT1;3 phosphorylation variants and control plants determined between day 0 and day 8 after transfer to MeA. Wild-type (Col-0), *qko*, *qko+35S-AMT1;3* and *qko+UBQ-AMT1;3* were used as control. Plants were precultured on 0.5 mM KNO_3 as sole N source for 7 daays before transplanted to agar plates containing 50 mM MeA in the presence of 0.5 mM nitrate. Growth rate of the primary root was used as measure for MeA uptake ability. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 10 – 12 individual plants per genotype.

In order to address the hypothesis whether phosphorylation of the C-terminus in AMT1;3 can uncouple ammonium uptake from ammonium sensing, the meristem length of the ten AMT1;3 variants was analysed when plants were exposed to MeA. Again, mimicking phosphorylation in the C-terminus downstream of T471 did not allow rescuing the meristem length of the PR (**Figure 11**). By contrast, mimicking phosphorylation at position T464 or T471 rescued the meristem length to a similar extent as in the *qko* mutant. Thus, phosphorylation downstream of T471 had no effect on the deactivation of AMT1;3 and maintained protein activity and repression of the meristem length. These results suggested that ammonium uptake is coupled with ammonium-mediated root growth repression.

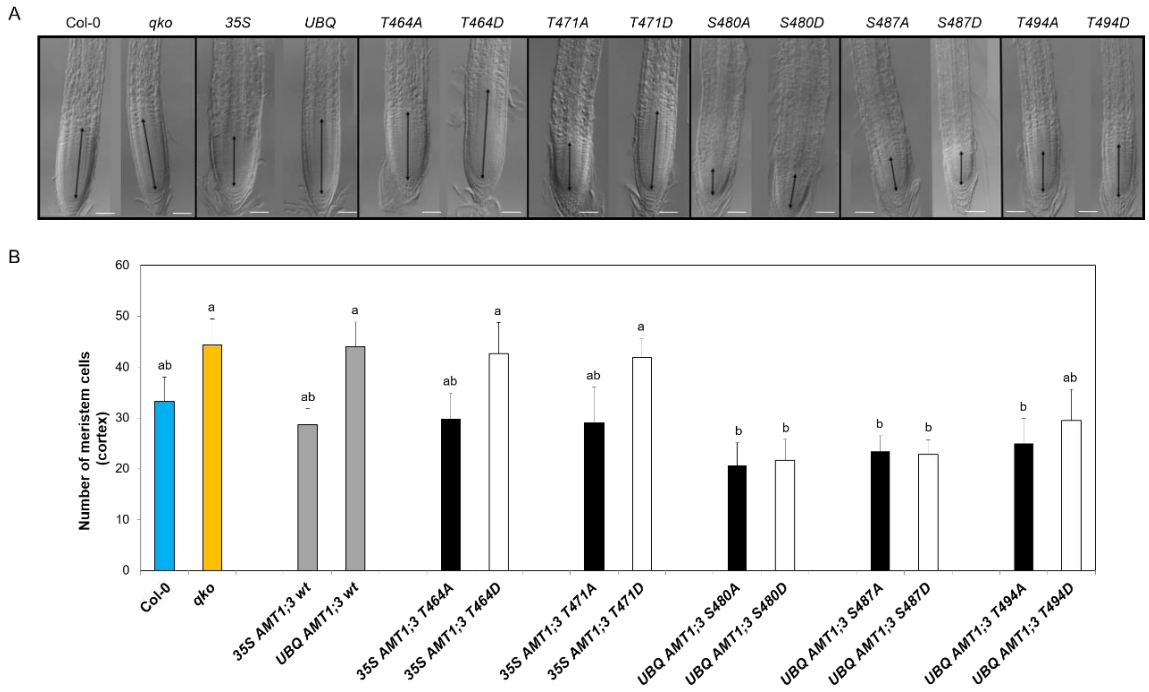

Figure 11. Modified C-termini in AMT1;3 define metylammonium sensitivity in qko lines expressing AMT1;3 phosphorylation variants

Ten AMT1;3 phosphorylation variants (*qko+35S-AMT1;3-T464A, qko+35S-AMT1;3-T464D, qko+35S-AMT1;3-T471A, qko+35S-AMT1;3-T471D, qko+UBQ-AMT1;3-S480A, qko+UBQ-AMT1;3-S480D, qko+UBQ-AMT1;3-S487A, qko+UBQ-AMT1;3-S487D, qko+UBQ-AMT1;3-T494A, qko+UBQ-AMT1;3-T494D*) were grown on agar plates containing methylammonium (MeA) to verify critical phosphorylation sides in the C-terminus crucial to confer methylammonium uptake. **(A)** PR meristem length of the ten tested AMT1;3 phosphorylation variants and control plants 5 days after growing on MeA containing agar medium. Arrows indicate meristem of the PR. Representative images from 20 plants per treatment were shown. Scale bar is 50 µm **(B)** Number of cortex cells in PR meristem of the ten tested AMT1;3 phosphorylation variants and control plants determined after 5 days after transfer to MeA. Wild-type (Col-0), *qko*, *qko+35S-AMT1;3* and *qko+UBQ-AMT1;3* were used as control. Plants were precultured on 0.5 mM KNO_3 as sole N source for 7 daays before transplanted to agar plates containing 50 mM of MeA in the presence of 0.5 mM nitrate. Number of cortex cells in the primary root meristem was used as measure for MeA uptake ability. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 16 – 20 individual plants per genotype.

4.1.4 The absence of high affinity ammonium transporters can be compensated for by elevated ammonium supply to induce higher-order LR formation

Like shown in Yuan et al. (2007a) and Lima et al. (2010), the Arabidopsis quadrouple knock-out *qko* shows very low ammonium uptake capacity compared to wild-type Col-0 plants. To verify if higher-order LR branching depends directly on ammonium sensing or signalling mediated by one of the AMTs or by ammonium itself, roots were exposed to different N treatments in horizontally-splitted agar plates. Fifteen days after transfer, the density of first-order LRs formed in response to 0.8 mM ammonium were not different between wild-type and *qko* plants (**Figure 12C, G, I, J**). Also, the root growth response to nitrate or N deficiency showed no significant differences between these two genotypes (**Figure 12A, B, E, F, I, J**). Thus, ammonium-induced changes in root architecture depended on AMT-mediated ammonium uptake. Since the loss of LR branching in *qko* under localized ammonium supply led to less ammonium uptake (**Figure 5**), a nutritional effect of ammonium-derived nitrogen had to be considered. In this case a higher concentration of locally supplied ammonium should restore LR branching in *qko* due to ammonium uptake via low-affinity transport systems (Loqué and von Wirén, 2004). Indeed, increasing external ammonium supply to 2 mM ammonium increased the density of higher-order LRs in *qko* to the level of wild-type plants (**Figure 12H, K, L**).

Additionally, elevated ammonium concentrations were locally supplied to wild type and *qko* in the vertically-splitted agar system. In presence of 0.8 mM ammonium higher-order LR formation in *qko* was strongly reduced in comparison to the wild type. However, increasing external ammonium up to 2 mM enhanced the formation of higher-order LRs in *qko* also in the vertically-splitted agar system (**Figure 13D, E**). In wild type plants, higher-order LR formation did not increase under elevated local ammonium supply (**Figure 13D, E**). Taking together, elevated local ammonium supply restored higher-order LR formation in *qko*. Thus, LR formation depends not on the presence of high affinity ammonium transporters *per se*, but on the amount of absorbed ammonium.

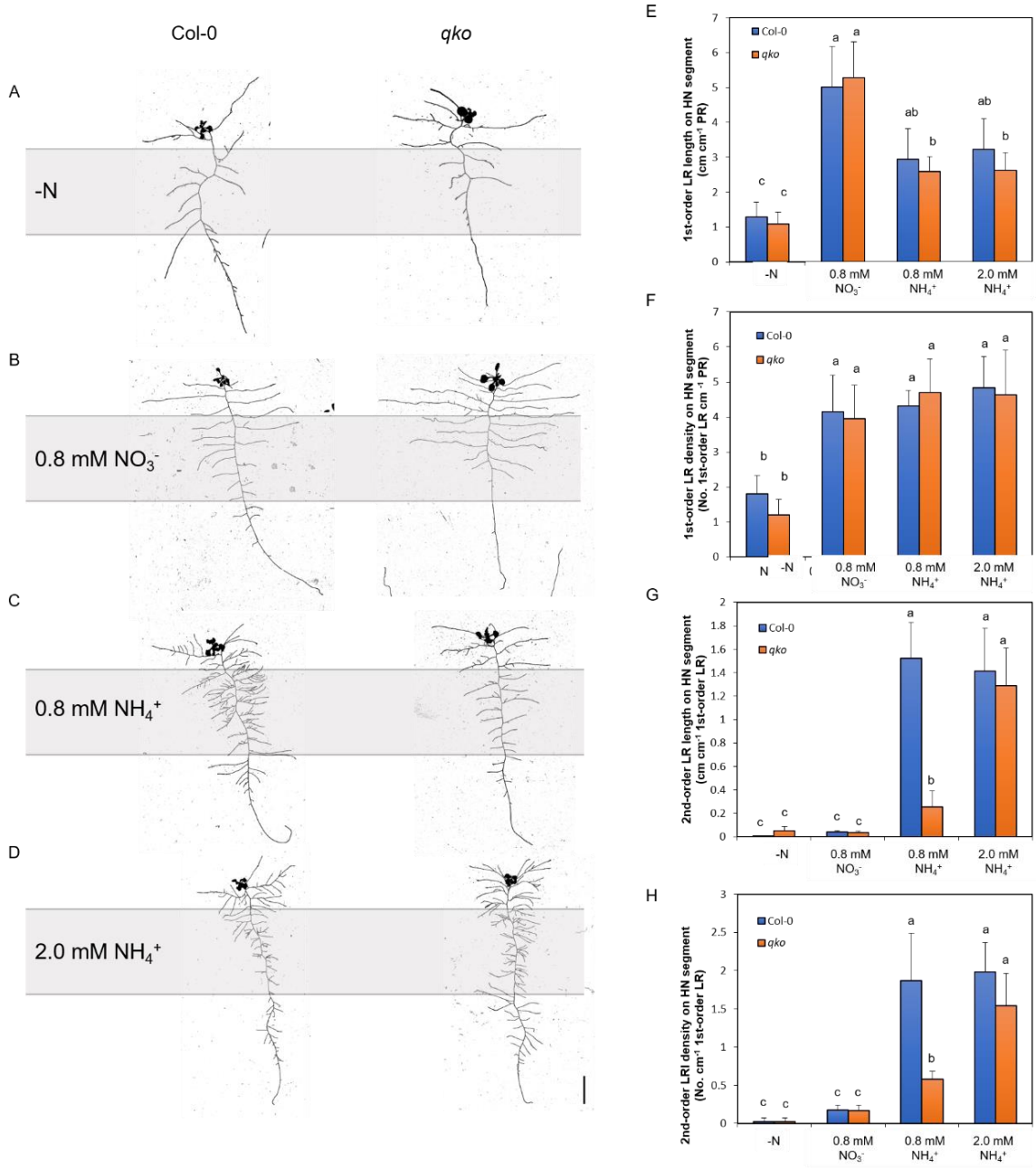

Figure 12. Higher-order LR branching in qko exposed to elevated ammonium concentrations in horizontally-splitted agar plates.
(A) to **(L)** shows LR development of *Arabidopsis thaliana* wild-type Col-0 and *qko* (*amt1;1, amt1;2, amt1;3, amt2;1*) grown on horizontally-splitted agar plates. **(A)** to **(D)** Root system architecture of Col-0 and *qko* on different local nitrogen supplies with the nitrogen source in the middle plate segment: **(A)** minus nitrogen, **(B)** 0.8 mM nitrate, **(C)** 0.8 mM ammonium and **(D)** 2.0 mM ammonium. Representative images from 8-12 plants per treatment are shown. Scale bar is 1 cm. **(E)** to **(H)** Individual root traits of Col-0 and *qko* in the horizontally-splitted agar system: **(E)** first-oder LR length, **(F)** first-order LR density, **(G)** second-order LR length and **(H)** second-order LR density. Plants were grown for 15 days on horizontally-splitted agar plates after 10 days pre-culture on 0.5 mM nitrate as sole N source. In minus N treatments, the middle compartment was supplied with 0.8 mM KCl. Upper and lower low nitrogen segments contained 5 µM KNO_3. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 8 – 14 individual plants per nitrogen treatment and genotype.

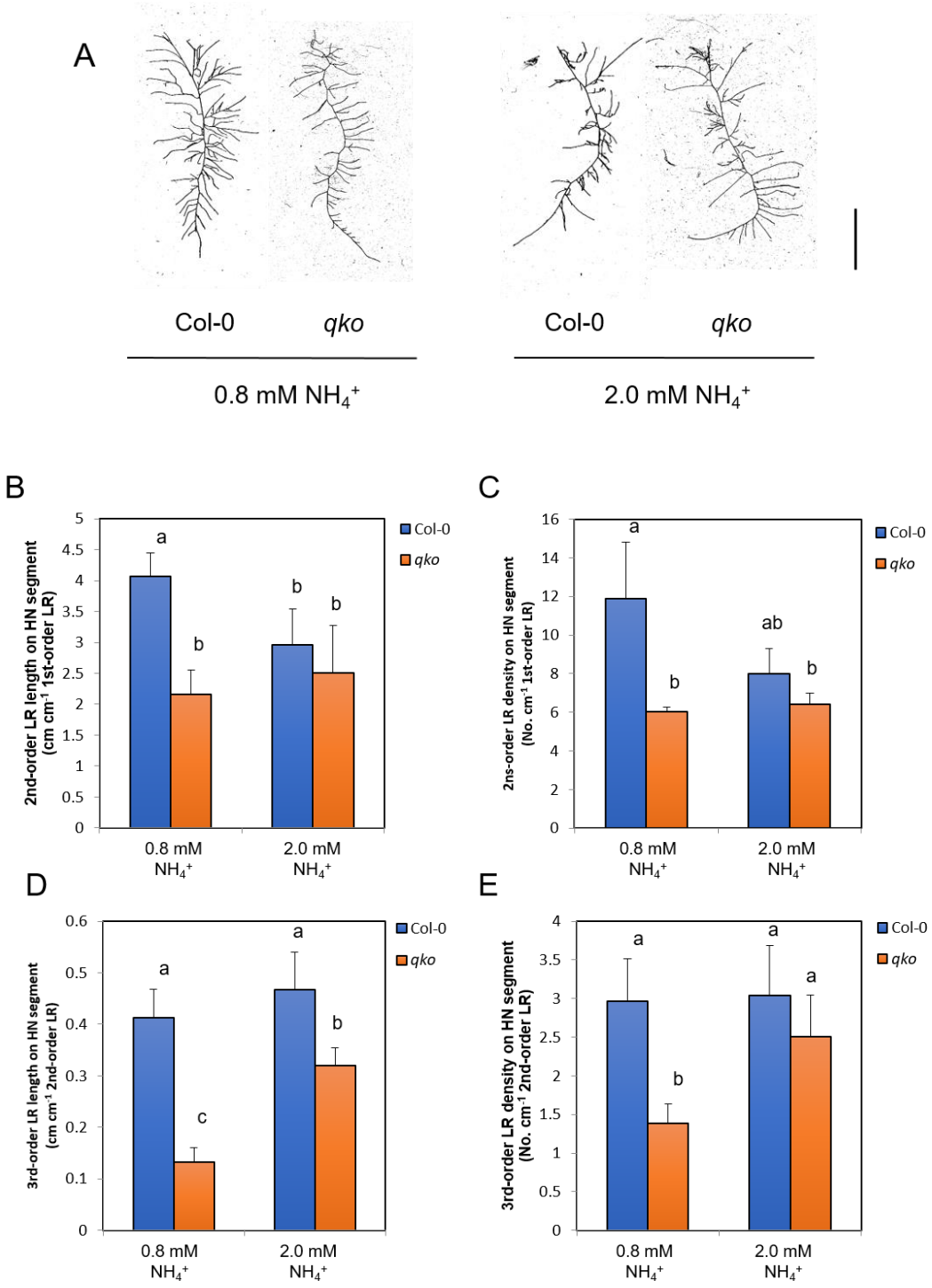

Figure 13. Higher-order LR branching in *qko* exposed to locally elevated ammonium concentrations.

(A) Phenotype of a first-order lateral root of wild-type and *qko* plants after its exposure to 0.8 or 2.0 mM ammonium. Scale bar is 1 cm. Representative images from 8 – 10 plants per treatment are shown. **(B-C)** Length and density of second-order lateral roots, and **(D-E)** length and density of third-order lateral roots of Col-0 and *qko* (*amt1;1, amt1;2, amt1;3, amt2;1*) plants grown on vertically-splitted agar plates. Plants were grown for 15 days on vertically-splitted agar plates with the first LR growing into an HN compartment containing 0.8 mM NH_4Cl or 2.0 mM NH_4Cl. Remaining root systems were grown in 5 µM KNO_3 (LN). Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 8 – 10 individual plants per nitrogen treatment and genotype.

4.2 Ammonium triggers lateral root emergence

Since increased LR branching may due to enhanced LR initiation or LR emergence, the question was addressed, if these two processes are affected by local ammonium supply. LRs in Arabidopsis are derived from pericycle founder cells positioned adjacent to the two protoxylem poles (Casimiro et al., 2001; Blakely et al., 1982). Seven developmental stages that precede LR emergence have been described by Malamy and Benfey (1997). The developmental stages of higher-order LR initials were grouped in 3 clusters, the first group included stage I till IV, the second group included stage V till VII and the third group represented already emerged LR initials (stage VIII), which were penetrating the epidermis of the parental root. To investigate at which step of higher-order LR formation ammonium plays a crucial role the auxin reporter line *DR5::GFP* was used to visualize LR initials at already early stages like Stage I. The reporter was introduced in the wild-type and *qko* background to compare the development of LR initials over time in the two genotypes. One week after transfer to local ammonium treatment, only a few higher-order LRs were emerged and the third-order LR density at all developmental stages showed no difference between wild-type and *qko* (**Figure 14A**). 10 days after transfer, a significant increase of emerged LRs was observed in wild-type plants but was absent in *qko* (**Figure 14B**). 12 days after transfer, almost three times more third-order LRs were emerged in wild-type plants, whereas in *qko* many LR initials were arrested in early developmental stages (**Figure 14C**).

Thus, local ammonium supply accelerated higher-order LR emergence in dependence of AMT-dependent ammonium uptake (**Figure 14D**). When the density of total LR initials (composed of both LRP and visible LRs) was used as a parameter to determine the probability of LR initiation, there was no significant difference between wild-type and *qko* at 7 days and 12 days after transfer while there was a slight increase in wild-type 10 days after transfer to ammonium (**Figure 14D**). This indicated that LR initiation was not regulated by local ammonium supply. Taken together, in the AMT knock-out mutant *qko*, LR emergence rather than LR initiation is largely impaired under local ammonium treatment (**Figure 14D**). These results show that local ammonium supply accelerates LR emergence and that this cell biological process is a major reason for enhanced higher-order LR branching in ammonium-supplied roots.

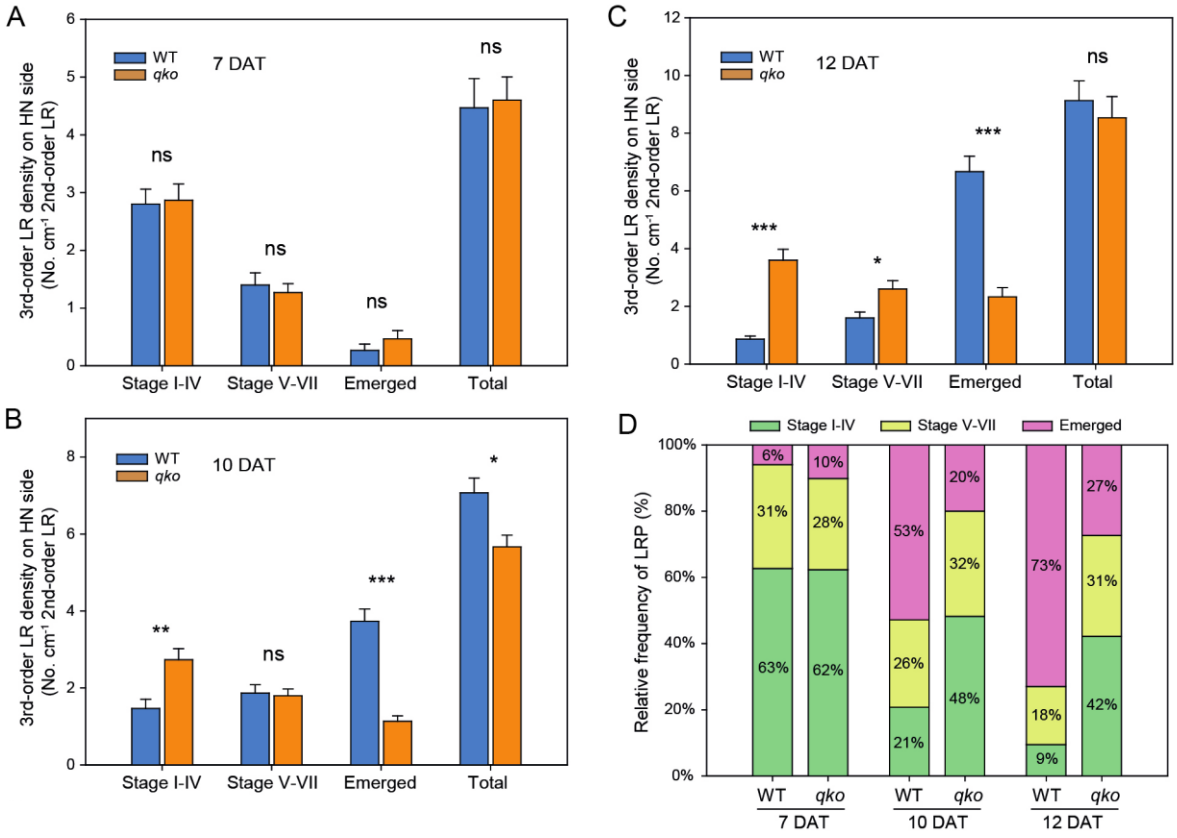

Figure 14. LR emergence in wild type Col-0 and qko exposed to local ammonium.
(A-C) Density of initiated and emerged third-order LR of wild-type and *qko* plants exposed to localized supply of ammonium after 7 **(A)**, 10 **(B)** or 12 **(C)** days. After a preculture on 0.5 mM nitrate plants were transplanted to vertically-splitted agar plates, in which first-order LRs grew into a high nitrogen (HN) compartment containing 0.8 mM NH_4Cl while the remaining root system continued to grow in 5 µM KNO_3 (LN). Third-order LRs were grouped in three groups of developmental stages containing stage I-IV, stage V-VII and those being emerged (stage VIII). **(D)** Relative frequency of LR primodia (LRP) in defined developmental stages at each time point. The number of LR initials was counted in the first three second-order LRs in a distance of 0.5 mm from the parental first-order LR at 7, 10 and 12 d after transfer to the vertically-splitted agar plates. Bars represent mean values (± SE) and asterisk denote significant differences among means at * P<0.05 ** P<0.01 *** P<0.001 (Student`s t-test), n = 15 individual plants per time point.

4.3 The N treatment mediates the auxin homeostasis in the root

4.3.1 Ammonium triggers auxin accumulation in the root vasculature

In plants, auxin acts as a dominant factor that tightly controls almost all stages of LR development (Péret et al., 2009a). Especially, the lateral distribution of auxin into the surrounding cells of emerging LR primordia is essential to promote LR emergence (Péret et al., 2009b). In order to investigate the role of auxin in local ammonium-induced LR emergence, *DR5:GFP* reporter in Col-0 background was used to monitor auxin responses in higher-order LRs under different nitrogen treatments. In second-order LR tips, auxin reporter expression was at a similar level in N deficienct and ammonium-treated roots,

while it increased upon exposure to nitrate (**Figure 15A-C**). By contrast, a markedly enhanced *DR5:GFP* activity was detected in the vasculature of second-order LR that were exposed to ammonium compared with those treated with nitrate (**Figure 15C**). In addition, reporter activity in the surrounding cells of emerging third-order LR primordia was also higher in ammonium-treated roots than nitrate-treated roots (**Figure 15B, C**). This observation showed that localized ammonium supply largely increases auxin distribution in the root vasculature and in the surrounding cells of emerging higher-order LRs, which in turn may stimulate higher-order LR emergence. Surprisingly, the highest auxin reporter expression was found in N-deficient roots, but there the accumulation of auxin was restricted to the vasculature and not present in outer cell layers (**Figure 15A**).

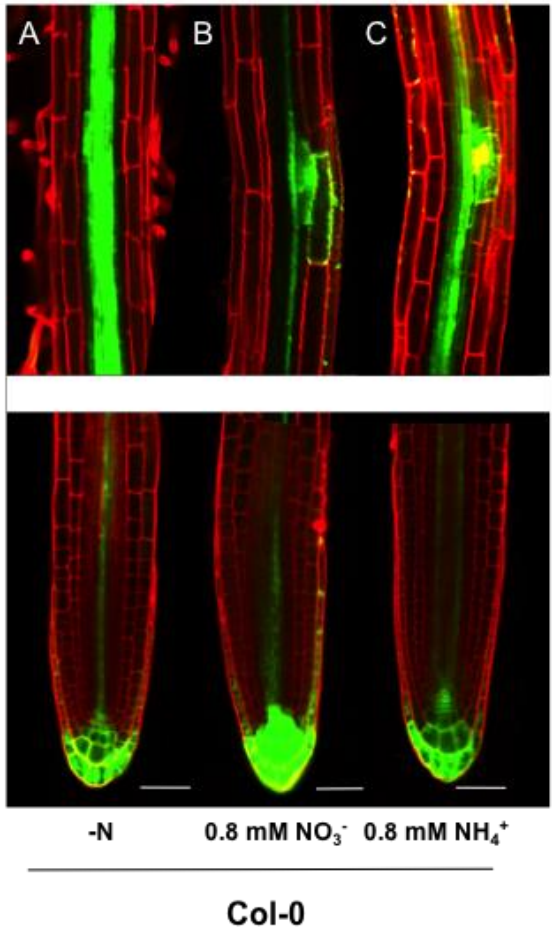

Figure 15. Auxin accumulation in roots in response to nitrogen supply.
GFP-derived fluorescence in second-order lateral roots (LR) of wild-type *Col*-0 *DR5::GFP* in response to different N treatments. 10 days-old plants were grown for 12 days on vertically-splitted agar plates with the first LR growing into a HN compartment containing **(A)** 0.8 mM KCl, **(B)** 0.8 mM KNO_3 or **(C)** 0.8 mM NH_4Cl. Remaining root system was grown in 5 µM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days before transplanting to vertically-splitted agar plates. Images from second-order LRs were taken by confocal laser scanning microscopy after 12 days on the local N treatments to detect auxin localization. Shown are represetative images from 12 individual plants per nitrogen treatment. Bars represent 50 µm.

To confirm the stimulatory effect of local ammonium on auxin distribution, the *DR5:GFP* construct was introduced into the *qko* background. In ammonium-exposed second-order LR tips, auxin distribution patterns were highly similar in wild-type and *qko* plants (**Figure 16A and B**). However, *DR5:GFP* expression was strongly enhanced in the vascular tissues of second-order LR and in the surrounding tissues of emerging third-order LR primordia of wild-type (**Figure 16A**) but not of *qko* plants (**Figure 16B**). The high degree of concordance between LR branching phenotypes and auxin distribution patterns indicates that local ammonium supply may stimulate higher-order LR emergence via modulating auxin distribution in roots.

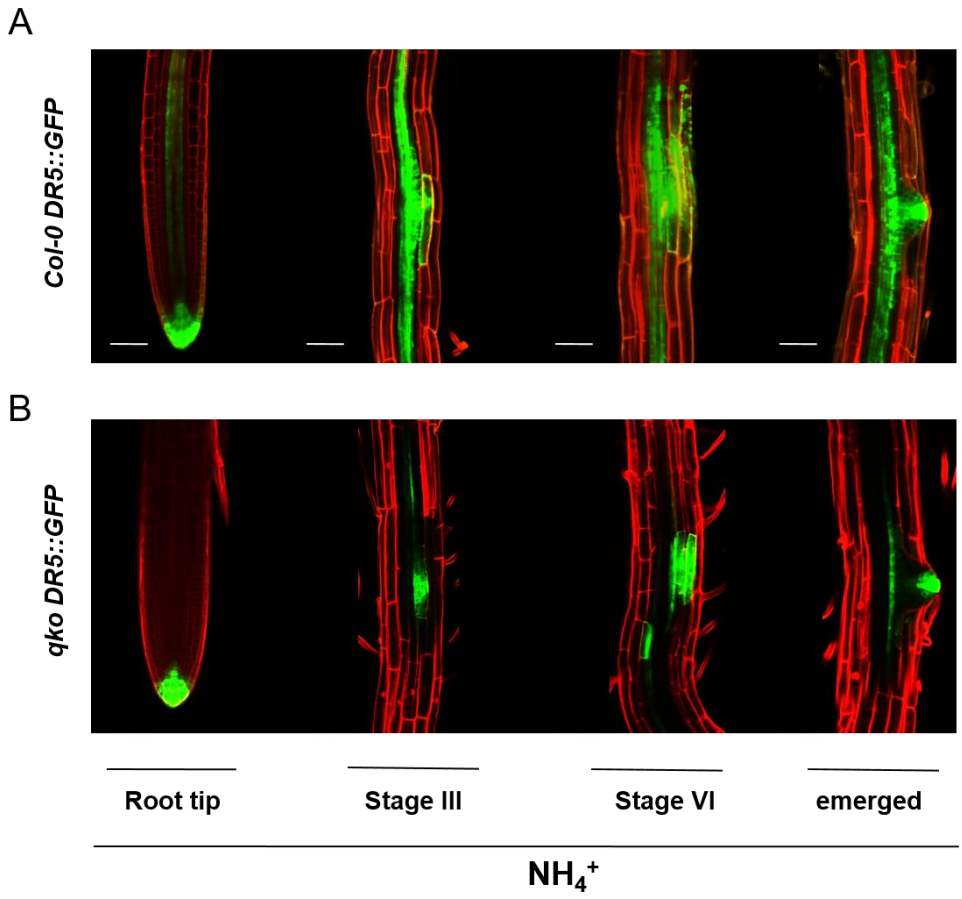

Figure 16. Auxin accumulation in roots of wild-type and *qko* plants under localized ammonium supply.
GFP-derived fluorescence in roots of **(A)** *Col-0 DR5:GFP* and **(B)** *qko DR5:GFP* grown on vertically-splitted agar plates under local ammonium supply analysed after 12 days by confocal microscopy. Pictures were taken of second-order LR tips and third-order LR initials at stage III, stage VI and when LR were emerged. 10 days-old plants were grown for 12 days on vertically-splitted agar plates with the first LR growing into a HN compartment containing 0.8 mM NH_4Cl. Remaining root system was grown in 5 µM KNO_3 (LN). Plants were precultured on 0.5 mM KNO_3 as sole N source for 10 days before transplanting to vertically-splitted agar plates. Images from second-order LRs were taken by confocal laser scanning microscopy after 12 days on the local ammonium treatment to detect auxin localization. Shown are represetative images from 15 individual plants per genotype. Bars represent 50 µm.

To determine whether the auxin accumulation in the root vasculature is triggered by exogenous or endogenous ammonium, intracellular ammonium concentrations were increased by adding MSX to the roots of *qko DR5::GFP*. In the presence of MSX, ammonium can not be assimilated and intracellular ammonium concentrations increase (Rhodes et al., 1986). No significant changes in the GFP signal intensity were detected in roots exposed to N-free medium or nitrate-containing medium in the presence of MSX in comparison with the control treatments (**Figure 17A, B, D, E**). In contrast, the MSX treatment clearly increased GFP-derived fluorescence in ammonium treated *qko DR5::GFP* plants. Nevertheless, roots treated with ammonium or mitrate with additional MSX did not reach a similar level of auxin reporter expression as roots grown on N-free medium (**Figure 17E, F**). This indicates that intracellular ammonium promotes auxin accumulation in the root vasculature independent of a direct involvement of AMT-mediated ammonium uptake.

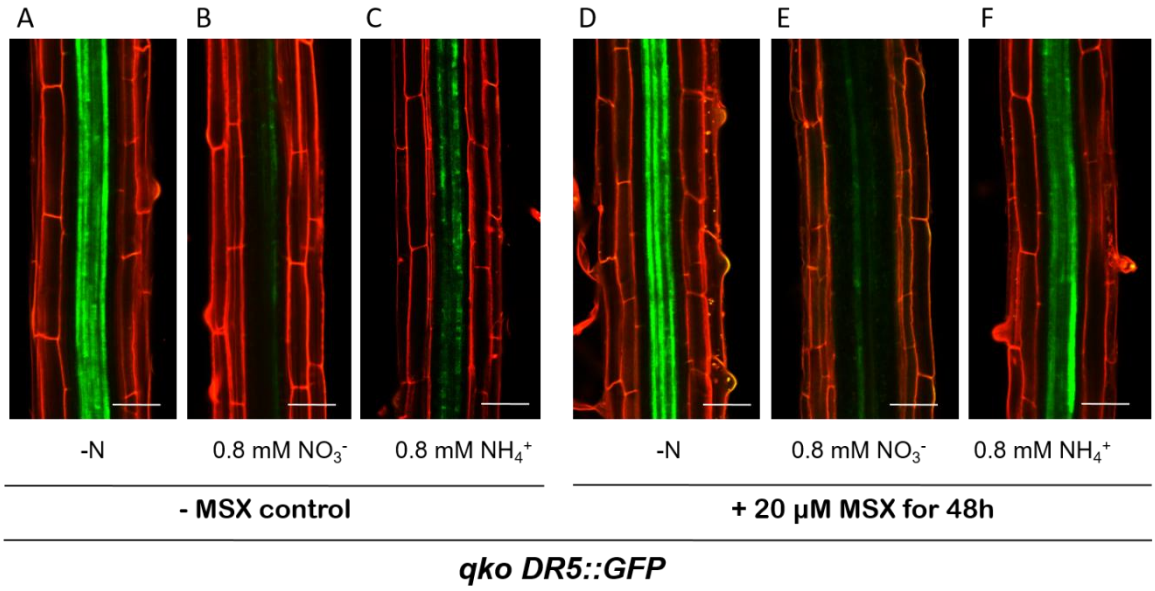

Figure 17. A glutamine synthetase inhibitor enhances auxin accumulation in the root vasculature of *qko* plants grown on local ammonium supply.
GFP-derived green fluorescence of *qko* plants expressing *DR5:GFP*. **(A)** Second-order lateral roots (LR) of *qko DR5::GFP* grown on N deficiency, **(B)** local nitrate or **(C)** ammonium, **(D)** Second-order LR of *qko DR5::GFP* grown on N deficiency in the presence of metheonine sulfoxamine (MSX), **(E)** local nitrate in the presence of MSX or **(C)** local ammonium in the presence of MSX, Plants were grown for 12 days on vertically-splitted agar plates with the first LR growing into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. After ten days on vertically-splitted agar plates plants were grown in the absence **(A-C)** or presence of 20 µM MSX for 48 hours **(D-F).** Plants were precultered for 10 days on 0.5 mM nitrate before transferred to vertically-splitted agar plates with the different treatments. Images from second-order LRs were taken by confocal laser scanning microscopy after 12 days on the different treatment to detect auxin dependent GFP intensity. Shown are represetative images from 12 individual plants per treatment. Bars represent 50 µm.

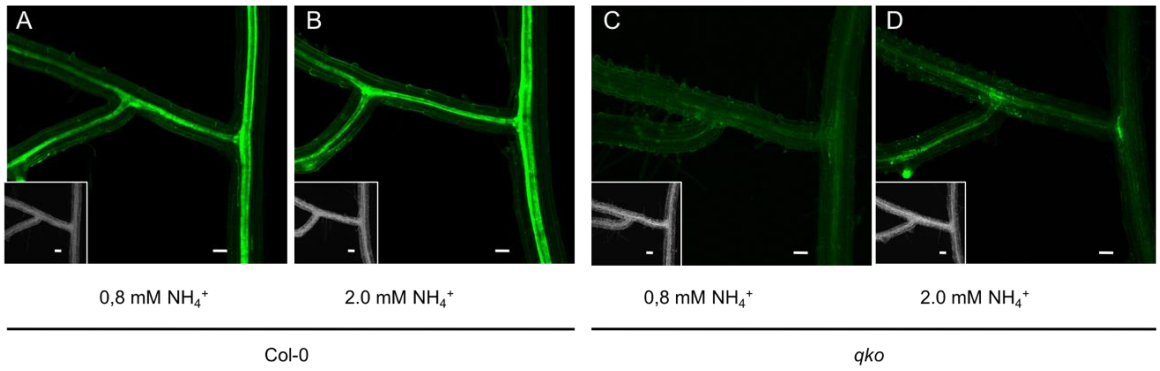

Figure 18. Auxin accumulation in the root vasculature of wild-type and qko plants grown on different concentrations of local ammonium supply.
GFP-derived fluorescence in second-order lateral roots (LR) of wild-type *Col-0 DR5::GFP* **(A and B)** and *qko DR5::GFP* **(C and D)** grown on vertically-splitted agar plates with elevated local ammonium supply. **(A)** *Col-0 DR5::GFP* grown on local 0.8 mM NH_4Cl, **(B)** *Col-0 DR5::GFP* grown on local 2.0 mM NH_4Cl, **(C)** *qko DR5::GFP* grown on local 0.8 mM NH_4Cl, **(D)** *qko DR5::GFP* grown on local 2.0 mM NH_4Cl, Plants were grown for 12 days with the first-order LR growing into an HN compartment containing 0.8 mM NH_4Cl or 2.0 mM NH_4Cl. Plants were precultered for 10 days on 0.5 mM nitrate before transferred to vertically-splitted agar plates with the different ammonium treatments. Images from second-order LRs were taken by confocal laser scanning microscopy after 12 days on the different treatments to detect auxin dependent GFP intensity. Shown are represetative images from 10 individual plants per nitrogen treatment. Bars represent 50 µm.

Activity of the *DR5::GFP* reporter was then monitored at two different levels of ammonium supply. In the Col-0 background, GFP signal intensity was only slightly higher at 2.0 mM than at 0.8 mM ammonium (**Figure 18A, B**). However, in *qko DR5::GFP* the GFP-derived fluorescence in the root vasculature was much stronger under elevated ammonium supply (**Figure 18D**). This was most likely due to a higher internal ammonium concentration in the root tissue and is independent of AMT-mediated ammonium transport.

4.3.2 Auxin homeostasis is influenced in an ammonium specific manner

In order to confirm the results obtained by using the auxin reporter line *DR5:GFP*, concentrations of auxin and auxin-related metabolites were measured by UPLC-MS/MS in Arabidopsis shoots and roots cultivated on localized ammonium or nitrate supply and compared with those of roots grown under N deficiency. It was possible to detect the active form IAA, its putative precursors, its metabolic intermediates or storage forms and its inactivated forms. Unfortunately, measurements had to be based on one biological replica for each root or shoot sample only, because 20 to 30 indivitual plants were needed to be pooled for one sample. Therefore, these results only show trends and have to be interpreted with caution. Auxin accumulation was affected in a nitrogen form-dependent manner in all three differently harvested plant parts (**Figure 19**). Regarding the two auxin

precursors, ammonium led to the highest IAN and IAM accumulation in the root (**Figure 19A, B**). Interestingly, IAM concentration showed the same pattern like IAA, suggesting that the IAA synthesis via the IAM parthway (Sugawara et al., 2009) is the most likely IAA synthesis parthway involved in the IAA accumulation on local ammonium supply (**Figure 19B and C**). However, IAN concentrations were still higher than those of IAM when nitrogen treatments were compared (**Figure 19A**). The presence of nitrate led to a slightly higher MeIAA accumulation than ammonium (**Figure 19D**). In parallel, N deficiency promoted less reversible inactivation of IAA towards IAAMe, whereas irreversible inactivation towards OxIAA increased (**Figure 19D, E**). The inactivation by the promotion towards the IAAla pathway played a negligible role but nevertheless, was higher in N-deficient roots than in those supplied with nitrogen (**Figure 19F**). Remarkably, the metabolically active auxin form IAA increased significantly in N-deficient roots compared to nitrate and ammonium treated roots (**Figure 19C and I**). However, ammonium increased the IAA concentration in comparison to nitrate drastically (**Figure 19C**).

In contrast, the reminiscent root system, consisting of the PR and LR system, grown on the low N compartment (LN) presented decreased IAN, IAM and IAA concentration (**Figure 19G, H, I**). The auxin precursors IAN and IAM were increased in roots growing on the LN compartment when nitrate was supply to the HN compartment (**Figure 19G and H**). Where the storage form IAAMe was most abundant in the LN compartment of local nitrate treated plants, the active form of auxin was under the limit of detection (**Figure 19I**). In contrast, in the remaining root system of plants locally supplied with ammonium or of plants grown completly under N-deficient conditions the active auxin form was detectable (**Figure 19I**). The storage form IAAMe and the inactivated OxIAA were increased in the reminiscent root system of nitrate treated plants (**Figure 19J and K**). IAAla concentration in the roots grown on the LN comparment showed nearly the same pattern and concentrations like in roots grown on the HN comparment. (**Figure 19F and L**).

The shoot auxin concentration of the differentially treated Arabidopsis plants growing in the vertically-splitted agar plates was highest in the nitrogen deficient plants (**Figure 19M, N, O, P, Q, R**). All measured auxin forms were at least two-fold higher in shoots suffering from nitrogen deficiency in comparison to nitrate treated plants. A local ammonium supply led to the overall lowest auxin concentration in the shoot tissue, being an indicator for enhanced shoot-to-root auxin transport or reduced auxin synthesis.

Taking together, results obtained with the reporter line *DR5:GFP* schowed a simular pattern like results from UPLC-MS/MS measured in roots grown on the different local N supplies.

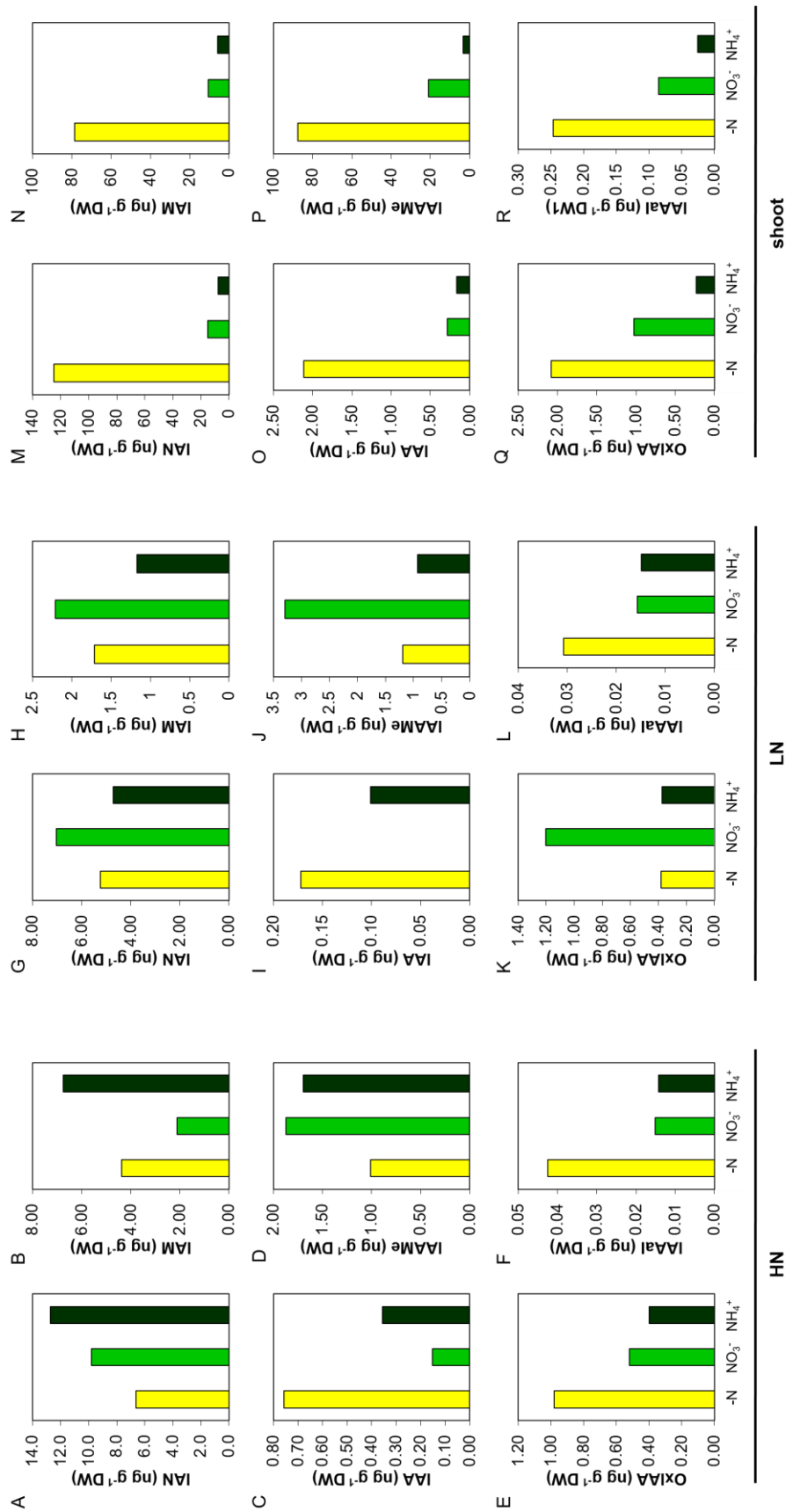

Figure 19. Concentration of auxin and auxin-related metabolites in response to local N supply.
Concentration of different naturally occuring auxin forms measured in different organs of wild-type (Col-0) plants grown in vertically-splitted agar plates. **(A-F)** Metabolite concentrations in first-order LRs grown on different local N supply (HN), **(G-L)** in the remaining root system from the low-N (LN) compartment, and **(M-R)** shoots. Wild-type were precultured for 10 d and after then transferred to local ammonium or nitrate supply for 15 days or grown in N deficiency before roots and shoots were sampled for analysis by UPLC-MS-MS of precursors (IAN, IAM), bioactive (IAA), storage (IAAMe, IAAla) and inactivated auxin forms (OxIAA). Each bar represents two technical replicates with 20 - 30 roots or shoots pooled into one biological sample. IAN, indole- 3-acetonitrile; IAM, indole-3-acetamide; IAA, indole-3-acetic acid; IAAMe, indole-3- acetic acid methyl ester; IAAla, indole-3- acetyl-L-alanine; OxIAA, 2-oxoindole-3- acetic acid.

4.3.3 The auxin reporter *DR5:GFP* is not exclusively induced by IAA

To test the substrate specificity of the inducibility of the DR5-dependent reporter to IAA the *qko DR5::GFP* line was used and nine different naturally occurring auxin forms in Arabidopsis were applied to the root tissue. The auxin forms IAA, IAAMe and IAAEt led to a strong GFP signal (**Figure 20A, B, F, G**), whereas the auxin forms IAN, IAM, IPyA, IAAala, IAAGlu and IAAOx did not (**Figure 20C, D, E, H, I, J**). The strongest GFP signal was observed after external application of IAAMe (**Figure 20F**). IAA led to a weak GFP signal when 10 µM (**Figure 20A**) were supplied while 1 mM IAA (**Figure 20B**) increased the GFP signal in the root vasculature, but not to the same intensity like IAAMe and IAAEt. The DR5-driven GFP signal in the root vasculature appeared to be stronger induced by the more diffusible auxin forms IAAEt and IAAMe than by IAA, which may be explained by the amount of auxin reaching the root vasculature by diffusion. Furthermore, IAAMe and IAAEt led to a more dispersed GFP signal, which was observed not only in the root vasculature but also in the surrounding root tissue. Additionally, IAAMe and IAAEt are more stable than IAA and therefore stay in higher amounts in the medium than IAA. To conclude, the DR5 promotor is not exclusively induced by external IAA.

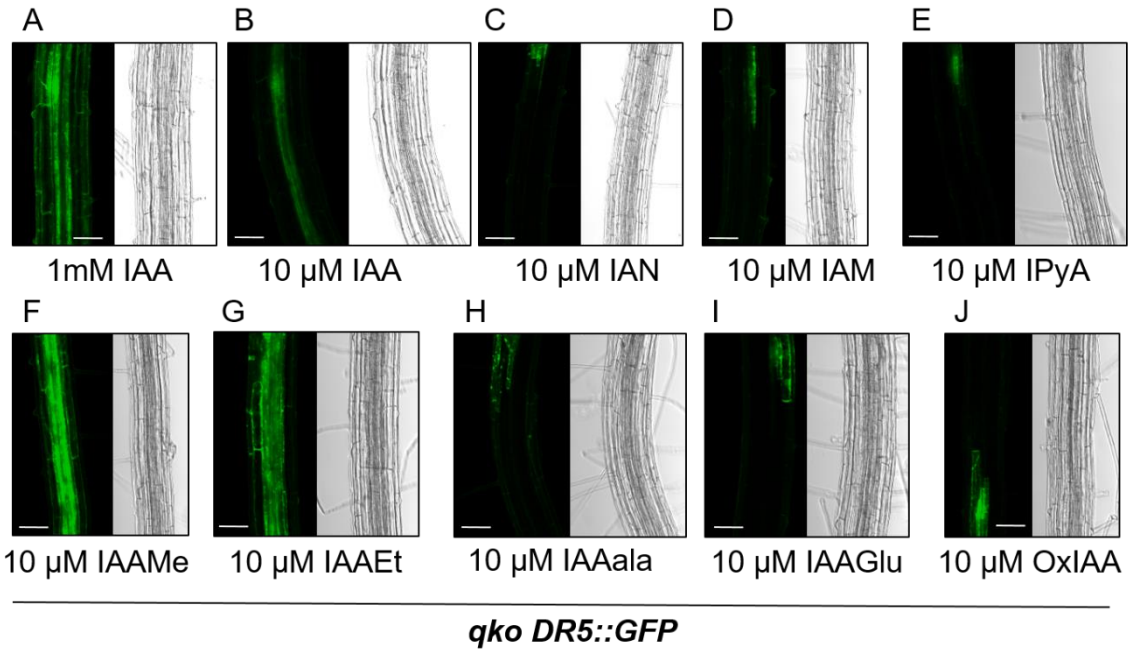

Figure 20. The auxin reporter *DR5:GFP* is not specific to IAA.
(A-J) GFP-derived fluorescence in second-order lateral roots (LR) of *qko DR5::GFP* in response to different externally supplied auxin forms. Plants were grown 7 days on ½ MS medium and treated with 1mM IAA **(A)**, 10µM IAA **(B)**, 10µM IAN **(C)**, 10µM IAM **(D)**, 10µM IPyA **(E)**, 10µM IAAMe **(F)**, 10µM IAAEt **(G)**, 10µM IAAala **(H)**, 10µM IAAGlu (I) or 10 µM OxIAA (J) over night. Images were taken with a confocal microscope 12 h after application of the IAA forms to the agar plates and the DR5 driven GFP signal was compared among the nine different auxin forms. Shown are represetative images from 5 individual plants per applied IAA form. Bars represent 50 µm.

4.3.4 Auxin is transported in the phloem of the root vascular system

Auxin is generally transported throughout the plant by two pathways, most IAA is transported away from the source tissues, like the meristem of young leaves or flowers, by a bulk flow in the mature phloem. In addition, a slower, carrier-mediated cell-to-cell directional transport moves auxin in the vascular system, i.e. xylem parenchyma from the shoot towards the root tip (Goldsmith, 1977). These two pathways seem to be connected at the level of phloem loading in leaves (Marchant et al., 2002) and phloem unloading in roots (Swarup et al., 2001). In this study, different marker lines were compared to verify, in more detail, in which root tissue auxin is transported. Therefore, a xylem-pole pericycle marker *E3754* (**Figure 21B**) (Bergmann et al. 2013) and a phloem maker *SUC2*, a plasma-membrane sucrose-H^+-symporter were used (**Figure 21C**) (Truernit and Sauer 1994) to compare the localization of the GFP signals with those of *Col-0 DR5::GFP* (**Figure 21A**). GFP-derived fluorescence in *Col-0 DR5::GFP* were overlapping with the GFP signals of the xylem-pole pericycle marker *E3754* in the xylem parenchyma in the LR branching zone of the second-order LR (**Figure 21B**) and the phloem maker *SUC2* localized in the ploem in the LR branching zone of the second-order LR and third-order LR (**Figure 21C**). The comparison of the three different maker lines led to the conclusion that auxin is not exclusively restricted to the phloem, but was also detected in the xylem parenchyma in second-order LRs.

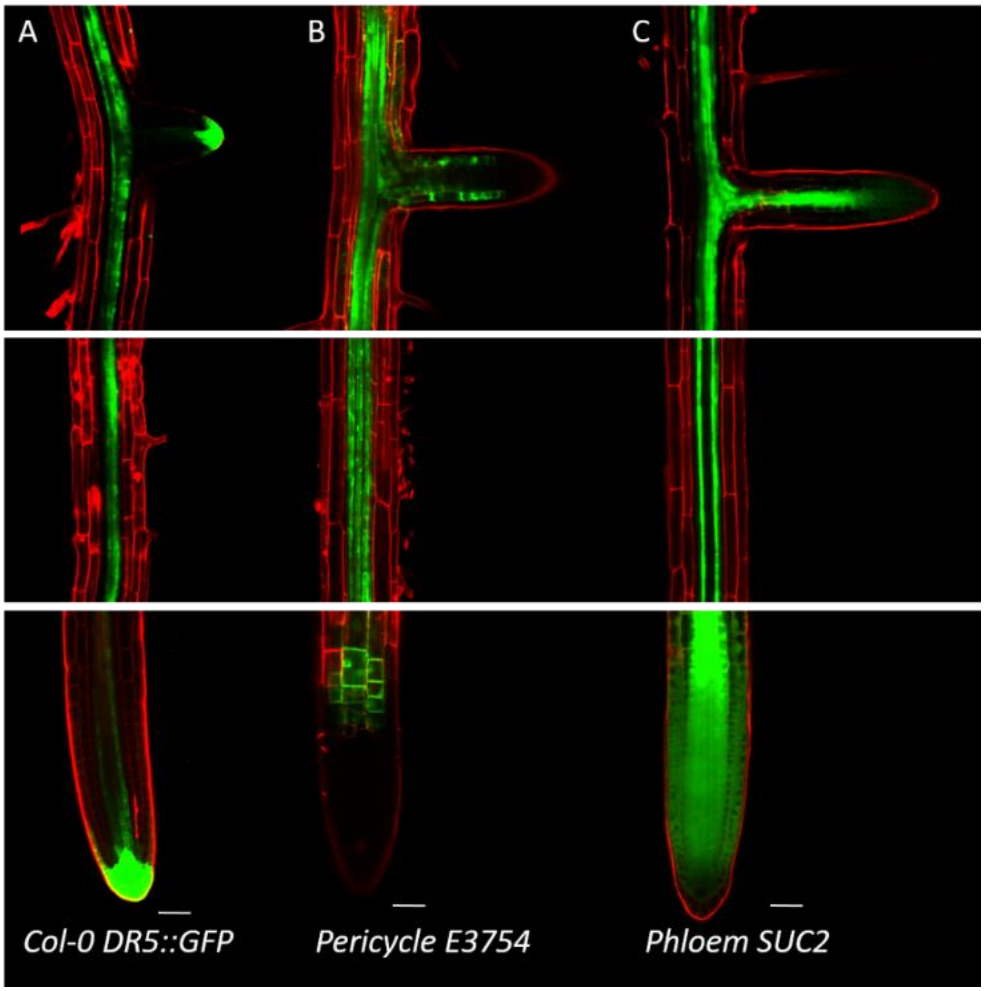

Figure 21. Localization of auxin-dependent GFP in the root vasculature of second-order LR as monitored by DR5::GFP.
GFP-derived fluorescence in second-order LR of *Col-0 DR5::GFP* **(A)**, the xylem-pole pericycle marker *E3754* **(B)** and the phloem maker *SUC2* **(C)** grown on ½ MS medium. Images were taken with a confocal microscope and DR5 driven GFP signal was compared among the three different reporter lines 10 days after germination. Shown are represetative images from 10 individual plants per reporter line. Bars represent 50 µm.

4.4 Altered shoot to root auxin transport facilitates higher-order lateral root branching on local ammonium

4.4.1 Higher-order lateral root branching is independent of de novo synthesis of IAA in the root

Auxin accumulation in ammonium-treated roots may be due to two possible mechanisms: either local auxin biosynthesis in roots or shoot-to-root auxin transport. In order to verify what mechanism triggers auxin-mediated higher-order LR branching in ammonium-rich patch, different auxin-related chemical inhibitors were used. Auxin biosynthesis is catalyzed by a tryptophan (Trp)-dependent two-step pathway in Arabidopsis (Mashiguchi et al., 2011). Firstly, Trp is converted to indole-3-pyruvate (IPA) by members of the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family, like TAA1 and by TAR2 (TRYPTOPHAN AMINOTRANSFERASE RELATED 2) (Stepanova et al., 2008; Tao et al., 2008). Then, IPA is catalyzed by members of the YUCCA (YUC) flavin monooxygenase family to generate indole-3-acetic acid (IAA). L-Kynurenine (Kyn) has been reported as a competitive inhibitor of TAA proteins and therefore represses local auxin biosynthesis in plants (He et al., 2011). To analyze the role of local auxin synthesis, a series concentration of Kyn was added to the HN-side of vertically-splitted agar plates to interrupt auxin biosynthesis in ammonium-treated roots (**Figure 22A**). First-order LR growth in the ammonium patch was largely repressed by increasing concentrations of Kyn (**Figure 22B and C**), which is in agreement with the essential role of local auxin biosynthesis in sustaining a general LR development. Surprisingly, third-order LR formation was not affected by Kyn supply (**Figure 22B to E**), whereas first-order LR length were decreased up to 50% due to Kyn-concentrations higher than 5 µM. Thus, local auxin biosynthesis appeared not necessary to trigger the higher-order LR branching in local ammonium supply.

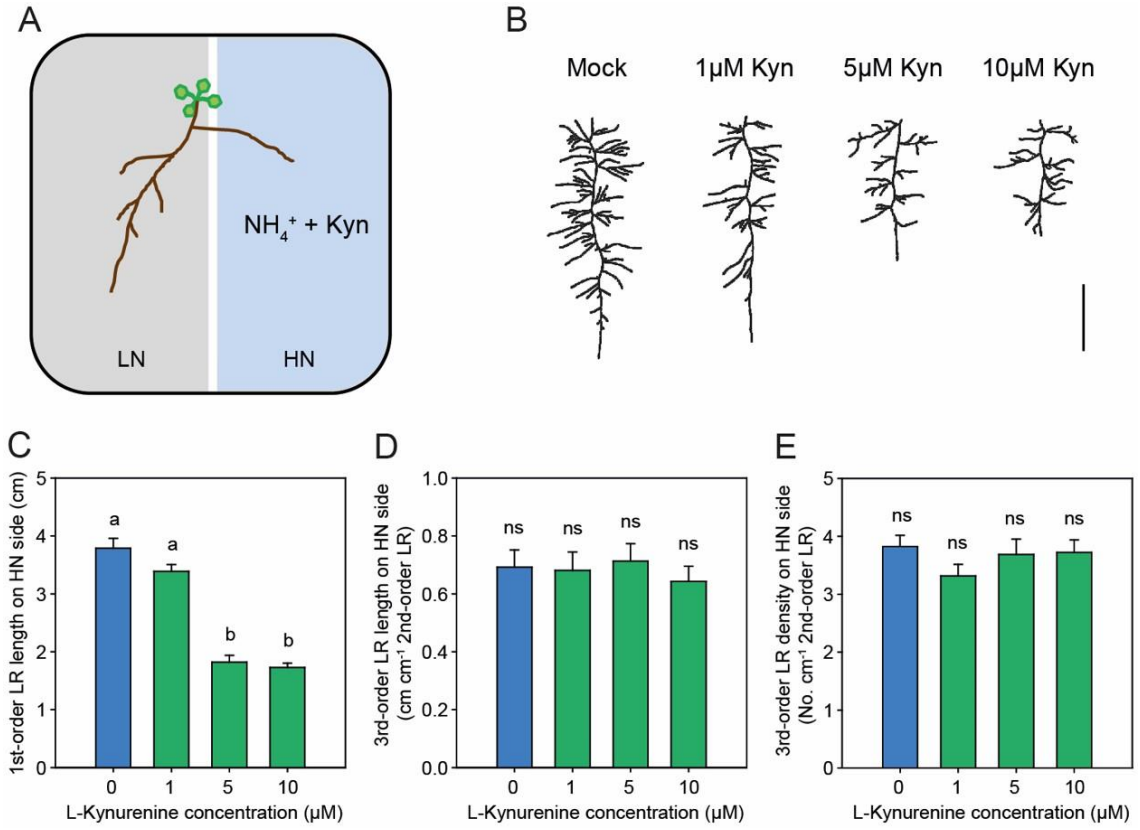

Figure 22. Higher–order LR formation on local ammonium supply in presence of an auxin synthase inhibitor.
(A) Schematic representation of the vertically-splitted agar plate setup for the L-Kynurenine (Kyn) experiment. Blue color indicates the agar segment containing L-Kynurenine. **(B)** Representative image of first-order LRs of 12 individual *Arabidopsis thaliana* wild-type plants growing in the presents of the IAA synthesis inhibitor Kyn. Scale bar is 1 cm. **(C)** First-order LR length on HN plate side, **(D)** third-order LR length on HN plate side, **(E)** third-order LR density on the HN plate side. Plants were grown on vertically-splitted agar plates for 15 days with the first-order LR growing into a HN compartment containing 0.8 mM NH_4Cl or 0.8 mM NH_4Cl plus 1µM, 5µM or 10µM Kyn. The mock control was supplied with the same amount of DMSO at a concentration of 0.05% (v/v). PR grew in 5 µM KNO_3 (LN), Scale bar represents 1 cm. Plants were precultured for ten days in the presents of 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 12 plants per treatment.

The key genes in the IPyA parthway-dependent IAA biosynthesis, *TAA1*, *TAR2*, and the 11 known *YUCCAs* were selected for qRT-PCR to monitor changes in their transcript levels in response to local ammonium supply. After 15 days on local ammoniuim, nitrate or nitrogen-free medium no significant differences were found regarding *TAR2* regulation between the three different N-treatments (**Figure 23B**). In contrast *TAA1* is upregulated by the to inorganic N-forms nitrate and ammonium (**Figure 23A**). YUC1, YUC2, YUC4 and YUC6 are required for the development of floral organs and vascular tissues (Cheng et al., 2006) and were mostly repressed by a local ammonium supply (**Figure 23C, D, and F**). YUC1, YUC4, YUC10 and YUC11 are essential for embryogenesis and leaf formation (Cheng et al., 2007) and were all repressed by a local ammonium supply. YUC3, YUC5,

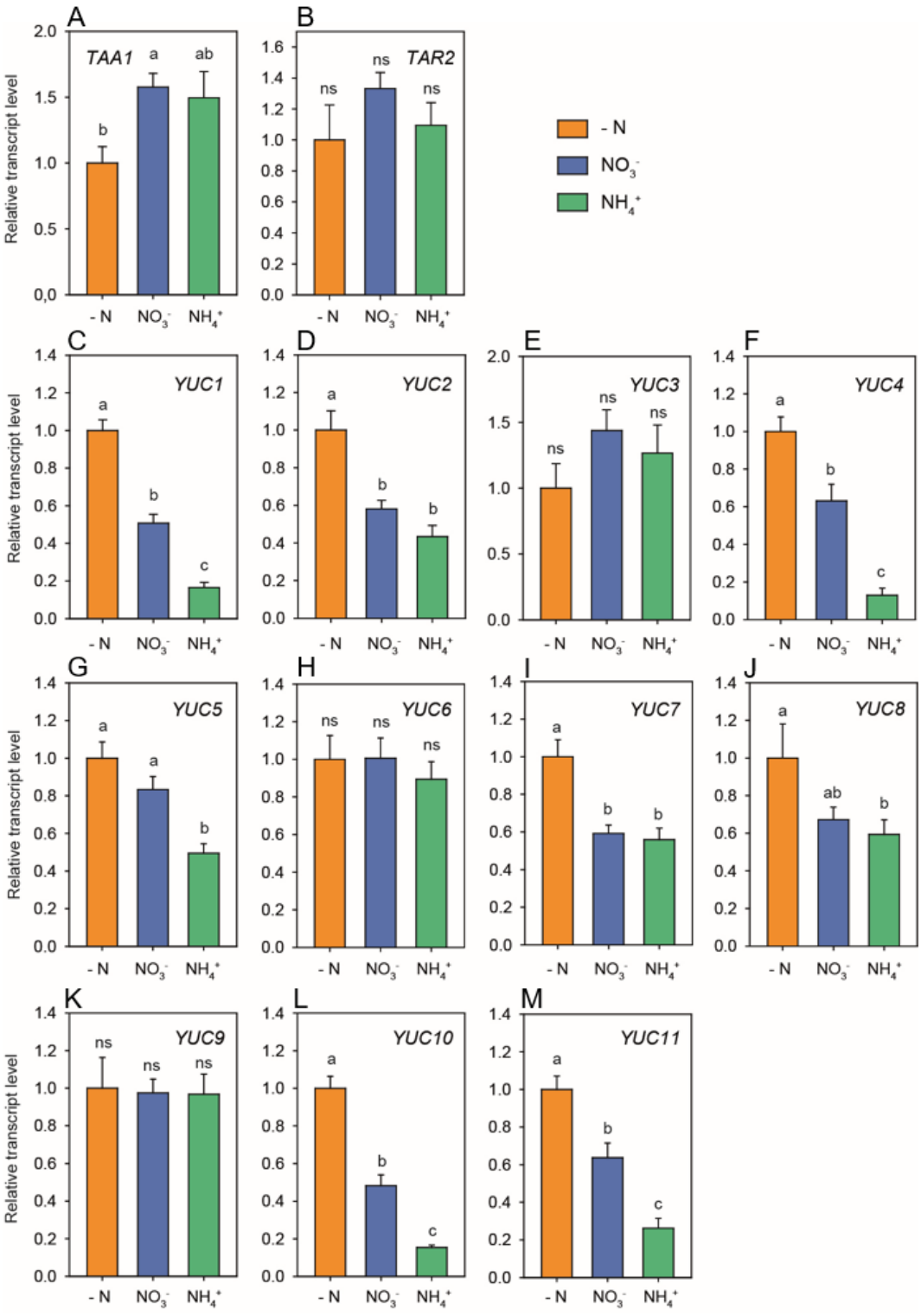

Figure 23. Transcript levels of genes involved in de-novo synthesis of auxin under supply of different nitrogen forms.

Relative expression levels of genes involved in the de-novo synthesis of auxin in the root vasculature of *Arabidopsis thailiana.* Relative transcript levels of **(A)** *TAA1,* **(B)** *TAR2,* **(C)** *YUC1,* **(D)** *YUC2,* **(E)** *YUC3,* **(F)** *YUC4,* **(G)** *YUC5,* **(H)** *YUC6,* **(I)** *YUC7,* **(J)** *YUC8,* **(K)** *YUC9,* **(L)** *YUC10 and* **(M)** *YUC11* in first-order LR of Arabidopsis wild-type plants after 15 days of growth on vertically-splitted agar plates with locally-supplied 0.8 mM NH_4Cl, KNO_3 or KCl. Plants were precultured 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 4 biological replicates per treatment.

YUC7, YUC8, and YUC9 function in the root gravitropic response and in root development (Chen et al., 2014). Where *YUC5*, *YUC7* and *YUC8* were repressed by ammonium and nitrate, the expression of *YUC3* and *YUC9* were not affected (**Figure 23E and K**). Taken together, the de-novo synthesis of IAA in the root vasculature appeared to be not transcriptionally upregulated by local N, neither by nitrate nor ammonium. Only N deficiency led to an upregulation of the IPyA parthway, which has been already reported by Ma et al. in 2014. This induction of the IPyA parthway was indicated mainly by the upregulation of different *YUCCA* genes.

To further investigate, whether higher-order LR branching on local ammonium is depending on TAR2, the *tar2* mutant was grown on vertically-splitted agar plates. The mutant *tar2* showed the same RSA like the wild-type for all N treatments (**Figure 24A, B**), indicating that the de-novo synthesis of IAA in the root vasculature is not involved in ammonium-dependent higher-order LR branching.

Additionally to the analysis by qRT-PCR and *tar2*, the reporter line *TAR2pro:TAR2::GFP* was used to clarify whether TAR2 is regulated at the protein level. As expected, TAR2 was upregulated under N deficiency as shown by an increased GFP signal, whereas nitrate and ammonium did not lead to an increased GFP fluorescence (**Figure 24C and D**). Taking together, ammonium-induced auxin accumulation in the root vasculature is not mediated by de-novo synthesis of auxin via the IPyA pathway.

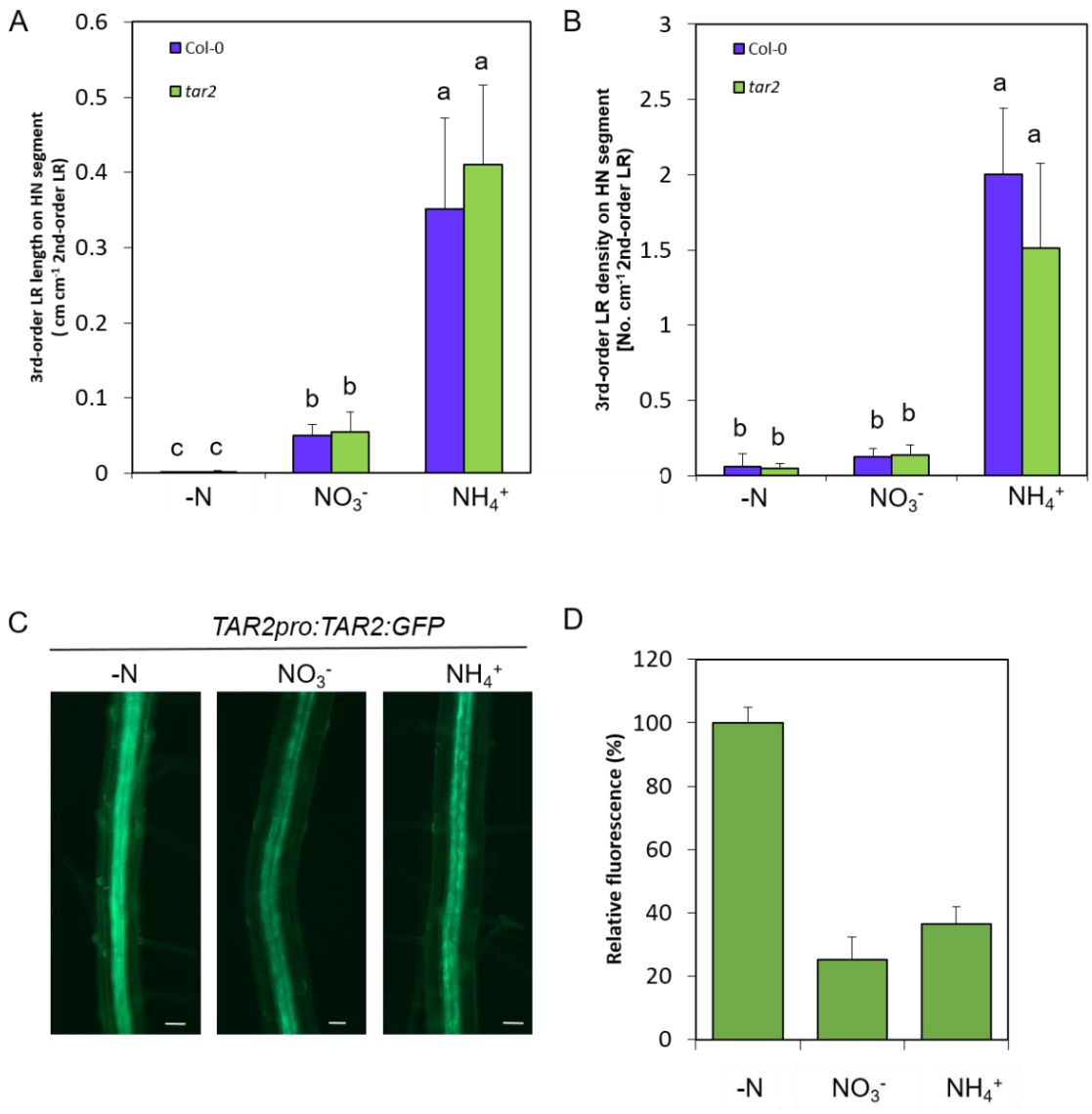

Figure 24. TAR2 is not involved in higher-order LR branching in response to local ammonium.

Development of first-order LR of 25 days-old *Arabidopsis thaliana* wild-type Col-0 and *tar2* mutant plants grown on different local N supplies on vertically-splitted agar plates. **(A)** Third-order lateral root (LR) length and **(B)** third-order LR density of wild-type (Col-0) and *tar2* after grown for 15 days on vertically-splitted agar plates. First-order LRs grew into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO3 or 0.8 mM NH4Cl. Remaining root system grew in 5 µM KNO_3 on low nitrogen (LN) compartment. Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values ± SD; n = 10-12 individual plants per treatment. Different letters denote significant differences among means at P<0.05 (Tukey's test). **(C)** GFP-derived fluorescence of *proTAR2:TAR2::GFP* in second-order LR branching zone grown on high nitrogen (HN)-side of vertically-splitted agar plates on nitrogen deficiency, local nitrate or local ammonim. First-order LRs grew into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH4Cl. PR grew in 5 µM KNO_3 on low nitrogen (LN) compartment. Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source. Fluorescent images were taken by confocal microscope at 12 days after local N treatment. Representative images among 10 individual plants per treatment were shown. Scale bars are 50 µm. **(D)** Quantitative readout of GFP fluorescence intensity for *proTAR2:TAR2:GFP* lines. Bars represent mean values ± SD; n = 10 roots per treatment (GFP fluorescence intensity was measured at three positions in each root).

4.4.2 Rootward auxin transport is crucial for higher-order lateral root branching on local ammonium supply

Shoot-to-root auxin flow in plants is facilitated by several classes of auxin transporters (Petrášek and Friml, 2009). In order to verify if the shoot-to-root auxin transport is the signal to trigger higher-order LR branching in a local ammonium patch, the chemical inhibitor N-naphthylphtalamic acid (NPA) was applied to block shoot-to-root auxin transport. NPA is widely used as a polar auxin transport inhibitor, which disrupts protein trafficking of auxin efflux carriers (Geldner et al., 2001; Muday and DeLong, 2001).

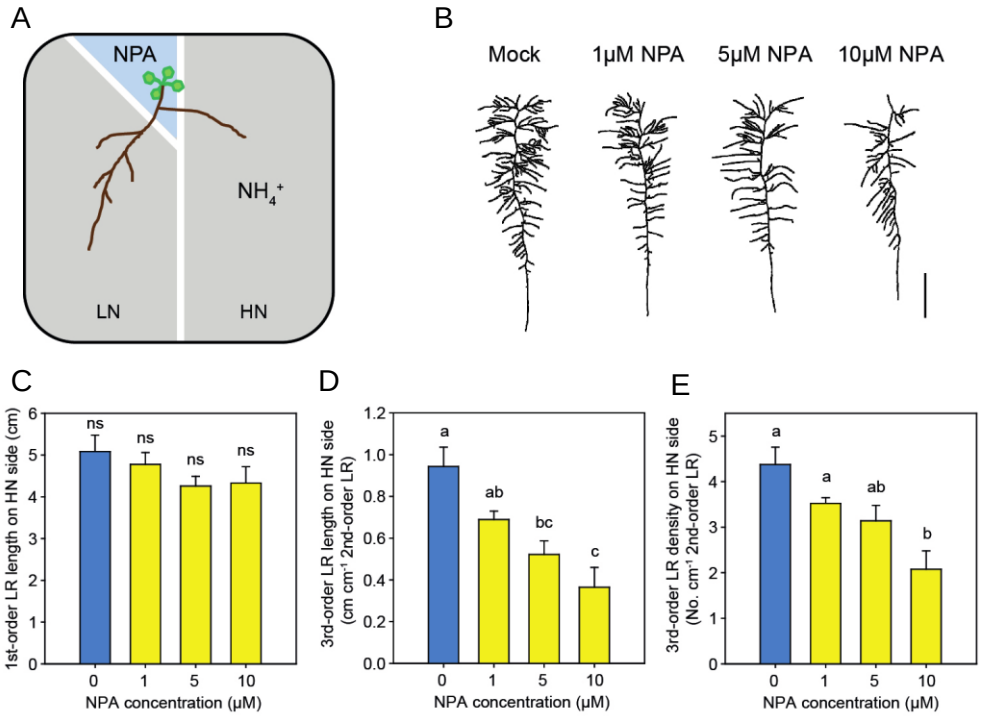

Figure 25. Auxin transport inhibitor NPA represses higher–order LR formation on local ammonium

(A) Experimental setup of N-naphthylphtalamic acid (NPA) treatment on Y-type splitted agar plates. Blue colour indicates the agar segment containing NPA. **(B)** Representative image of lateral root (LR) on HN-side under NPA treatment. Wild-type plants grown on a Y-type split agar plates for 15 days with a first^t-order LR growing on HN-side containing 0.8 mM NH_4Cl, while NPA was added to the top segment at indicated concentrations. The mock control contained 0.05% (v/v) DMSO. Plants were precultured for 10 days on 0.5 mM nitrate as sole N source. Scale bar is 1 cm. Quantitative higher-order LR traits under NPA treatment **(C)** first^t-order LR length on HN side, **(D)** third-order LR length on HN side, and **(E)** third-order LR density on HN side. Bars represent mean values ± SE; n = 12 plants per treatment. Different letters represent significant differences among means at $P<0.05$ (Tukey's test).

Therefore, a series concentration of NPA was supplied to the top part of Y-type split agar plates (**Figure 25A**), in which NPA was restricted to the shoot or the base part of PR (Figure 25A). Compared with the mock control, NPA treatment largely decreased third-order LR length and density (**Figure 25D and E**), indicating shoot-to-root auxin transport is required for triggering higher-order LR branching in the local ammonium treatment. First-order LR growth were not affected by the NPA treatment (**Figure 25C**).

To identify auxin transporters crucial for the formation of higher-order LRs on local ammonium supply several auxin transport mutants were screened on vertically-splitted agar plates. When the length and density of third-order LRs of auxin transport mutants on the HN plate side were monitored and compared, *pin2* mutant plants showed a enhanced higher-order LR formation compared to wild-type plants, which was indicated by an increased higher-order LR density (**Figure 26B**). PIN2 plays a crucial role in shootward auxin transport and distribution (Kleine-Vehn et al., 2008). The *pin3* and the *pin7* mutant showed the same higher-order LR density and length like the wild-type (**Figure 26A and B**). The PINFORMED proteins PIN3 and PIN7 participate in auxin transport in plant roots in a rootward direction (Friml et al., 2003; Blilou et al., 2005). PIN4 is localized in vascular initials and their derivatives, where it facilitates the directed auxin flow in roots through the vascular cylinder toward the root meristem (Friml et al., 2002). The *pin4* mutant line showed the strongest reduction in higher-order LR formation compared to all other tested auxin transporter mutant lines, shown by a strong reduction in higher-order LR length and density (**Figure 26A and B**). AUX1 belongs to the amino acid/auxin permease (AAAP) family of proton-driven transporters (Bennett et al., 1996; Young et al., 1999) and regulates lateral root development by facilitating the export of IAA from newly developing leaf primordia, IAA unloading in the primary root apex, and import of IAA into developing LRP. However, *aux1* is known to show impaired LR formation on medium containing ammonium in compination with nitrate as N source (Marchant et al., 2002). Therefore, a reduction in higher-order LR formation due to localized ammonium supply was expected, but the tested *aux1* mutant line showed no significant difference to the wild-type regarding RSA (**Figure 26**). In *Arabidopsis thaliana*, AUX1 belongs to a small multigene family comprising four highly conserved genes AUX1, LAX1, LAX2, and LAX3. All four members of this AUX/LAX family display auxin uptake functions. AUX1, LAX1, and LAX3 have been described to regulate distinct auxin-dependent developmental processes in roots (Peret et al., 2012). Nevertheless, *lax1* and *lax3* showed no reduced higher-order LR formation on local ammonium supply (**Figure 26**). NRT1;1 is a well-described nitrate transporter, which transports auxin under low nitrate availability. It has been proposed that NRT1.1 represses LR growth under low nitrate availability by promoting shootward auxin transport out of these roots (Krouk et al. 2010a). The *nrt1;1* mutant line showed neither enhanced nor

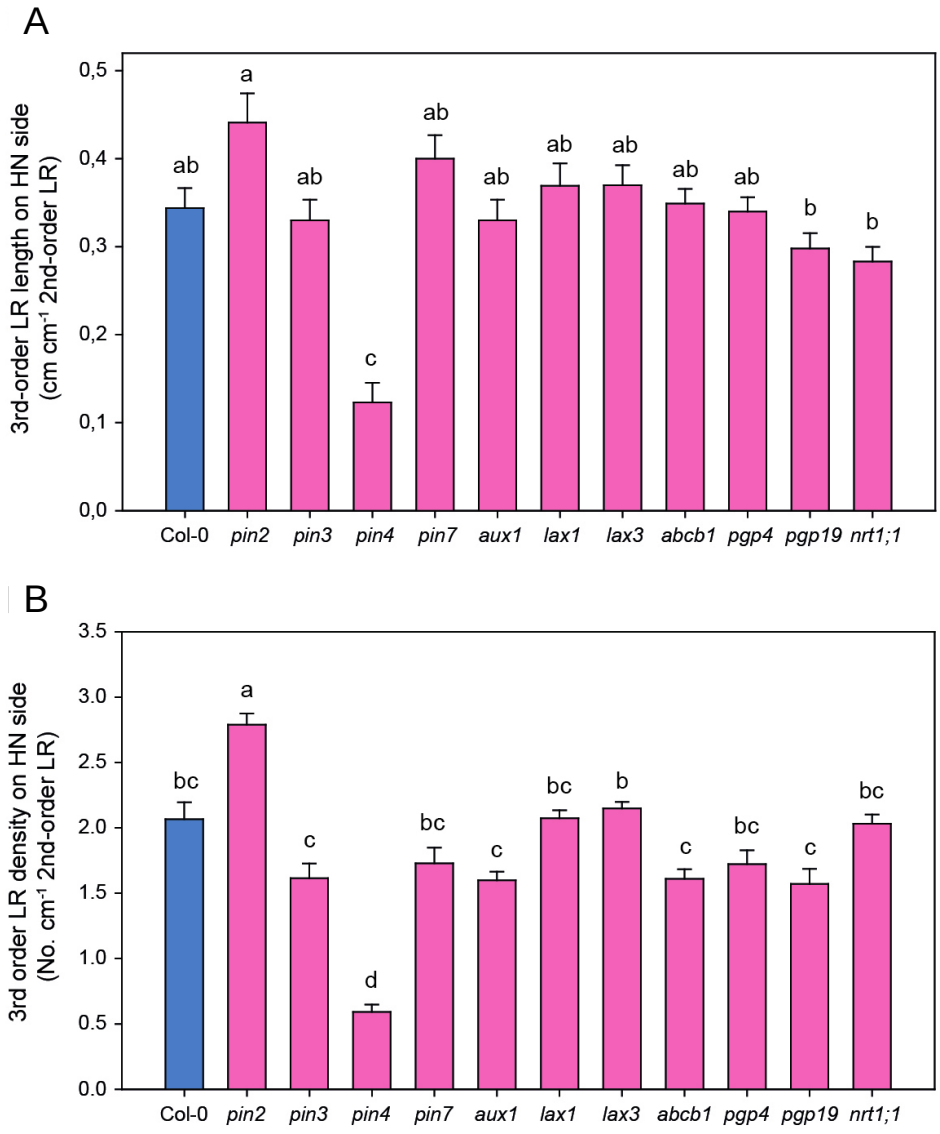

Figure 26. Screening of auxin transport mutants on local ammonium supply
Formation of third-order LR of *Arabidopsis thaliana* wild type (Col-0) and mutants related to polar and radial IAA transport. **(A)** Thrid-order LR length and **(B)** third-order LR density of Col-0, *pin2*, *pin3*, *pin4*, *pin7*, *aux1*, *lax1*, *lax3*, *abcb1*, *pgp4*, *pgp19* and *nrt1;1* were grown on vertically-splitted agar plates for 15 days. First-order LR growing into an HN compartment containing 0.8 mM NH_4Cl. PR growing in 5 µM KNO_3 (LN). Plants were precultured for 10 days on medium containing 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 8 – 10 individual plants per genotype.

reduced higher-order LR formation on local ammonium in comparison with wild-type plants (**Figure 26**). Data from Wang et al. 2013 supported a model, in which ABCB1-mediated lateral auxin efflux at the plasma membrane of endodermis, cortex and epidermis cells, enhances auxin concentration in the root apoplast. A deletion of *ABCB1* did not affect the higher-order LR formation on local ammonium (**Figure 26**). The polar transport of auxin

from cell to cell is also achieved through the coordinated process of efflux and influx transporters, which include P-GLYCOPROTEIN (PGP) encobesides PIN-type auxin transporters (Geisler et al., 2005; Petrásek et al., 2006; Cho et al., 2007). PGP4 and PGP19 are known for their auxin efflux activity in plant cells. The corresponding mutant lines *pgp4-1* and *pgp19-4*, however, did not show any reduction in higher-order LR formation (**Figure 26**).

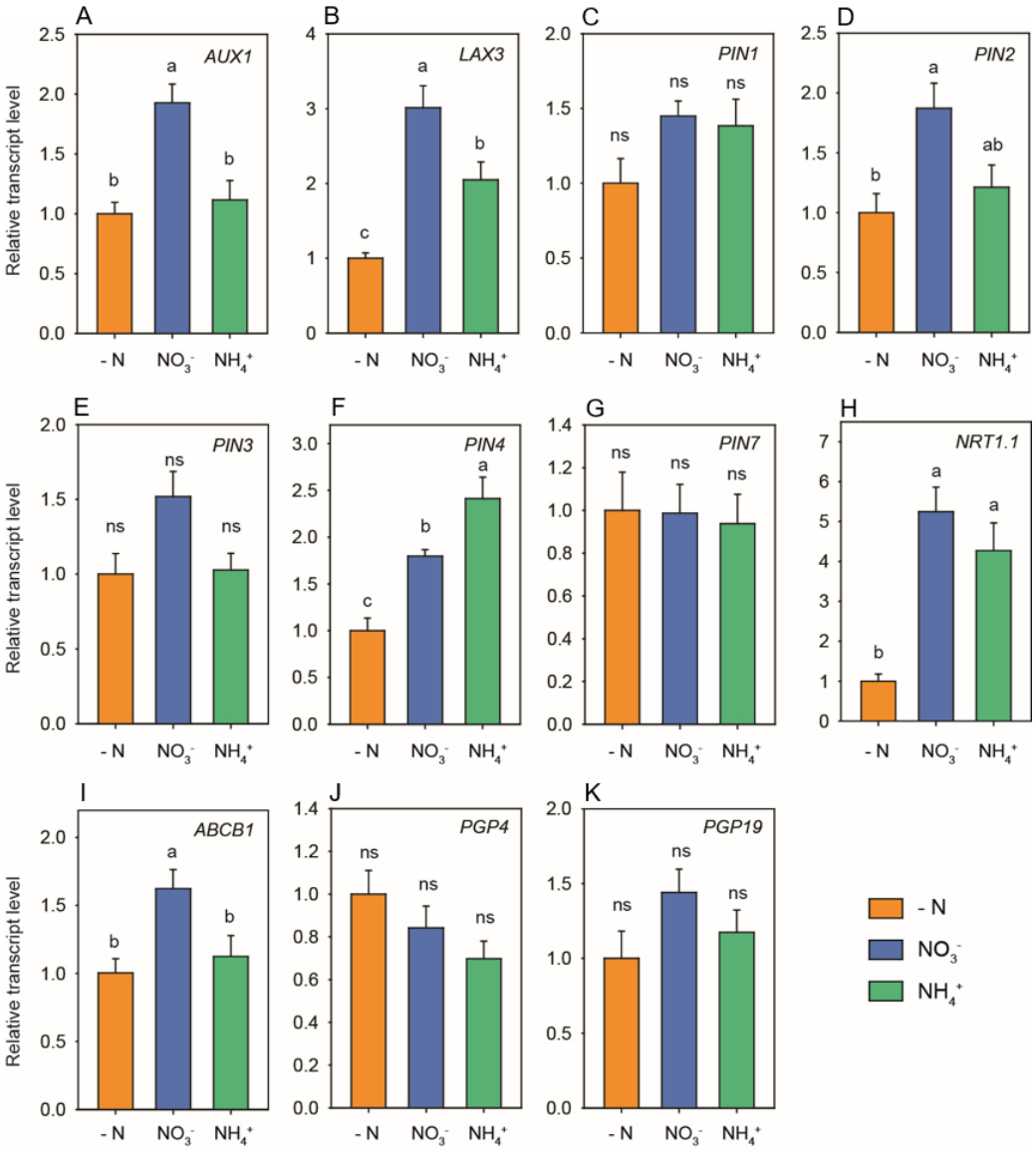

Figure 27. Transcript levels of auxin transport-related genes as affected by the supply of different nitrogen forms.
Relative transcript levels of **(A)** *AUX1,* **(B)** *LAX3,* **(C)** *PIN1,* **(D)** *PIN2,* **(E)** *PIN3,* **(F)** *PIN4,* **(G)** *PIN7,* **(H)** *NRT1;1,* **(I)** *ABCB1,* **(J)** *PGP4,* and **(K)** *PGP19* in first-order lateral roots (LR) of Arabidopsis wild-type plants after 15 days of growth on locally-supplied 0.8 mM NH_4Cl, KNO_3 or KCl. Plants were precultured 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 4 biological replicates per treatment.

In addition to the mutant screening, the corresponding auxin transporter genes were selected for qRT-PCR to monitor changes in their transcript levels in response to local ammonium supply. After 15 days on the different local N treatments, significant differences were found regarding gene regulation. *AUX1* and *LAX3* were enhanced under local nitrate supply, were N deficiency led to a repression of *LAX3* (**Figure 27A and B**), indicating the necessity of active radial auxin transport espessially in the presence of nitrate. Regarding the PIN genes, just *PIN2* and *PIN4* seems to be regulated by nitrate and ammonium. *PIN2* was strongly upregulated by nitrate and down regulated by N deficiency (**Figure 27F**). In contrast *PIN4* was significantly induced by ammonium (**Figure 27F**), reflecting the results from the mutant screening (**Figure 26A and B**). N deficiency repressed *PIN4* and *NRT1;1* expression (**Figure 27F**). The auxin transporter *ABCB1*, involved in radial auxin transport was induced by nitrate (**Figure 27I**), where PGP4 and PGP19 were not significantly affected by the testet N treatments (**Figure 27J and K**).

Since *pin2* showed an altered higher-order LR formation on local ammonium (**Figure 26B**), the role of PIN2 in the emergence process of higher order LRs was more deeply investigated. Actually, the *pin2* mutant line showed higher third-order LR density in all treatments but particularly enhanced LR density and length on locally supplied ammonium in comparison to wild-type (**Figure 28A and B**). This suggested that the emergence process of higher-order LRs profited from suppression of PIN2-dependent auxin transport. To observe the N-dependent regulation of the PIN2 protein, the reporter line *PIN2pro:PIN2:GFP* was used. The auxin exporter PIN2 was localized at the apical side of LR cap and epidermis cells in the the elongation zone of the root tip, like described by Abas et al. (2006). Nitrate led to a strong GFP signal, indicating high *PIN2* promotor activity and/or stable PIN2 protein levels (**Figure 28C, D**). Under N deficiency or ammonium supply PIN2-dependent fluorescence was low (**Figure 28C and D**). Actually, *PIN2pro:PIN2:GFP* expression or PIN2 stability on local ammonium depended on time and root age. In younger root tips, PIN2-derived fluorescence had higher intensity but decreased in more matured root segment that were in contact with ammonium for longer time (data not shown). Taking together, PIN2 appeared being repressed by ammonium mainly at the protein level, which was in agreement with enhanced third-order LR density in the *pin2* mutant.

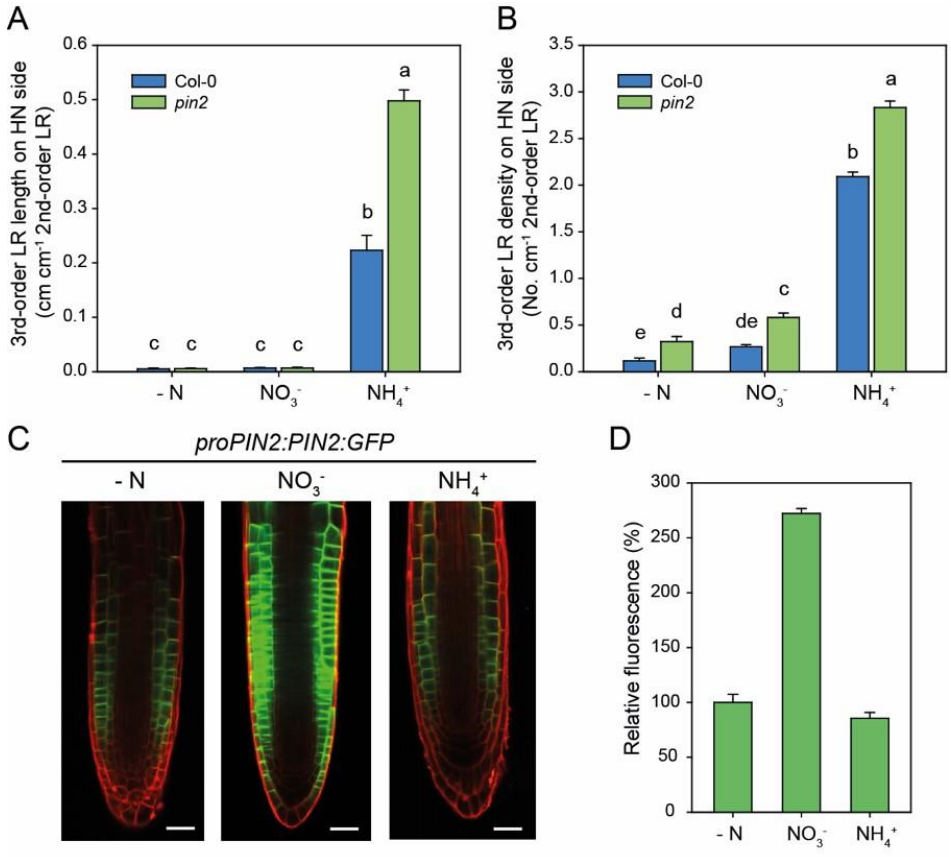

Figure 28. Influence of local N supply on regulation of PIN2 expression and higher-order lateral root formation in the pin2 mutant.

Development of first-order lateral roots (LR) of 25 days-old *Arabidopsis thaliana* wild-type Col-0 and *pin2* mutant plants grown on different local N supplies on vertically-splitted agar plates. **(A)** Third-order LR length, **(B)** third-order LR density in the high nitrogen (HN) compartment. First-order LRs grew into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. Primary roots grew in 5 µM KNO_3 in the low nitrogen (LN) compartment. Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 12 – 15 individual plants per treatment and genotype. **(C)** GFP-derived fluorescence in second-order LR from transgenic plants expressing *PIN2pro:PIN2:GFP* in overlay with propidium iodide-dependent red fluorescence of roots grown on the HN compartment on the vertically-splitted agar plate. Fluorescent images were taken by confocal microscope 12 days after transplating to local N treatments. Representative images from 15 individual plants per treatment are shown. Bars represent 40 µm. **(D)** Quantitative readout of GFP-dependent fluorescence intensity in epidermis cells at the meristematic zone of second-order LR tips expressed relative to the levels detected in –N-treated roots. (n = 10 root tips per treatment, on which the fluorescence was measured in at least three different positions). Bars are means ± SE.

In roots, PIN4 is localized in the proximal meristem, vascular initials, quiescent center and the stem cell niche, where it establishes a sink enhancing rootward auxin flow through the vascular cylinder toward the central root meristem (Friml et al., 2002). Since *pin4* showed a reduced higher-order LR formation (**Figure 26**) and an upregulation of *PIN4* was investigated by qRT-PCR (**Figure 27F**) on local ammonium, the role of PIN4 in the emergence process of higher order LRs was also more deeply investigated. The pin4 mutant line showed a drastically lower higher-order LR formation on locally supplied

ammonium in comparison to wild-type plants (**Figure 29A and B**), suggesting crucial impact of PIN4-dependent auxin flow on the emergence process of higher-order LRs in response to ammonium. However, N deficiency as well as nitrate supply were not leading to different intensities in higher-order-LR branching in the pin4 mutant line in comparison to the wild-type (**Figure 29A and B**).

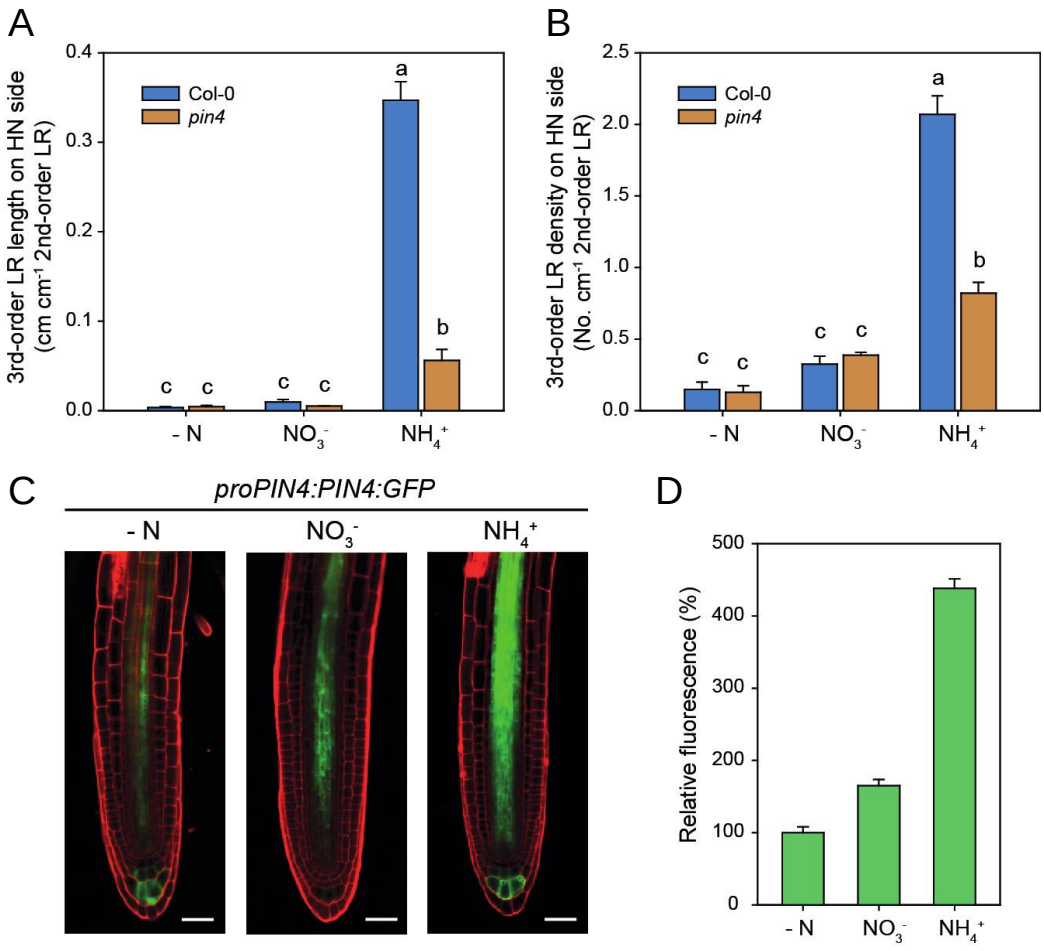

Figure 29. Influence of local N supply on regulation of PIN4 expression and higher-order lateral root formation in the pin4 mutant.
Development of first-order lateral roots (LR) of 25 days-old *Arabidopsis thaliana* wild-type Col-0 and *pin4* mutant plants grown on different local N supplies on vertically-splitted agar plates. **(A)** third-order LR length, **(B)** third order LR density in the high nitrogen (HN) compartment. First-order LRs grew into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. Primary roots grew in 5 µM KNO_3 in low nitrogen (LN) compartment. Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 12 – 15 individual plants per treatment and genotype. **(C)** GFP-derived fluorescence in second-order LR from transgenic plants expressing *PIN4pro:PIN4:GFP* in overlay with propidium iodide-dependent red fluorescence of roots grown on the HN compartment on the vertically-splitted agar plate. Fluorescent images were taken by confocal microscope 12 days after transplating to the local N treatments. Representative images from 15 individual plants per treatment are shown. Bars represent 40 µm. **(D)** Quantitative readout of GFP-dependent fluorescence intensity in epidermis cells at the meristematic zone of second-order LR tips expressed relative to the levels detected in –N-treated roots. (n = 10 root tips per treatment, on which the fluorescence was measured in at least three different positions). Bars are means ± SE.

To monitor the regulation of PIN4 at the protein level the reporter line *PIN4pro:PIN4:GFP* was used. However, compared to N deficiency or nitrate nutrition PIN4 expression was up-regulated by local ammonium supply from the apical meristem of second-order lateral roots throughout the stele (**Figure 29C and D**). Nitrate led to weaker PIN4 expression than ammonium in the root when roots of the same developmental stage were compared (**Figure 29C, D**). Interestingly, PIN4-derived GFP fluorescence decreased over time on local nitrate, when roots of different developmental stages were compared (data not shown). Mature root parts which were exposed to the nitrogen treatment for longer showed the effects of PIN4 repression much more intensively than young freshly developed roots. The under local ammonium expanded and intensified expression pattern indicated formation of a large capacity for rootward auxin fluxes.
Taken together, the auxin efflux carriers PIN2 and PIN4 appeared as critical components for triggering higher-order LR development in local ammonium. Local ammonium repressed PIN2 expression, while it induced PIN4 expression. These opposite effects may be due to their different role in cell type-specific auxin transport.

4.5 The impact of ammonium-dependent medium pH on auxin-depemdent higher-order lateral root formation

4.5.1 Higher-order lateral root branching on local ammonium depends on a low apoplastic pH

Even though the ammonium-dependent regulation of PIN2- and PIN4-mediated auxin transport had clear effects on higher-order LR formation under local ammonium supply, it remains open how short-distance auxin movement facilitates radial auxin distribution into the surrounding cells of developing LR primordia. To evaluate the impact of medium pH on higher-order LR formation under local ammonium supply, wild-type plants were pre-cultured for ten days on mild nitrogen deficiency with a medium pH of 5.8 and afterwards transferred to vertically-splitted agar plates with the first-order LR growing on ammonium at increasing medium pH. The first-order LR showed a clear response to the different medium pH. A medium pH of 4.5 and 5.0 led to a repression of the root system, especially the elongation of the first-order LR was inhibited (**Figure 30A and B**). The higher-order LR branching appeared to be equale between the different external pH values. A medium pH of 5.5 or higher did neither affect the elongation of the first-order LR nor the higher-order LR density (**Figure 30A, B and C**). To conclude, the medium pH was not determining higher-order LR branching on local ammonium supply,

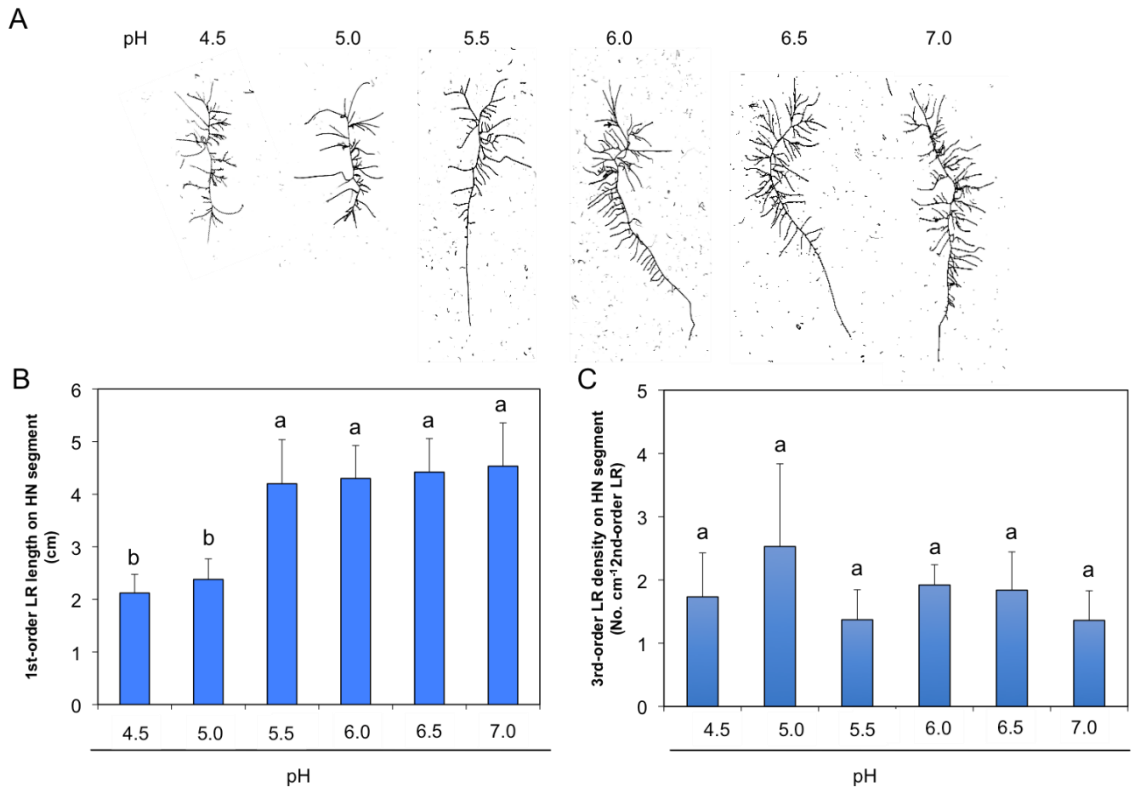

Figure 30. Medium pH does not alter higher-order LR formation in response to local ammonium.
Development of first-order lateral roots (LR) of *Arabidopsis thaliana* wild type Col-0 grown on local ammonium in vertically-splitted agar plates in response to different medium pH. **(A)** Representative image of first-order lateral roots grown in a high nitrogen (HN) compartment. Representative images from 15 individual plants per treatment are shown. **(B)** Third-order LR length in the high nitrogen (HN) compartment, **(C)** Third-order LR density on HN compartment. First-order LRs grew into a HN compartment containing 0.8 mM ammonium. The pH was bufferd by 5 mM MES to pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. PR grew in 5 µM nitrate on low nitrogen (LN) compartment. Plants were pre-cultured for 10 days on 0.5 mM nitrate as sole N source at a medium pH of 5.7. Bars represent mean values (± SD) and letters denote significant differences among means at $P<0.05$ (Tukey´s test), n = 10 - 12 individual plants per pH treatment.

Ammonium uptake leads to apoplastic acidification and cytosolic alkalinization induced by proton export across the plasma membrane (Murány et al., 1994; Kosegarten et al., 1997). To prevent pH changes of the agar medium in petri dishes the pH is mostly buffered by MES (Lima et al., 2010; Gruber et al., 2013). Here, medium pH was buffered at pH 5.7 using 5 mM MES. Then, the pH indicator bromocresol purple was added to observe changes in medium pH, and additionally, agar pieces of 2 by 2 cm were cut out near the roots to measure pH more accurately. Plants which were growing on nitrogen deficiency did not change medium pH after 15 days of growth, as the pH stayed at 5.7 like adjusted before (**Figure 31A, B**). In contrast, local nitrate and ammonium supply were leading to pH changes. Nitrate supply increased the pH slightly to pH 5.9 (**Figure 31A, B**), which was also indicated by a darker purplish color of the agar medium around the root on the HN

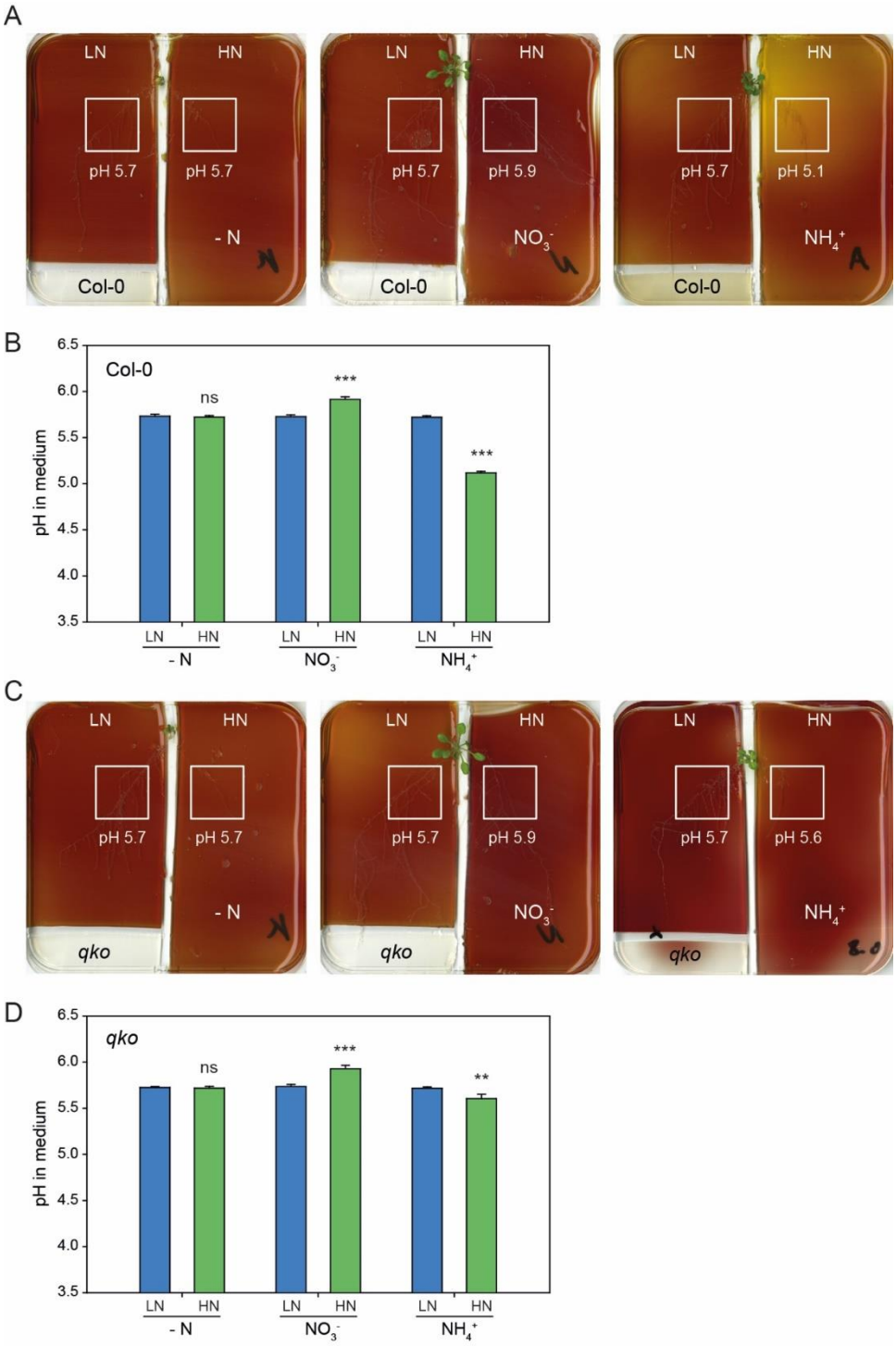

Figure 31. Changes in rhizosphere pH due to local supply of different N forms
Changes in medium pH after growth of Arabidopsis wild-type (Col-0) and *qko* plants on vertically-splitted agar plates in response to different local N supplies indicated by color change of bromocresol purple. **(A and C)** Agar plates stained by bromocresol purple over night after growth of Col-0 **(A)** or *qko* **(C)** on local supply of different N forms. Representative image from 5 individual agar plates per treatment and genotype are shown. Plants growing with their first-order lateral root in the high nitrogen (HN) compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl, while the primary root grew in 5 µM KNO_3 (LN). Plants were precultured for 10 days on 0.5 mM nitrate as sole N source before transplanting to the vertically-splitted agar plates. **(B)** pH of agar medium on the HN and LN compartment after cultivation of Col-0 **(B)** or *qko* **(D)**. For pH measurements 2x2 cm pieces of agar were cut out (white boxes), dissolved in water and pH measured by a pH meter. Bars represent mean values (± SD) and asterisks denote significant differences among means at ** P<0.01 or *** P<0.001 (Student`s t-test), n = 5 individual agar pieces per genotype and N treatment.

plate side. Ammonium led to an opposite effect and resulted in an acidification of the medium on the HN plate side indicated by a color change of bromocresol purple from purple to yellow (**Figure 31A**). The more accurate measurement confirmed a pH drop to 5.1 (**Figure 31B**). To sum up, first-order LR growing on vertically-splitted agar plates significantly changed medium pH even if the medium pH is buffered, whereby ammonium acidified the agar medium surrounding the LR. Note that the acidification effect by ammonium depends on AMT-mediated ammonium uptake, since the strong acidification of the medium pH is absent in the *qko* mutant (**Figure 31C, D**).

The genetically encoded sensor apo-pHusion has been developed to monitor pH changes in the apoplast of plant cells (Gjetting et al., 2012). The corresponding reporter line harbours a tandem concatenation of enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP1), which allows ratiometric pH measurements. With decreasing pH the GFP signal is decreasing as well, which means that a strong GFP signal just occurs under alkaline conditions while it disappears under acidic conditions (Gjetting et al., 2012). Here, the apo-pHusion reporter line was used to monitor apoplastic pH changes when roots grew in the three differend nitrogen treatments. A strong GFP signal appeared in the stele of N deficient roots, indicating the existence of alkaline apoplastic conditions in cells of the central cylinder (**Figure 32A and B**). In nitrate-treated roots, GFP fluorescence was observed in the vasculature and in outer cell layers, where nitrate uptake usually occurre (**Figure 32A and B**), suggesting that nitrate uptake leads to an extensive alkalinisation of the root apoplast. By contrast, local ammonium treatment largely repressed GFP signal intensity all over the second-order LRs, indicating a strong acidification of root apoplast in these tissues upon exposure to ammonium (**Figure 32A and B**). Taking together, while nitrogen deficiency and local nitrate supply led to an alkalinization of the root tissue at the site of higher–order LR emergence, local ammonium supply decreased the pH drastically, which implicates a connection between the emergence of LRs and an acidified apoplast in those root cells that overley LR initials.

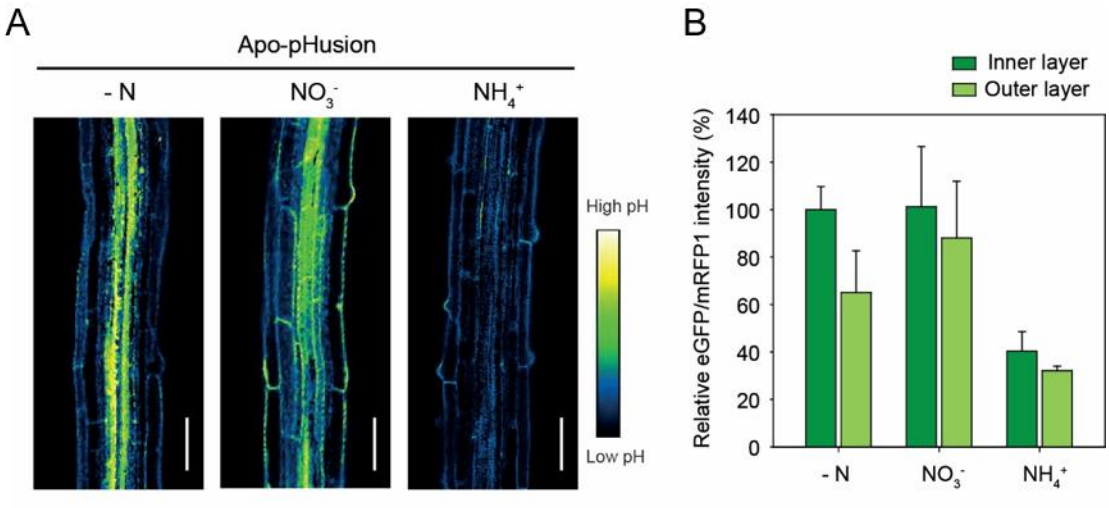

Figure 32. Apoplastic pH in root cells of second-order LRs grown in presence of different N forms.
(A) Fluorescence intensity ratio of eGFP/mRFP1 of the apo-pHusion reporter line in the branching zones of second-order lateral roots (LR) in response to different N supplies shown by false color code. Representative images from 15 individual plants per treatment are shown. Plants of the pH reporter line apo-pHusion were grown for 12 days on vertically-splitted agar plates with their first-order LR growing on high nitrogen (HN) containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. Primary roots grew in 5 µM KNO_3 (LN).12 days after transplanting from preculture plates containing 0.5 mM nitrate as sole N source fluorescence of the apo-pHusion protein was measured in the branching zone of second-order LRs grown on HN side by confocal microscope. Apoplastic pH is indicated by the fluorescence intensity ratio of eGFP/mRFP1. Representative virtual ratio images among 10 roots per treatment are shown. Scale bars are 50 µm. **(B)** Quantitative readout of apo-pHusion fluorescence intensity. The intensity ratio of eGFP/mRFP1 was calculated in the inner root layers (vasculature, pericycle) and in the outer root layers (endodermis, cortex, epidermis) and was expressed relative to the levels detected in the inner layer of –N-treated roots. (n = 10 root tips per treatment, on which the fluorescence was measured in at least three different positions). Bars represent mean values ± SD.

4.5.2 Apoplastic acidification led by ammonium uptake promotes radial auxin diffusion

Indole-3-acetic acid (IAA), the major physiologically active form of auxin in plants, is a weak acid with a pKa of 4.75, meaning that the membrane permeability of IAA is largely affected by pH. In acidic conditions, the protonated form of IAA (IAAH) can permeate membranes and enter cells via diffusion, while with increasing pH, IAA dissociates into the anionic form (IAA^-), which cannot permeate membranes (Goldsmith, 1977; Swarup and Péret, 2012). To verify the influence of N supply on pH-dependent auxin diffusion, external IAA was added into the HN side of the vertically-splitted agar plates. When external IAA was supplied at a concentration of 0.1 µM to N-deficient roots or nitrate-treated roots, higher-order LR branching in the wild type was not significantly changed (**Figure 33A and B**). By contrast, when external IAA was supplied to the roots exposed to local ammonium, higher-order LR branching in wild-type roots was largely stimulated (**Figure 33A and B**).

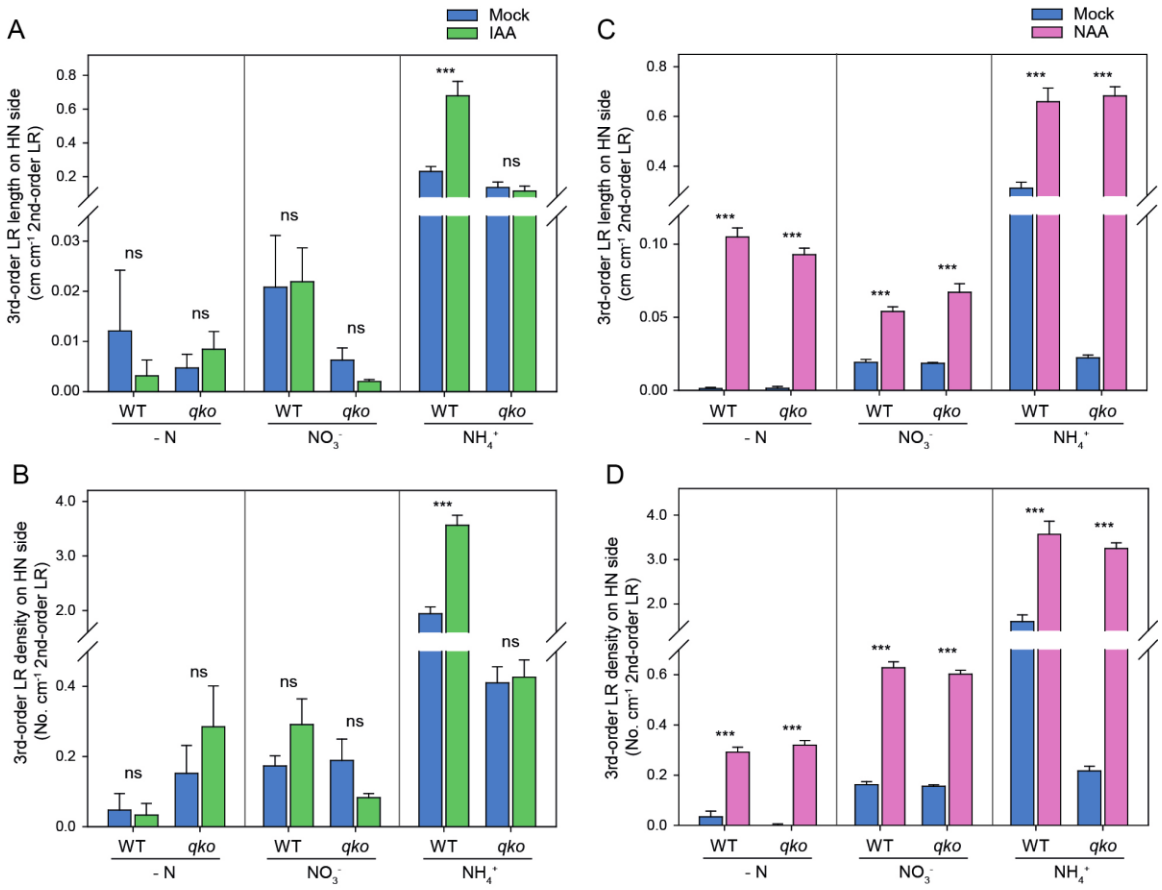

Figure 33. Higher-order lateral root branching after exposure to IAA or to the synthetic auxin analogue NAA.
Impact of externally supplied auxin on development of first-order lateral roots (LR) of *Arabidopsis thaliana* wild-type (Col-0) and *qko* grown on vertically-splitted agar plates in response to different local N supplies. **(A)** Third-order LR length and **(B)** third-order LR density on the HN plate side of Col-0 and *qko* grown under N deficiency or in presence of nitrate or ammonium with or without additional supply of 0.1 µM IAA, **(C)** third-order LR length and **(D)** third-order LR density on the HN plate side of Col-0 and *qko* grown under N deficiency or in presence of nitrate or ammonium with or without additional supply 0.1 µM NAA. Plants grew with their first-order LR into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl with or without added IAA or NAA. PR grown in 5 µM KNO_3 (LN). Lateral root growth on HN side were analysed 15 days after treatment. Plants were precultured for 10 days on 0.5 mM nitrate as sole N source. Bars represent mean values (± SE) and asterisks denote significant differences between mock control and IAA or NAA supply at * P<0.05, ** P<0.01 or *** P<0.001 (Student`s t-test), n = 10 individual plants per treatment and genotype.

The increased sensitivity of ammonium-treated roots to external IAA confirmed that apoplastic acidification in response to ammonium uptake stimulates radial auxin diffusion. When external IAA was supplied to *qko* plants, in which ammonium-induced apoplastic acidification is almost absent (**Figure 31C and D**), higher-order LR development was not significantly altered, irrespective of the type of N source supplied (**Figure 33A and B**). The lacking sensitivity of *qko* to external IAA indicated that pH-dependent auxin diffusion increases with apoplastic acidification, which in turn is associated with AMT-mediated ammonium uptake. To verify this conclusion, 1-naphthaleneacetic acid (NAA) was supplied, which is a lipophilic and membrane-permeable auxin analogue and can easily

premeate cell membranes by diffusion in a pH-independent manner (Swarup and Péret, 2012). As expected, highly diffusible NAA increased third-order LR length and density in ammonium-exposed wild-type roots to a similar extent as IAA but additionally in nitrate-exposed and N-deficient roots, confirming the insensitivity of NAA to N-dependent pH changes. Notably, in ammonium-exposed *qko* roots NAA restored third-order LR length and density up to the wild-type level (**Figure 33C and D**), emphasizing the importance of auxin protonation for higher-order LR development.

Auxin transport to the cortex cells has been described as key mechanism for LR emergence. To monitor auxin distribution in the root tissue, the auxin reporter lines *DR5v2-ntdTomato–DR5-n3GFP* and *DII VENUS* were used as tool to monitor radial auxin distribution. *DR5v2-ntdTomato–DR5-n3GFP* expresses a GFP reporter with a nuclear localization signal under a synthetic auxin-inducible promoter (Liao et al., 2015). In roots growing under N deficiency and in roots growing on local nitrate supply, the auxin-dependent GFP signal of *DR5-n3GFP* was mostly restricted to the vasculature (**Figure 34A and B**). In contrast, local ammonium supply promoted radial auxin distribution throughout the root were auxin reached besides other cell layers also the cortex cells (**Figure 34C and D**). In order to get a deeper insight into radial auxin movement in response to different local nitrogen supplies, the auxin reporter *DII-VENUS* was employed, which possesses a higher sensitivity towards auxin but decreases fluorescence in the presence of auxin. Under N deficiency or local nitrate supply, a nuclear-localized fluorescence signal was detected especially in the outer layer root tissues within LR branching zone, indicating the absence of auxin, and allowing to conclude that auxin distribution was restricted in inner root cells (**Figure 34C and D**). In contrast, when roots were exposed to local ammonium, *DII-VENUS*-dependent fluorescence markedly faded away in the outer cell layers, including cortex and epidermal cells, suggesting higher auxin abundance across the circumference of the ammonium-treated root (**Figure 34C and D**). Taken together, local ammonium supply allows enhanced radial auxin diffusion into outer cell layers, while N deficiency and local nitrate treatment trap auxin in the vasculature and represses radial auxin movement.

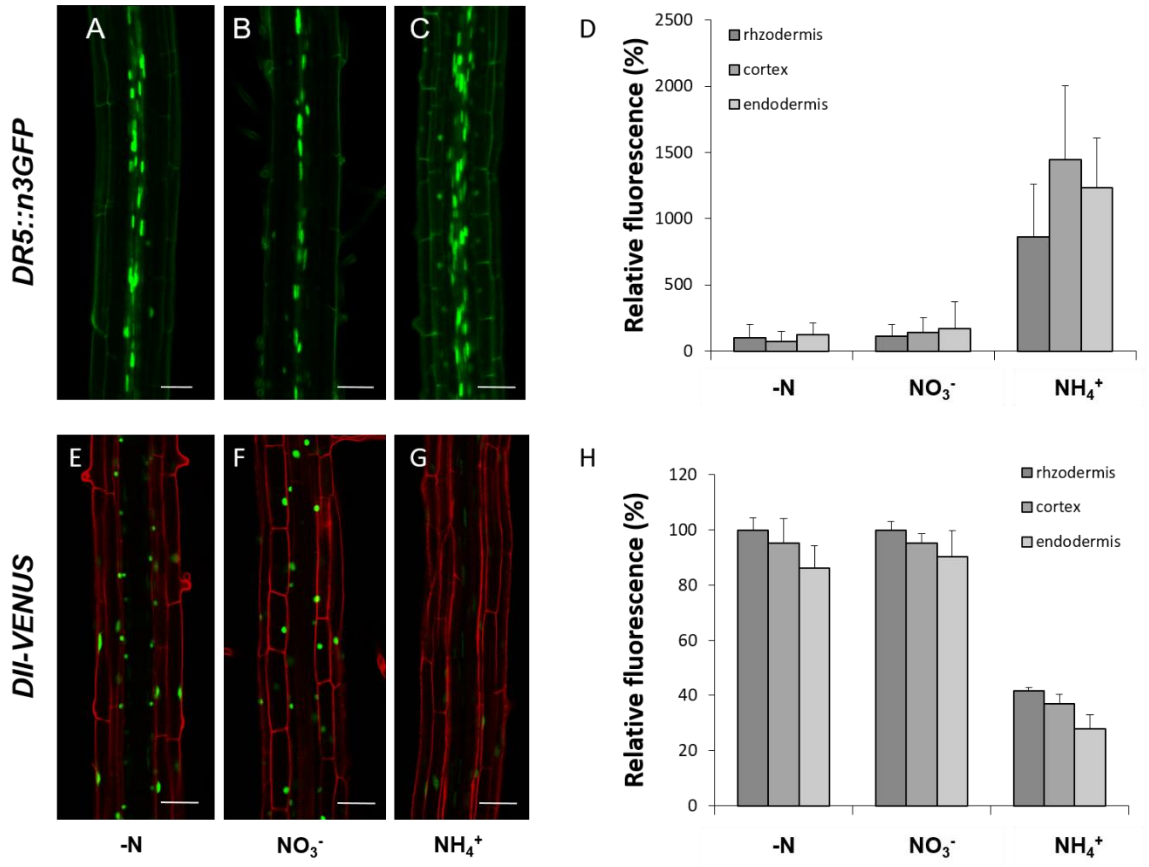

Figure 34. Lateral auxin distribution in second-order lateral roots supplied with different N forms.
(A-C) Expression of *DR5v2:n3GFP* in second-order LRs at the LR branching zone in response to **(A)** N deficiency, **(B)** nitrate or **(C)** ammonium supply. **(E-G)** Expression of *DII-VENUS* in second-order LRs at the LR branching zone in response to **(E)** N deficiency, **(F)** nitrate or **(G)** ammonium supply. Representative images from 15 individual plants per nitrogen treatment are shown. Bars represent 50 µm. **(D, H)** Quantitative readout of GFP-dependent fluorescence intensities in the rhizodermis, cortex and endodermis at the LR branching zone of second-order LR relative to the fluorescence detected in the rhizodermis of N-deficient roots for **(D)** *DR5v2:n3GFP* and **(H)** *DII-VENUS*. GFP-derived fluorescence was analysed 12 days after treatment. Bars represent means ± SD; n = 10 roots per treatment, on which the fluorescence was measured in at least three different positions. Images were taken by confocal microscope from second-order LR of the auxin reporter lines *DR5v2:n3GFP* and *DII VENUS* that were grown on vertically-splitted agar plates with their first LR growing into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. Primary roots were grown in 5 µM KNO_3 (LN). Plants were precultured for 10 days on 0.5 mM nitrate as sole N source.

Regarding the role of auxin in regulating LR emergence, the auxin influx carrier LAX3 facilitates lateral auxin distribution into root cells just overlaying the developing LR primordia, thus contributing to LR emergence (Swarup et al., 2008). In the *aux1 lax3* double mutant, LR emergence is largely impaired because of disrupted radial auxin transport, whereas LR initiation is not much altered (Swarup et al., 2008). It was thus hypothesized that IAA protonation in response to local ammonium supply can compensate for the loss of radial auxin movement in the *aux1 lax3* double mutant and restore LR emergence. Since visible first-order LRs are hardly formed in the *aux1 lax3* double mutant, it was impossible to analyse the LR phenotype of *aux1 lax3* in vertically-splitted agar

plates, in which a first-order LR needs to be guided into a separate compartment. Therefore, LR development of the *aux1 lax3* mutant was investigated in horizontally-splitted agar plates, where localized ammonium was only supplied to the middle agar compartment (**Figure 35A - C**).

In agreement with severely impaired LR emergence as reported previously (Swarup et al., 2008), visible LR formation of *aux1 lax3* under N deficiency or local nitrate supply was much lower than that of wild-type plants (**Figure 35**). However, local ammonium treatment in the middle segment partially restored LR development in *aux1 lax3*, especially second-order LR density, which reached wild-type level (**Figure 35G**). However, even first-order LR length and density was increased (**Figure 35D and E**). Meanwhile, the *arf7* arf19 double mutant, which shows a severe defect of LR initiation (Okushima et al., 2007), was also investigated under the same conditions (**Figure 35A - C**). Irrespective of the type of N source no visible LR were generated in *arf7 arf19* (**Figure 35**). Therefore, local ammonium supply restored LR emergence as being defect in *aux1 lax3*, but not LR initiation, being defect in *arf7 arf19,* indicating that ammonium-induced radial auxin diffusion can partially replace LAX3-mediated lateral auxin transport during LR emergence. These results further support the conclusion that local ammonium supply promotes radial auxin diffusion into the outer layer cells overlying the developing LR primordia, and thus leads to a stimulatory effect on LR emergence.

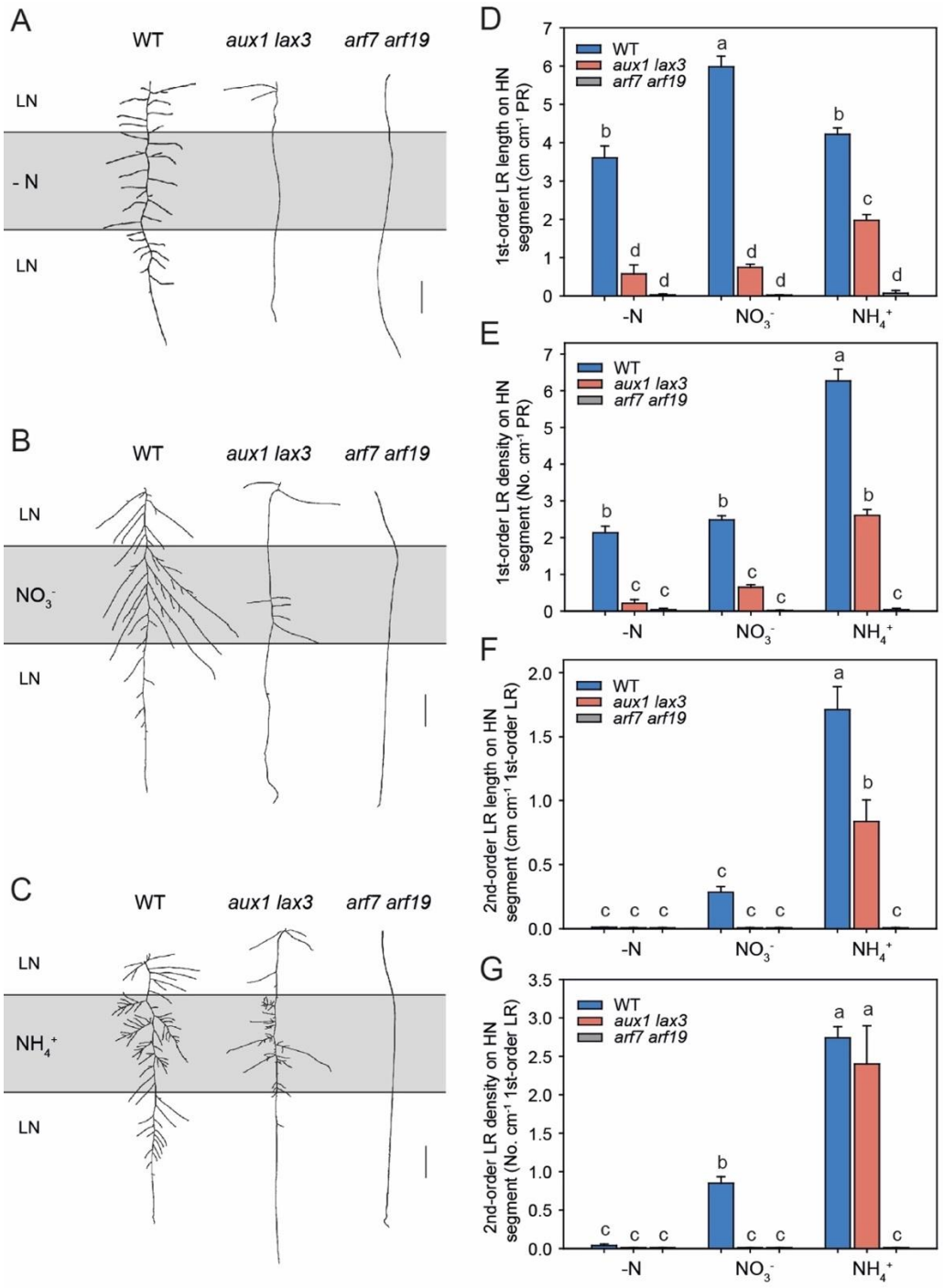

Figure 35. Higher-order lateral root formation in auxin signaling and auxin transport mutants.

(A-C) Root system architecture of wild-type (Col-0), or *aux1 lax3* and *arf7 arf19* double mutants grown on horizontally-splitted agar plates with their PR growing in 0.5 µM KNO_3 (LN) and a middle compartment (HN) containing **(A)** 0.8 mM KCl, **(B)** 0.8 mM KNO_3 or **(C)** 0.8 mM NH_4Cl. Scale bars are 1 cm. Representative images of roots from 10 individual plants per treatment and genotype are shown**. (D)** First-order LR length, **(E)** first-order LR density, **(F)** second-order LR length and **(G)** second-order LR density of Col-0, *aux1 lax3* and *arf7 arf19* in the middle plate segment. Bars represent mean values (± SE) and letters denote significant differences among means at P<0.05 (Tukey´s test), n = 10 individual plants per treatment. Plants were grown on horizontally-splitted agar plates with their PR growing into a HN compartment containing 0.8 mM KCl, 0.8 mM KNO_3 or 0.8 mM NH_4Cl. Plants were precultured for 10 days on 0.5 mM nitrate as sole N source.

The pH of the rhizosphere and the root apoplast is drastically influencing nutrient uptake and RSA of plants (Richardson et al., 2010; Haling et al., 2011). Since the increased auxin accumulation in N-deficient roots could not promote LR development (**Figure 4**), it appeared that a large amount of auxin was locked in the vasculature of N-deficient roots (**Figure 15A**). This raised the hypothesis that the alkaline apoplastic pH of N-deficient cells in the central cylinder traps auxin in the vasculature, and therefore represses LR development. To stimulate lateral diffucsion of auxin trapped in N-deficient root steles, medium pH was decreased from 5.7 to 5.0 to mimic the apoplastic acidification brought about by ammonium supply (**Figure 31A and B**).

In this experimental setup, wild-type plants were grown for seven days on pH 5.7 and for additional eight days on pH 5.0. Control plants were growing continuously on pH 5.7 for 15 days. At pH 5.7, higher-order LR formation was strongly reduced under nitrogen deficiency. However, decreasing the external pH down to 5.0 enhanced the formation of second- and third-order LRs drastically (**Figure 36A, D and E**). Thus, lowering pH locally was sufficient to restore higher-order LR formation even in N-deficient roots. This result reveals that auxin, trapped in the vasculature, can be released by external and presumably apoplastic acidification, leading to stimulation of higher-order LR development.

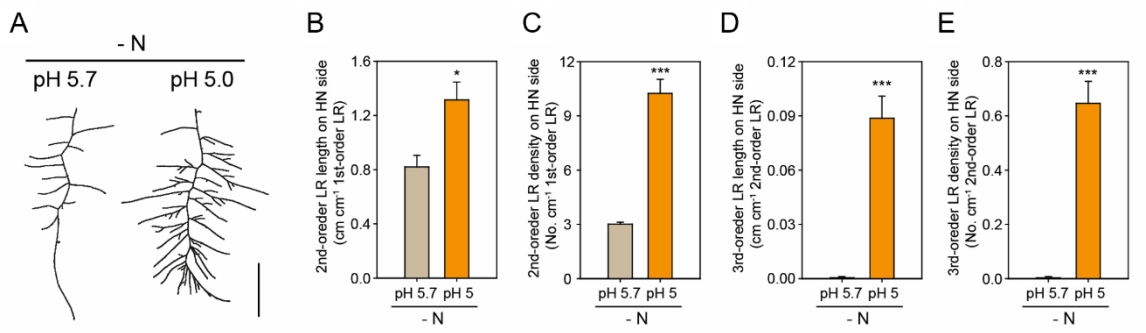

Figure 36. Higher-order LR formation in response to medium pH.
(A-E) Higher-order lateral root (LR) development under N deficiency at different medium pH. **(A)** Representative images of first-order lateral roots from 10 individual plants per treatment and genotype are shown. Scale bar is 1 cm. **(B)** Second-order LR length, **(C)** second-order LR density, **(D)** third-order LR length and **(E)** third-order LR density of LR from Col-0 plants. Col-0 plants were first grown for 7 days on vertically-splitted agar plates with 0.8 mM KCl in the first-order LR segment at pH 5.7, and then transferred to vertically-splitted agar plates where medium pH in first-order LR compartment was adjusted to 5.0 or 5.7 by 5 mM MES buffer. Remaining roots were growing in 5 µM KNO_3 with medium pH of 5.7. Plants were precultured on medium with a pH of 5.7 containing 0.5 mM nitrate as sole N source for 10 days before transplanting to vertically splitted agar plates. Lateral root growth in response to localized pH drop was measured at 8 days after transfer from vertically-splitted agar plates with pH 5.7 to vertically-splitted agar plates with pH 5.0. Bars represent mean values ± SE; n = 10 plants per treatment. Asterisk denote significant differences among different pH at * P<0.05 ** P<0.01 *** P<0.001 (Student's t-test).

5 Discussion

The plasticity of root system architecture (RSA) is tightly regulated by internal and external sensing and signalling mechanisms, some of which depend on water and nutrient availability in the soil (Toorchi et al., 2002; Trachsel et al., 2009: Gruber et al., 2013. Among the nutritional signals, the external availability of inorganic N is a major determinant to shape root system architecture, whereby local ammonium specifically stimulates LR branching (Drew, 1975; Lima et al., 2010). However, the molecular mechanisms underlying the induction of LR branching by ammonium have remained largely unknown. The work presented here provides a mechanism for the adaptation of root system architecture to ammonium and links local ammonium availability with changes in auxin distribution in the plant, which acts as a signal for lateral root branching

5.1 Ammonium acts as a signal for root branching

5.1.1 Local nitrogen supply affects root system architecture in a nitrogen form-dependend manner

The two inorganic nitrogen forms ammonium and nitrate lead to a completely contrasting root system architecture (RSA), if supplied locally. Whereas nitrate leads to a more elongated root system with long LRs, which allows foraging for the mobile nitrogen source nitrate, ammonium leads to a more compact highly branched root system with short LRs and a higher number of roots of different orders (Zhang and Forde, 1998; Remans et al., 2006; Lima et al., 2010; Li et al., 2010; Araya et al., 2016; **Figure 4D, F, G, H and I**). Since the different inorganic N forms alter root system architecture in different ways, the distinct mechanisms that integrate external N signals into the root developmental program where of particular interest. Targeted growth responses of lateral roots towards a spatially localized nutrient source differ among nutrients and their impact on cell biological processes (Drew, 1975; Giehl and von Wirén, 2014). Localized patches of nitrate, phosphate and iron all increase lateral root length. Thereby nitrate primarily causes a higher rate of cell production in the LR meristem, whereas iron enhances cell elongation (Linkohr et al., 2002; Zhang et al., 1999; Giehl et al., 2012). There are several examples in which nutrients interfer with auxin distribiution in roots. Localized iron enhances AUX1-mediated auxin import, which triggers lateral root elongation into an iron-rich patch (Giehl et al., 2012). Nitrate availability modulates auxin distribution in LR tips via the dual-affinity nitrate and auxin transporter NRT1.1/NPF6.3 (Krouk et al., 2010b). In the absence of

nitrate, NRT1.1 facilitates auxin influx into lateral root tips, which brings auxin out of LR apices and represses LR growth. However, the presence of nitrate inhibits the auxin transport capacity of NRT1.1, thus allowing auxin to remain in lateral root apices and promote lateral root elongation (Krouk et al., 2010b). This NRT1.1-mediated auxin transport mechanism has been suggested to prevent futile root growth into an N-deficient environment (Mounier et al., 2014). Additionally, NRT1;1 facilitated auxin export out of LR primordia, which represses LR emergence under low nitrate. Under sufficient nitrate supply auxin accumulates in the LR primordia, where it induces LAX3-mediated auxin transport into LR overlaying endodermis and cortex cells to faccilitae LR emergence (Krouk et al., 2010; Bouguyon et al. 2015: Swarup et al., 2008).

So far, only localized ammonium supply has been shown to increase the number and density of higher-order LRs while repressing their elongation. It was suggested that the higher-order LR branching on local ammonium depends on a sensing and signalling cascade involving high affinity ammonium transporters of the AMT-family (Lima et al., 2010). The Arabidopsis AMT quadruble knock out *qko*, which is defective in four root-expressed high affinity ammonium transporters (*amt1;1, amt1;2, amt1;3, amt2;1*) shows very low capacity for high-affinity ammonium uptake, irrespective of whether ammonium is supplied to the whole root system or locally (Yuan et al. 2007a; Lima et al. 2010; **Figure 5).** Furthermore, the *qko* shows a drastic reduction in higher-order LR formation on local ammonium (**Figure 4E-I**, **Figure 12G, K, L** and **Figure 13**; Lima et al., 2010) and it was assumed, that low internal ammonium concentrations are the reason for the decreased higher-order LR formation (Lima et al., 2010). To compensate for the lack off high affinity ammonium transport in *qko*, the ammonium concentration in the media was increased up to 2 mM, which resulted in an increase of higher-order LRs in horizontally- as well as in vertically-splitted agar plates and reached wild-type level (**Figure 12H, K, L** and **Figure 13**). It was concluded that in *qko*, higher-order LR branching was enhanced by increased internal ammonium concentrations. An explanation for this higher-order LR formation in *qko* on elevated ammonium concentration is the fact, that ammonium is not exclusively transported by high affinity ammonium transporters (HATS) but also transported by low affinity ammonium transporters (LATS), as described in yeast (Loqué and von Wirén, 2004). The potassium channel AKT1 is a likely candidate to be involved in low-affinity ammonium uptake in plants (Spalding et al., 1999). The observation that elevated ammonium supply induces LR branching in *qko*, probably by using low-affinity membrane transport systems, indicates that, LR branching on local ammonium can be independent of the AMTs, if ammonium concentrations in the medium are high. Important to consider, ammonium is not only taken up from the substrate, but is also generated in numerous endogenous metabolic processes, including nitrate reduction (Hawkesford et al., 2012;

Bittsánszky et al., 2015). Thus, also other N sources can trigger higher-order LR branching as well.

Surprisingly, Lima et al. (2010) made another observation than those observed here. These authors reported that *qko* showed no enhanced higher-order LR formation on elevated ammonium supply, even though the root ammonium concentrations reached the same level as that in wild-type plants supplied with 0.8 mM ammonium. Based on this, they proposed that AMTs may be involved in the sensing and signalling of ammonium and rejected a simple nutritional effect by the amount of ammonium being taken up. However, the reason for such contrasting observations may originate from experimental factors influencing higher-order LR branching in *qko*. For example, Lima et al., (2010) used up to 10 plants per plate in the horizontally-splitted agar plate system, which may lead to rapid ammonium depletion and a dilution of ammonium in the agar medium. In contrast, if just one plant per plate was grown in the horizontally-splitted agar plate, LR branching became evident (**Figure 12D**). This could be the reason, why *qko* showed higher-order LR branching in the experiments conducted here in comparision to the one reported by Lima et al., (2010).

To conclude, higher-order LR branching in an ammonium patch does not strictly relie on high affinity ammonium transport systems, because the lack of AMTs in *qko* can be compensated for by elevated external ammonium supply, facilitating ammonium uptake via low-affinity ammonium transport sysems. Indicating, ammonium itself is the crucial factor in ammonium-dependent higher-order LR formation.

5.1.2 Different expression patterns and posttranscriptional regulation of AMT1;1 and Amt1;3 explain the crucial role of AMT1;3 in higher-order lateral root branching on local ammonium

In 2010 Lima et al. showed that local ammonium supply stimulates higher-order LR branching in an AMT1;3-dependent manner. Higher-order LR branching on local ammonium supply was lower exclusively in *amt1;3*, while single AMT gene deletions in the other ammonium transporter genes *AMT1;1*, *AMT1;2*, or *AMT2;1* did not affect this root trait significantly. Especially the fact that AMT1;1 had no impact on higher-order LR branching was surprising, because of its co-localization in outer root cells and highly similar biochemical transport properties to AMT1;3. To investigate the reason for this difference, the *AMT1;1pro-AMT1;1gene:GFP* and *AMT1;3pro-AMT1;3gene:GFP* lines were grown on local ammonium supply. Whereas, the fluorescence of both AMT gene GFP fusions dercreased in more basal parts of the PR (Loque et al., 2006), the

fluorescence of the *AMT1;3pro-AMT1;3gene:GFP* line appeared strong in the LR branching zone of second-order LRs on local ammonium (**Figure 7E and F**). Interestingly, the AMT1;3 localization in the plasma membrane of primordia overlaying endodermal and cortical cells is mediating cellular ammonium intake exactly at the place, where ammonium uptake is facilitating LR formation.

Besides their partially different localization AMT1;1 and AMT1;3 showed a dramatic difference when it came to their abundance in the tissue of second-order LRs on local ammonium. *AMT1;1* was repressed on local ammonium, in contrast to *AMT1;3*. In the absence of nitrogen or when nitrate was supplied locally both plasma membrane localized AMTs were induced (**Figure 6** and **Figure 7**). Since ammonium uptake from the rhizosphere into the root tissue has to be tightly regulated to avoid toxic effects of elevated cytosolic ammonium concentrations (Loqué and von Wirén, 2004), the regulation of AMT-type ammonium transporters is controlled at different levels, in particular at the transcriptional or posttranslational level (Crowford and Forde, 2002). In Arabidopsis *AMT1;1* and *AMT1;3* are induced by resupply of ammonium (Loqué et al., 2006), and in tomato (*AMT1;2*) or rice (*AMT1;2* or *AMT1;1*) AMTs were induced by a resupply of ammonium as well (Lauter et al., 1996; von Wirén et al., 2000; Sonoda et al., 2003b). In Arabidopsis *AMT1;1* transcript levels appear to respond more directly or faster to external ammonium supply than the transcripts of the other AMTs, which is a indicator for the tight regulation of AMT1;1 (Gazzarrini et al., 1999; Rawat et al., 1999; Shelden et al., 2001; Sohlenkamp et al., 2002). The repression of *AMT1;1* depends on a local nitrogen signal (Gansel et al., 2001) and glutamine, the first amino acid produced during nitrogen assimilation, has been proposed as the molecular trigger to down-regulate *AMT1;1* (Rawat et al., 1999; Schlenkamp et al., 2002). Lima (2010) tested whether ammonium induced third-order LR formation was caused by a higher expression level of *AMT1;3* in the split-root agar system. Therefore, AMT gene expression during third-order LR development on local ammonium was monitored in a time-course. *AMT1;1* transcript levels strongly increased and had their peak at the second day after transfer to vertically-splitted agar plates. From the third day on, transcript levels of *AMT1;1* decreased dramatically. In contrast, *AMT1;3* transcript levels remained at a constantly low level during the whole period of third-order LR formation on vertically-splitted agar plates, which coincided with the stable protein abundance of AMT1;3 at the epidermal and cortical cells at the root tip and the LR branching zone also in presence of ammonium (**Figure 6F** and **Figure 7E, F**). To which extent the presence of AMT1;1 or AMT1;3 is influenced by their transcriptional regulation or is a result of different protein stability is not yet clear. Lower transcript levels, and at the same time, higher protein levels of AMT1;3, may be seen as a first hint that the AMT1;3 protein is more stable in the presence of ammonium. By using horizontally-splitted

agar plates a similar result was obtained, *AMT1;1pro-AMT1;1gene:GFP* in the PR was repressed after 7 days on local ammonium, whereas *AMT1;3pro-AMT1;3gene:GFP*-dependent fluorescence was not affected (**Figure 8**). The nitrogen status-independent *AMT1;3* expression was also shown by Yuan et al. (2007b), where transcript levels as well as protein levels of a *35S:AMT1;3* construct introduced in the *amt1;1* background were not repressed in the presence of ammonium, indicating that there is no posttranscriptional regulation via RNA interference (RNAi) like described for *AMT1;1*. The presence or absence of AMT1:3 affects besides higher-order LR branching also the elongation of the PR in response to local ammonium. In the *amt1;3* mutant no repression of PR elongation could be measured (**Figure 9C and D**). AMT1;3 seems to be crucial for the repression of PR elongation, if the PR hits an ammonium patch. In contrast, in the *amt1;1* mutant PR elongation was repressed like in wild-type when ammonium was supplied to the PR root tip (**Figure 9**).

Besides the transcriptional regulation, there is evidence, that AMT-type ammonium transporters are target of posttranscriptional regulation. If nitrogen deficient plants were resupplied with ammonium, the ammonium influx showed a faster time-dependent repression relative to *AMT1;1* transcript levels in Arabidopsis roots (Rawat et al., 1999). Furthermore, Yuan et al. (2007b) showed that the mRNA stability of *AMT1;1* transcripts changed in a N-dependend manner in Arabidopsis and tobacco. The mRNA abundance of *35S:AMT1;1* transcripts increased under N deficiency and responded rapidly to ammonium or nitrate resupply in roots of tobacco. These studies showed that the ammonium uptake is uncoupled from the *AMT* expression and is most likely the result of a possible posttranscriptional regulation of these high affinity transporters. Interestingly, N-dependent posttranscriptional regulation was not found for 35S-driven transcript levels of AMT1;3, which reflects the different regulation of the closly related AMT1;1 and AMT1;3. There is extracellular ammonium sensing and downstream regulation of ammonium transport described for AMT1;1 which is co-localized with AMT1;3 in outer root cells. Transport activity of AMT1;1 is allosterically regulated via phosphorylation of its C-terminal domain. AMT1 proteins form a trimeric complex, in which the cytosolic C-terminus of each monomer is able to attach to the pore region of the neighbouring subunit to trans-activate or –inactivate ammonium transport. Phosphorylation of a highly conserved threonine residue in the C-terminus of any individual monomer relieves on the interaction between the C-terminus and the conducting pore, mediating trans-inactivation not only of the individual monomer but also of the complete trimeric complex (Loqué et al., 2007). Under low ammonium supply, the critical threonine residue T460 in the C-terminal domain of AMT1;1 is dephosphorylated, whereas elevated ammonium supply confers phosphorylation and thereby inactivation of the AMT1;1 trimer (Lanquar et al., 2009).

Neither ammonium assimilation products, such as glutamine, nor an MSX-induced increase of intracellular ammonium can induce T460 phosphorylation, implying that the phosphor-dependent inactivation of AMT1;1 is specifically triggered by extracellular ammonium (Loqué et al., 2007; Straub et al., 2017).

Hence, it has been proposed that AMT1;1 itself acts as a transceptor and recruits a kinase from the cytosol upon extracellular ammonium binding (Lanquar et al., 2009). This kinase has been indentified as CALCINEURIN B-LIKE INTERACTING PROTEIN KINASE23 (CIPK23; Straub et al. (2017)). The characterization of CIPK23 was a big step to understand ammonium sensing and signalling events in plants. Rapid C-terminal phosphorylation of AMT1;1 and AMT1;2, but not of AMT1;3, upon ammonium resupply has been found. AMT1;1 phosphorylation upon ammonium resupply has been confirmed in a phosphoproteomics approach (Engelsberger and Schulze, 2012).

Yuan et al. (2013) verified wether the phosphorylation at position T464 in the C-terminus of AMT1;3, which is the corresponding position to T460 in the C-terminus of AMT1;1, had a similar function in the allosteric regulation of ammonium transport. By expressing two AMT1;3 variants, T464A and T464D, in the yeast strain DL1 or the *qko*, both defective in high-affinity ammonium uptake (Marini et al., 1997; Loqué et al., 2007; Lima et al., 2010), they showed that just wild-type AMT1;3 and *AMT1;3TA* were able to facilitate ammonium uptake in the yeast mutant and Arabidopsis mutant if grown on ammonium as sole N source. However, there are four additional predicted phosphorylation sides in the C-terminus of AMT1;3, threonine residues at position 471 and 494 as well as the serine residues at position 480 and 487. To investigate which phosphorylation sites may have an impact on the activation and de-activation of AMT1;3, methylammonium (MeA) was used as toxic ammonium analogue to test the AMT1;3 activity in the ten related phosphorylation-variants, in which the threonine or serine is replaced by alanine to mimic permanent phosphorylation (rendering AMT1;3 inactive) or by asparagine to mimic permenant de-phosphorylation (rendering AMT1;3 active). These ten AMT1;3 variants, *T464A*, *T464D*, *T471A*, *T471D*, *S480A*, *S480D*, *S487A*, *S487D*, *T494A* and *T494D* were expressed in the background of *qko* to avoid possible side effects from the other three root-expressed AMTs. The results of the experiment and earlier studies done by Yuan et al. (2013) using MeA indicated that just the phosphorylation sites at position 464 and 471 had a regulatory effect on ammonium uptake, in contrast to the phosphorylation sites at position 480, 487 and 494. It appears that also in AMT1;3 the position, at which the phosphorylation of the C-terminus occurs, must be close to the end of the last trans-membrane-spanning domain (Loqué et al., 2007). The plants with a constitutively active AMT1;3 showed clear toxicity symptoms on MeA and had repressed root growth with impaired PR elongation and LR formation (**Figure 10** and **Figure 11**). Whereas ammonium is the signal for

posttranslational regulation of AMT1;1 by phosphorylation (Loque et al., 2007), the signal which regulates AMT1;3 has not yet been found.

Interestingly, trans-inactivation by C-terminal phosphorylation functions not only within homotrimeric complexes as shown for AMT1;1 (Loqué et al., 2007), AMT1;2 (Neuhäuser et al., 2007), and AMT1;3, but also in heterotrimeric AMT1 complexes, which increases the chance for regulatory signal exchange between the different AMT isoforms within a trimer (Yuan et al., 2013). However, the prominent role of AMT1;3 in ammonium-dependent higher-order LR branching (Lima et al., 2010) is still unclear and seems independent of the regulation in the heterotrimer by AMT1;1, especially on localized ammonium supply. Unfortunately, it seems that AMT1;1 and AMT1;3 play important roles as core components in different signaling pathways. Their co-localization and physical interaction are crucial for the coordination of ammonium acquisition and might be equally important for ammonium foraging in response to systemic nitrogen signals reflecting the nitrogen status within the plant or the external ammonium availability in the rhizosphere. Since a pore mutation and C-terminal mutations in the AMT1;3 protein not only abolished the ammonium uptake capacity but also impaired the formation of third-order LRs (Lima, 2010), ammonium-dependent LR branching appeared to depend on a functional AMT1;3 protein. These observations suggested that conformational changes in the C-terminus of the AMT1;3 protein might be involved in ammonium-dependent signalling. Since AMT1;3 can be trans-inactivated by AMT1;1 when assembled in a heterotrimeric AMT1 complex, the ammonium uptake on local ammonium supply maybe mainly restricted by phosphorylation of AMT1;1. If AMT1;1 is transcriptionally repressed as consequence of ammonium uptake, there is probably a shift from AMT1;1-AMT1;3 heterotrimeric complexes to homotrimeric complexes with AMT1;3, which are not phosphorylated in response to an ammonium signal. Hence, influx of ammonium into the root cells can be maintained. This may explain why on local ammonium supply, AMT1;3 is the crucial high-affinity ammonium transporter and downstream sensing and signalling processes, ultimately leading to higher-order LR branching. Besides transcriptional and posttranscriptional regulation of AMT1;3 by local ammonium supply, there is additional evidence for posttranslational regulation of AMT1;3 by endocytosis. In 2013, Wang et al. used total internal reflection fluorescence microscopy, which allows single-molecule imaging of labelled proteins at the plasma membrane surface. They figured out that under nitrogen deficiency or low-ammonium conditions, AMT1;3 showed a relatively long-lived residence time in the plasma membrane. However, immediately after ammonium addition, AMT1;3 proteins cluster were formed that disappeared from the plasma membrane to become internalized by the endocytic pathway. This mechanism restricts ammonium uptake rapidly and thereby prevents ammonium toxicity. This feedback mechanism acts

besides other mechanisms such as ammonium assimilation or ammonium compartmentation into the vacuole to prevent ammonium toxicity within the root (Liu and von Wirén, 2017). Since the regulation of AMT1;1 and AMT1;3 in response to ammonium supply differs between these two high-affinity transporters, it is not surprising that one of them, i.e. AMT1;3 plays a major role in higher-order LR branching on local ammonium supply while AMT1;1 has hardly any impact on this root trait.

Taken together, the epidermal and cortical localization also in presence of ammonium seem to be a key to allow AMT1;3 mediating ammonium uptake into those cells that locate directly above developing LR primordia. In this case, AMT1;3 acts merely as ammonium transporter mediating cellular ammonium intake adjacent to LR primordia rather than as ammonium transceptor converting the substrate passage into a developmentally active signal.

5.2 Altered auxin distribution triggers higher-order lateral root branching in an ammonium patch

5.2.1 Higher-order lateral root branching on local ammonium coincides with auxin accumulation in the root vasculature

The molecular mechanisms triggered by ammonium itself, leading to higher-order LR branching in an ammonium patch, are linked to an altered auxin distribution in the root tissue of second-order LRs.

Auxin acts as a growth signal not only at the cellular but also at the whole-plant level, and is able to transmit systemic responses of the plant to different biotic or abiotic stimuli (Casimiro et al., 2001; Bhalerao et al., 2002). The cell-to-cell transport of auxin involves export and import processes across the plasma membrane, whereas membrane transport can be either secondary active or passive. Besides, auxin is one of the best described molecular signals triggering changes in RSA (Bennett 2015), rendering it a promising candidate to discover sensing and signalling processes mediating ammonium-dependent changes in root morphology.

Measuring concentrations of different auxin species by LC-MS-MS of plants growing on vertically-splitted agar plates provided a first impression about the changes in auxin homeostasis in dependence of nitrogen treatments. In the shoots, it was noted that N-deficient plants had highest concentrations of all detected auxin species in comparison to nitrogen-supplied plants (**Figure 19M-R**), leading to the hypothesis that N-deficient plants have either overall enhanced auxin biosynthesis in the shoot, retarded auxin degradation

or restricted transport to the root (Ma et al., 2014). In another study, shoot-supplied ammonium was found to lead to high auxin concentrations in the shoot. Moreover, shoot-supplied ammonium was proposed to inhibit LR emergence in roots by interfering with AUX1-dependent auxin transport from shoot to root. (Li et al. 2011). This ammonium-dependent regulatory mechanism on LR emergence appears to be unrelated to the ammonium-induced LR emergence characterized here, because local ammonium supply promoted LR emergence (**Figure 4** and **Figure 14**) and AUX1 appeared not being involved in this process (**Figure 26** and **Figure 35**).

The tryptophan-dependent synthesis of IAA is catalyzed via three major routes involving the production of different precursors, which are either indole-3-acetonitrile (IAN) or indole-3-acetamide (IAM) or indole-3-pyruvic acid (IPyA) (Ljung et al., 2001; Pollmannet al., 2003; Mashiguchi et al., 2011; Ma et al., 2014). Auxin inactivation occurs by oxidation of the ring structure of IAA, generating the catabolite 2-oxoindole-3-acetic acid (OxIAA) (Normanly et al., 2010). The majority of conjugated IAA, detected by LC-MS-MS is bound to various sugars, sugar alcohols, amino acids or even proteins or is present in methylated form (Bartel and Fink, 1995; Yang et al., 2008). Those conjugated auxin forms have been described as storage or long-distance transport forms of the active auxin form IAA (Normanly et al., 2010). The concentrations of IAN, which has been reported as the most abundant detectable precursor of IAA (Sugawara et al., 2009; Novak et al., 2012), and IAM, which can be directly synthesized from OxIAA or indirectly from OxIAA via IAN (Sugawara et al., 2009), were increased in roots growing into the ammonium patch (**Figure 19A and B**). At the same time, IAN and IAM concentrations on the LN plate side and in shoots of ammonium-treated plants were reduced (**Figure 19G, H, M and N**). The high abundance of the precursers IAN and IAM in the ammonium-treated root parts in comparison to roots growing under -N conditions suggested an increased IAA synthesis involving the IAN or IAM pathway. Even if IAN levels were slightly higher, only IAM levels showed the same changes in its concentration as IAA (**Figure 19B and C**). If IAN and IAM were transported from shoots to roots, was not investigated here. In Arabidopsis, the indole-3-pyruvic acid (IPyA) pathway has been suggested as the major biosynthetic pathway of auxin (Mashiguchi et al., 2011; Ma et al., 2014), however, since IPyA is highly unstable and easily degraded during extraction under acidic conditions (Novak et al., 2012) it could not be measured in this study. As an alternative indication for the inducibility of the IPyA pathway by ammonium, expression levels of the IPyA-biosynthesis genes *TAA1/TAR2* and the 11 so far known *YUCCA* genes were determined. Quantitative RT-PCR showed that local ammonium most likely repressed the *de novo* synthesis of auxin in the root by the IPyA parthway by downregulating most of the *YUCCA* genes (**Figure**

23C-M), where *TAR2* seemed to be not affected at the transcripional level by the different N treatments used in this study (**Figure 23B**).

The active auxin form IAA, which is known to be directly involved in the LR emergence process (Orman-Ligeza et al., 2016), was present at higher concentrations in ammonium-treated root parts in comparison to nitrate-treated root parts (**Figure 19C**), suggesting that ammonium elicits a signalling pathway, which leads to the synthesis of the active form of auxin in ammonium treated root parts.

The conjugated auxin form IAAMe has been suggested to be one of the storage or transport forms of IAA, supported by the observation that this type of IAA inactivation is reversible (Yang et al., 2008). Concentrations of IAAMe were almost 50% higher in roots supplied with local N, than in roots grown under N deficiency, indicating that the transport of auxin in the form of IAAMe was enhanced in roots reaching the N source (**Figure 19D**). There is strong evidence, that ammonium-treated plants facilitate auxin transport into those root parts reaching the N source, because IAAMe concentrations on the LN side of ammonium-treated plants was as low as in –N treated-plants on HN or LN. In contrast, nitrate-treated plants showed increased IAAMe concentrations in the whole root system (**Figure 19D, J**), suggesting that under nitrate IAA becomes more rapidly methylated for transport or storage. It has to be kept in mind that the results have to be interpreted with caution, because of missing biological replicates in this experiment. Nonetheless, since 20 or more roots were pooled for one sample, the data obtained from this experiment may provide a first indication for the changes in IAA homeostasis in response to local ammonium.

To verify the results obtained by LC-MS-MS, the IAA-inducible reporter line *DR5:GFP* was used. Subjecting the *DR5:GFP* reporter line to different auxin forms showed GFP-derived fluorescence also under externally supplied IAAMe (**Figure 20F**). Thus, it remains open to what extent this synthetic reporter can be related to the active auxin form IAA. Anyway, *DR5:GFP* expression indicated that auxin accumulated in the vasculature of LRs locally exposed to ammonium (**Figure 15C, Figure 16A** and **Figure 18A and B**). Remarkably, this enhanced auxin accumulation was much weaker in the *qko* mutant (**Figure 16B**), which failed to form a highly branched root system under local ammonium supply (**Figure 4E, H, I, Figure 12G, Figure 13A**), implying that internal ammonium alters auxin accumulation in roots. Indeed, inhibition of ammonium assimilation by short-term MSX treatment enhanced the GFP signal in the *qko* background (**Figure 17**), indicating that cytosolic ammonium concentration determines the auxin accumulation in the root vasculature independent of the contribution of AMT-sdependent ammonium uptake. Elevated cytosolic ammonium concentrations in roots promote ammonium translocation to the shoot, probably because ammonium assimilation into glutamine is saturated (Guan

et al., 2016). If external ammonium concentrations were low, root- and shoot-ammonium concentrations in *qko* were significantly decreased in comparision to wild-type plants (Lima et al., 2010; Figure 5). There is a possibility that the ammonium concentaration in the root or shoot is affecting the auxin accumulation in the root. Since, the *gln1;2* mutant, defective in ammonium assimilation in roots, shows increased ammonium concentrations in the root and shoot (Guan et al., 2016), it has been suggested that MSX treatment also increases besides root-ammonium concentrations also shoot-ammonium concentrations. Increased shoot- and root-ammonium concentrations in MSX-treated *qko DR5:GFP* led to increased GFP signal intensity on local ammonium (**Figure 17**). Additional evidence that increasing root- or shoot concentrations of ammonium promote auxin accumulation in the root tissue is seen in the fact that elevated ammonium supply increased GFP fluorescence intensity in the root vasculature of *qko DR5:GFP*, which was very weak under low ammonium supply (**Figure 18C and D**). It has to be kept in mind that the difference in GPF fluorescence intensity between *wt DR5:GFP* and *qko DR5:GFP* could be a result of different insertion sites of the reporter construct in the genomic DNA, because both lines are originating from different transformation events and have not been generateted by crossing. The comparision between both lines would be more reliable if the two reporter lines would be originating from the same crossing population.

The strong auxin response of ammonium-treated roots of wild-type plants was comparable with the auxin accumulation in N-deficient roots (**Figure 15A and C**), whereas the nitrogen form nitrate induced a much weaker auxin accumulation in the root tissue (**Figure 15B**). The auxin accumulation in the N-deficient roots was even stronger than in ammonium-treated roots, since these high auxin levels in the root vasculature are brought about by enhanced auxin biosynthesis in the root. This *de novo* synthesis is catalysed by the IPyA-pathway involving the tryptophan amino transferases TAA1, TAR2 and 11 different YUCCAs, where YUC3, YUC5, YUC7, YUC8 and YUC9 function in root gravitropic responses and root development (Ma et al., 2014). However, ammonium-triggered auxin accumulation in the root vasculature appeared not to rely on *de novo* synthesis of auxin. Supporting evidence for this conclusion was achieved by using Kyn. L-Kynurenine (Kyn), which is a competitive substrate to tryptophane, inhibits auxin biosynthesis by TAA1/TAR-type enzymes in plants (He et al., 2011). In the presence of Kyn higher–order LR branching on local ammonium supply was not affected, whereas first-order LR elongation was strongly inhibited (**Figure 22**). Additionally, neither the mutant *tar2* showed deviant root morphology to the wild-type on local ammonium (**Figure 24A and B**), nor the reporter line *TAR2pro-TAR2:GFP* responded to local ammonium (**Figure 24C and D**).

These results indicated that ammonium-induced root branching depends mainly on the transport of shoot-derived auxin and not on auxin *de novo* synthesis in the root vasculature.

5.2.2 Local ammonium supply modulates root system architecture due to altered shoot-to-root auxin transport

Altered shoot or root concentrations of ammonium could trigger a signal leading to enhanced shoot-to-root auxin transport. Several lines of evidence suggested that rootward auxin transport plays a critical role in triggering LR branching under local ammonium availability. The auxin transport inhibitor NPA blocks active auxin transport by disrupting protein trafficking of auxin efflux carriers (Geldner et al., 2001; Muday and DeLong, 2001). When NPA was supplied to the base of ammonium-treated first-order LRs higher-order LR formation decreased drastically (**Figure 24B, D and E**). This implies that auxin efflux carriers may be the targets of local ammonium supply to generate an altered rootward auxin transport. In fact, it has been shown that shoot-derived auxin is especially important for stimulating LR emergence in Arabidopsis seedlings (Bhalerao et al., 2002) and therefore, an enhanced auxin transport from the shoot to the root was expected to occur in ammonium-treated plants. Auxin is transported in relatively short time over wide distances from shoots to root apices via the vascular system (Baker, 2000). Thereby, auxin can be translocated via the phloem or via polar transport from cell-to-cell via the xylem parenchyma (Rashotte et al., 2000; Swarup et al., 2001; Marchant et al., 2002) By comparing the localization of a reporter line of the *SUC2* promoter-, driving a plasma-membrane sucrose-H^+ symporter expressed in the phloem of the root vasculature, with the localization of the auxin reporter *DR5:GFP*, both lines showed a similar localization pattern in the root vasculature (**Figure 21C**), although DR5-dependent fluorescence was not exclusively located to the two phloem strands. This diffusive fluorescence may indicate a part of the auxin was transported via the xylem parenchyma.

Phenotypic screening of auxin-related mutants pointed out that among different auxin carriers PIN2 and PIN4 played key roles, because their corresponding mutant lines showed aberrant LR formation under local ammonium. Furthermore, their corresponding reporter lines indicated that PIN2 and PIN4 were regulated at the protein level by local ammonium (**Figure 26B**, **Figure 28** and **Figure 29**). In fact, ammonium regulated PIN2 and PIN4 in an opposite manner. Localized ammonium supply up-regulated *PIN4* expression and PIN4 abundance in second-order LRs (**Figure 27F** and **Figure 29C, D**), which may enhance the driving force to facilitate rootward auxin flow in roots (Friml et al.,

2002). Whether N-deficient roots were independent of polar auxin transport from the shoot to the root remains open. At least, the contribution of PIN4 should be low, because PIN4 reporter expression was weak under N deficiency (**Figure 27F** and **Figure 29C, D**). In agreement with this, the *pin4* mutant showed largely decreased higher-order LR branching under local ammonium treatment (**Figure 26A, B** and **Figure 29A, B**).

PIN2 plays a crucial role for auxin transport and distribution from the root meristem to the root elongation zone (Kleine-Vehn et al., 2008). The PIN2 reporter line showed weaker GFP fluorescence in second-order LRs on local ammonium supply (**Figure 28C, D**). The repression of the auxin flow from the root meristem to the root elongation zone could lead to an accumulation of auxin in the root meristem of second-order LRs, resulting in a back pressure of auxin, which may contribute to auxin accumulation in the root vasculature of ammonium-supplied roots. Additionally, it was shown by the *DR5:GFP* reporter in the background of the *pin2* mutant that the absence of PIN2 results in an accumulation of auxin in the tip of LR primordia, coinciding with enhanced LR emergence (Swarup et al., 2008). As expected, the mutant line showed enhanced higher-order LR branching on local ammonium supply (**Figure 26B** and **Figure 28A, B**), which supports the hypothesis that auxin accumulation in the root vasculature and/or in the LR primordia promotes LR emergence. It has been already suggested previously that the repression of PIN2 alters auxin distribution in root apices exposed to ammonium and that this causes a reduction in root elongation (Liu et al., 2013; Zou et al., 2013). PIN2 appears to be a general stress target as it is involved in the adaptation of roots to abiotic stress (Baluška et al., 2010; Liu et al., 2013). While ammonium uptake leads to an acidification of the root tissue, nitrate uptake has an opposite effect and causes alkalinization (Maynard and Barker, 1969; Haynes, 1986; Marschner, 1986: **Figure 32A and B**). Alkalinization increases shootward auxin transport and PIN2 abundance in the root apex (Xu et al. 2012), which coincides with the observations in nitrate-treated roots, where *proPIN2:PIN2:GFP* was strongly expressed (**Figure 28C**) and auxin concentrations in roots were low (**Figure 15B** and **Figure 19C**), most likely due to enhanced shootward auxin transport.

Interestingly, the other PINs seemed not to be involved in higher-order LR branching on local ammonium, since their expression was not affected by local ammonium supply and the related mutant lines showed no difference to the wild-type in higher-order LR branching (**Figure 26** and **Figure 27C, E, G**).

All these results indicate that the down-regulation of PIN2 and up-regulation of PIN4 synergistically generate a driving force to promote shoot-to-root transport of auxin to establish elevated auxin levels in the root vasculature that finally contribute to LR emergence under localized ammonium supply.

5.2.3 The lateral root emergence process is altered in higher-order lateral root branching on local ammonium due to enhanced radial auxin diffusion

Unexpectedly, N-deficient roots exhibited an even higher auxin accumulation in the root vasculature than ammonium-exposed roots (**Figure 15A, C** and **Figure 19C**). Nevertheless, LR elongation or branching was still repressed compared with that of local nitrate- or ammonium-treated roots (**Figure 4**). It appears that large amounts of auxin were trapped in the vasculature of N-deficient roots, prohibiting auxin to interfere with LR development (**Figure 15A, Figure 34A, D, E and H**). These observations imply that, beyond the absolute amount of auxin in the root vasculature, an additional mechanism facilitating radial auxin distribution, is required for directing auxin into outer root cells to promote LR emergence.

As first step in LR formation, a LR initial is formed during the initiation process, which involves auxin signalling in pericycle cells, the swelling of LR founder cell and endodermis degradation by cell wall-remodelling genes. The latter process allows the freshly formed LR initial to cross the endodermal cell layer. Then, the LR initial has to penetrate through the cortical cell layer for LR emergence. A combined approach of root morphological analysis and mathematical modelling has suggested that ammonium promotes LR emergence rather than initiation (Araya et al., 2016). Indeed, monitoring the development of LR primordia at different stages in wild-type and *qko* confirmed that local ammonium supply promotes LR emergence rather than LR initiation (**Figure 14**).

However, the emergence process requires auxin influx into the cortex, which depends on the auxin influx transporters AUX1 and LAX3 (Swarup et al., 2008). As shown by the high-resolution auxin reporters *DR5v2:n3GFP* and *DII-VENUS*, localized ammonium supply not just increased auxin accumulation in the root vasculature but also enhanced radial auxin distribution into outer cell layer in the LR branching zone (**Figure 34C, D, G and H**). This is not a typical PIN-mediated auxin movement (Swarup and Péret, 2012; Petrášek and Friml, 2009) and therefore most likely depends on other auxin transport mechanisms.

Active radial auxin transport relies mainly on auxin importers like AUX1 (Bennett et al., 1996) or LAX-type proteins, such as LAX1 and LAX3. However, Wang et al. (2013) supported a model in which ABCB1-mediated lateral auxin efflux at the plasma membrane of epidermis, cortex and endodermis cells, enhances auxin concentration in the root apoplast, which could be a prerequisite for LR emergence. In the present screening approach, several auxin transporters involved in rootward, shootward or radial auxin transport were tested. Surprisingly, except for PINs no other auxin transporters tested in this study showed significantly altered LR branching in their corresponding mutant lines

when grown on local ammonium (**Figure 26**). Furthermore, *LAX3* and *ABCB1* expression was not altered by localized ammonium treatment (Figure 27B and I). It thus seems that LAX3- and ABCB1-mediated radial auxin movement is not involved in the root morphological change in response to local ammonium supply, indicating the existence of a LAX3-independent mechanism that mediates radial auxin distribution and stimulates LR emergence. Actually, AUX1 has also been suggested to be involved in rootward auxin transport and LR primordia emergence (Swarup et al. 2001; Marchant et al. 2002; Laskowski et al. 2008), since its expression was observed in the vasculature of the parental roots (Laskowski et al. 2008). However, AUX1 expression was not altered by localized ammonium, as well (**Figure 27A**). Interestingly, *AUX1*, *LAX3* and *ABCB1* were all induced by local nitrate (**Figure 27A, B and I**), supporting the findings that LR emergence, in the presence of nitrate, relies on active radial auxin transport facilitated by those auxin transporters. Here, localized ammonium largely restored higher-order LR formation in the double mutant *aux1 lax3*, defective in LR emergence, but not in the double mutant *arf7 arf19* with impaired LR initiation (**Figure 35**). This further supports the stimulatory role of local ammonium in LR emergence due to altered radial auxin transport and not to altered auxin signalling.

Taking together, these findings provide a first hint that higher-order LR branching on local ammonium depends on a radial auxin transport mechanism, which is at least independent of active radial auxin transport facilitated by the auxin transporters tested in this study.

5.3 Acidification of the apoplast as consequence of ammonium uptake results in radial auxin diffusion promoting lateral root emergence

5.3.1 Ammonium uptake-facilitated auxin protonation leads to an auxin bypass replacing active radial auxin transport by passive auxin diffusion

Ammonium uptake is associated with a strong acidification of the rhizosphere, while exclusive nitrate nutrition causes rhizosphere alkalinization (Maynard and Barker, 1969; Haynes, 1986; Marschner, 1986: **Figure 31**). For the sake of a balanced rhizosphere pH that allows plants best to manipulate the pH according to their needs, most plants grow best on a mixture of nitrate and ammonium avoiding negative effects of ammonium on root growth (Goyal et al., 1982a, 1982b). As nitrate is co-transported with protons across the plasma membrane, nitrate uptake consumes protons in the rhizosphere that otherwise accumulate during ammonium uptake. The nitrate reduction in the cell produces hydroxide

ions that capture the protons produced by ammonium assimilation (Marschner, 1986; Van Beusichem et al., 1988). Interestingly, ammonium nutrition triggered higher-order LR branching even when the medium pH was increased (**Figure 30A and C**), leading to the conclusion that rhizosphere acidification by the root was dominant over the buffering capacity of the medium. Additionally, agar plates stained by bromocresol purple indicated clearly acidification of the rhizosphere of roots growing into the ammonium patch by color change from purple to yellow (**Figure 31**). Subsequent pH measurements confirmed a pH drop from 5.7 to 5.1 after 15 days on ammonium (**Figure 31B**). Nevertheless, first-order LR elongation was restored by a more alkalized medium pH and increased almost twofold between pH 4.5 and 7.0 (**Figure 30B**). These observations suggested that the medium or rhizosphere pH has a strong impact on LR elongation even in the presence of ammonium, whereas LR branching is primarily triggered by the apoplastic pH. One reason for this differential response may lie in the localized expression of AMTs, which determine the extent of ammonium uptake and subsequent apoplastic acidification. In particular localized expression of AMT1;3 in the presence of ammonium and in the LR branching zone of second-order LRs mediates apoplastic acidification at those sites where LR primorida have been formed and await emergence (**Figure 7E and F**). Ammonium affects the expression of a broad spectrum of genes involved in external stress responses (Patterson et al., 2010), and a large part of these genes respond primarily to the ammonium-dependent acidification of the root apoplast, because 20–41% of the ammonium-responsive genes are also upregulated by low external pH. Besides the impact of ammonium on the induction of different sensing and signalling pathways by induction or repression of certain genes, ammonium affects RSA also due to a physiological change in the root tissue. Several lines of evidence reveal that pH-dependent auxin diffusion may facilitate radial auxin distribution under local ammonium supply promoting higher-order LR emergence. The core effect is that a change of apoplastic pH can affect auxin diffusion, because membrane-permeability of IAA is modulated by pH value. Specifically, low pH promotes the protonation of IAA leading to increased auxin diffusion, while high pH stimulates the dissociation of IAAH that makes auxin transport dependent on membrane transporters (Goldsmith, 1977; Swarup and Péret, 2012). If the apoplastic pH drops from 5.9 in the case of nitrate-supplied roots to 5.1 after 15 days on ammonium (**Figure 31B**), the amount of diffusible IAAH increases from less than 7% to more than 30%, enhancing the probability of radial auxin diffusion dramatically (**Figure 34C, D, G and H**; Swarup and Perét, 2012). There are other examples indicating how auxin diffusion and related developmental processes are influenced by the change of apoplastic pH (Steinacher et al., 2012; Swarup and Péret, 2012). For instance, overexpression of the H^+-pyrophosphatase AVP1 increased protein abundance and activity of P-type ATPases,

leading to a decrease of apoplastic pH, which finally enhanced auxin diffusion into the cytosol and promoted auxin-related developmental processes (Li et al., 2005). Similarly, the CATION EXCHANGERs CAX1 and CAX3 may also affect auxin diffusion by changing apoplastic pH. In *cax1, cax3* single or double mutants, auxin-related guard cell movement is blocked by elevated apoplastic pH due to repressed auxin diffusion. Interestingly, low pH treatment can fully rescue the auxin-deficient phenotype in these mutants, emphasizing the influence of apoplastic pH on auxin diffusion in plant cells (Cho et al., 2012). Like shown in this study, the distribution of auxin in the outer layer cells of LR branching zone, as shown by *DR5v2:n3GFP* and *DII-VENUS*, highly coincided with apoplastic pH (**Figure 32** and **Figure 34**), directly revealing that local ammonium promotes radial auxin movement into outer cell layers, whereas under N deficiency auxin remained trapped in the root vasculature.

Strikingly, the different sensitivity of wild-type and *qko* roots to external IAA supplementation confirms the stimulatory effect of local ammonium on auxin diffusion. When external IAA was added to wild-type plants, the stimulation on LR branching occurred only in response to ammonium. By contrast, in *qko* ammonium-dependent stimulation of LR branching by external IAA was almost absent (**Figure 33A, B**), indicating that ammonium stimulates auxin diffusion in an AMT-dependent manner. The IAAH analog NAA stimulated higher-order LR branching irrespective of the supplied nitrogen form and independent of a functional high-affinity ammonium transporter (**Figure 33C, D**), supporting that IAA protonation is a prerequisite for enhanced LR emergence.

In support of this hypothesis trapped auxin in the vasculature of N-deficient roots can be activated by local acidification. In N-deficient plants, TAR2-mediated local auxin biosynthesis generates large amounts of auxin in the root vasculature (**Figure 15A** and **Figure 19C**; Ma et al., 2014), but this auxin remains trapped in the stele as long as the apoplastic pH is alkaline (**Figure 15A**, **Figure 32A, B** and **Figure 34A, D, E, H**). Intrestingly, low pH treatment significantly recovered higher-order LR formation in N-deficient roots (Figure 36), indicating that pH decrease increases radial auxin diffusion for subsequent LR development even in N-deficient plants. It seems that local ammonium evokes a LAX3-independent radial auxin diffusion process to stimulate LR emergence. Due to a defective radial auxin transport process in the *aux1 lax3* double mutant, LR emergence is largely impaired, showing a LR-less phenotype (Swarup et al., 2008). However, visible LR formation in *aux1 lax3* double mutant was markedly restored by local ammonium supply (**Figure 35**). Meanwhile, AMT1;3, the key ammonium transporter for triggering LR branching (Lima et al., 2010), is also expressed in the cortical and epidermal cells of the LR branching zone (Loqué et al., 2006; Yuan et al., 2007a), overlapping with the localization of LAX3 in the outer layer root cells (Swarup et al., 2008). These results

suggest that AMT-dependent apoplastic acidification under local ammonium supply facilitates an alternative radial auxin movement that partially replaces the role of LAX3 in LR emergence.

Furthermore, external abiotic stimuli like ammonium lead to the production of reactive oxygen species (ROS) (Patterson et al., 2010; Mittler et al., 2011), which occurs downstream of auxin-mediated signal transduction pathways (Correa-Aragunde et al., 2013; Ivanchenko et al., 2013; Joo et al., 2001; Ma et al., 2014). ROS are involved in several signalling cascades during plant development (D'Haeze et al., 2003; Ishibashi et al., 2013; Joo et al., 2001; Mori et al., 2001: Ros Barcelo, 2005; Joo et al., 2001; Passaia et al., 2013) and act during LR formation as well (Correa-Aragunde et al., 2013; Li and Jia, 2013; Manzano et al., 2014). The action of ROS in the process of LR formation is strongly coupled to auxin responses (Correa-Aragunde et al., 2013; Ma et al., 2014). Ammonium is known to affect genes involved in the production and scavenging of reactive oxygen species (ROS) (Patterson et al., 2010; Bloch et al., 2011; Xia et al., 2015). In roots or leaves, ammonium supply increases the levels of H_2O_2, as shown in tomato (Fernández-Crespo et al., 2015), Arabidopsis (Patterson et al., 2010), and rice (Xia et al., 2015). The production of ROS in the root apoplast depends on several classes of enzymes, including respiratory burst oxidase homologs (RBOH) and class III peroxidases (Sagi and Fluhr, 2006; Shapiguzov et al., 2012). Those enzymes act in the apoplast of epidermal and cortical cells during LR emergence. Especially, the induction of several *RBOH* genes and ROS-related genes are associated with increased LR emergence due to the activation of cell wall-remodeling enzymes. Surprisingly, ROS treatment can overcome impaired LR emergence in the *aux1 lax3 double* mutant (Orman-Ligeza et al., 2016) like locally supplied ammonium (**Figure 35C-G**).

All these findings suggest that ammonium leads to an acidification of the apoplast, which is causing auxin protonation-dependent radial auxin diffusion. As consequence, auxin reaches the LR primordia underlying rhizodermal and cortical cells independent of secondary active auxin transport by LAX3 or AUX1. Finally, cell wall-remodeling genes are induced by auxin-dependent ROS signaling, which results in LR primordia emergence.

5.4 Conclusion

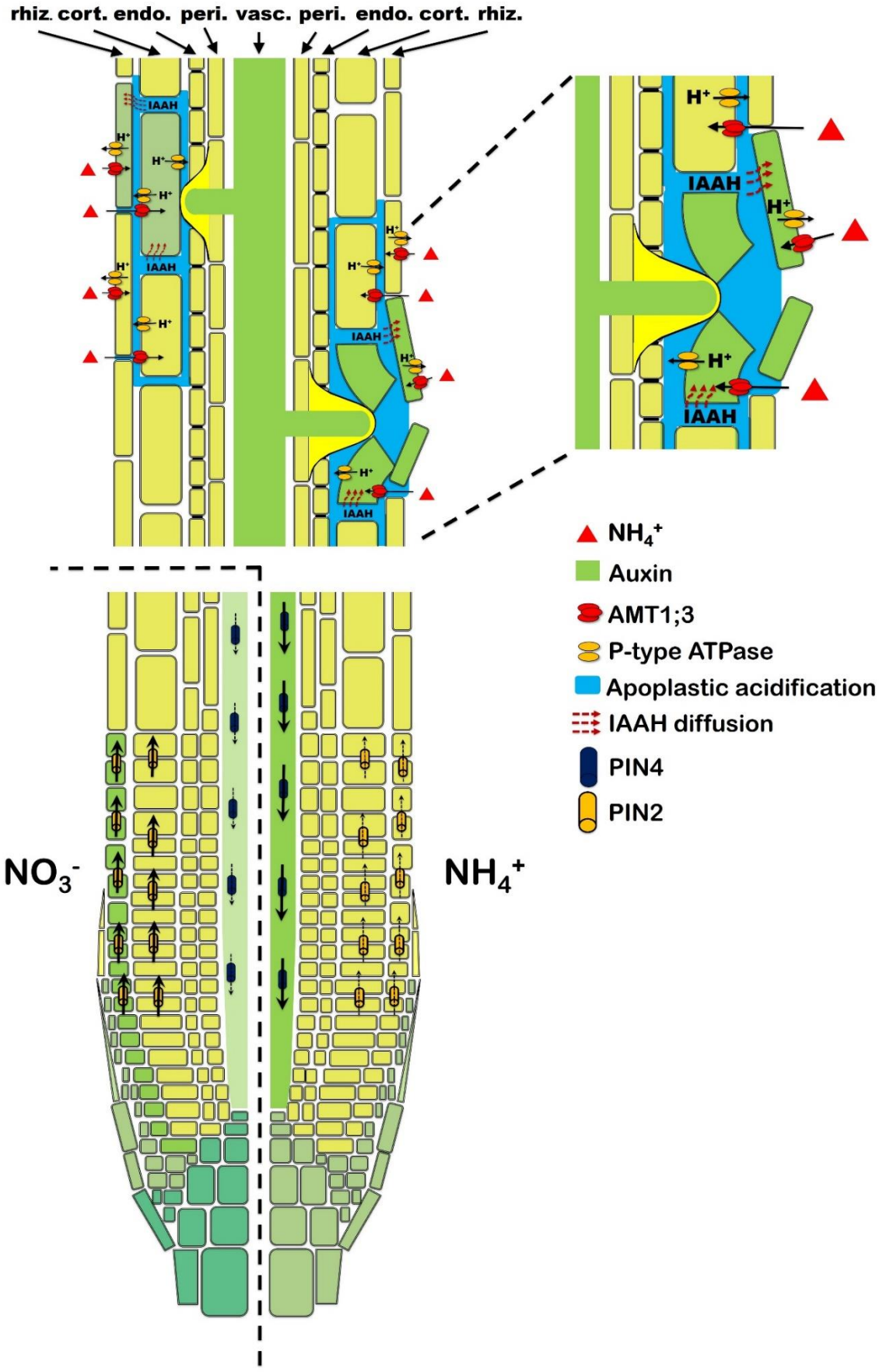

Figure 37. Higher-order LR formation in response to medium pH.
The model summarizes the situation in ammonium treated roots if supplied locally. When ammonium is taken up by AMT-type ammonium transporters in the lateral root branching zone, proton secretion maintains the anion-cation balance within the root cells. The acidification of the apoplast enhances IAA protonation, which enhances passive IAAH diffusion. Hence, IAAH reaches the lateral root (LR) primodium overlaying endodermis and cortex cells, it causes cell wall loosening and activation of cell wall-remodelling genes. As a consequence, LR emergence is strongly promoted within an ammonim patch extending the surface area of the root system near the N source. Furthermore, local ammonium or nitrate alter the by PIN2 and PIN4 facilitated polar auxin transport in an opposite manner. Local ammonium is repressiing PIN2 by at the same time inducing PIN4, which leads to an accumulation of auxin in the root vasculature. Local nitrate induces PIN2 and facilitates thereby an enhanced shootward auxin transport to the root elongation zone, which deplets the auxin concentration in the root vasculature.
rhiz.: rhizodermis, cort.: cortex, endo.: endodermis, peri.: pericycle, vasc.: vasculature

Based on the current study, a model is proposed that explains how local ammonium triggers LR branching via modulating PIN-mediated polar auxin transport and pH-dependent radial auxin diffusion (**Figure 37**). Localized ammonium supply represses PIN2 expression and up-regulates PIN4 expression in LR tips, which promotes shoot-to-root auxin transport and leads to auxin accumulation in the vasculature of the LR branching zone. This process generates an auxin source for subsequent auxin diffusion. Meanwhile, proton release as a consequence of AMT1;3-mediated ammonium uptake leads to apoplastic acidification, auxin protonation and enhanced radial auxin diffusion, directing auxin from the vasculature to outer cell layers. The influence of local ammonium on auxin distribution synergistically stimulates LR emergence, shaping a highly branching root system.

With this model, the present study lays a new basis to understand how localized N availability modulates auxin distribution in plants and subsequently reprograms LR development to alter root system architecture in a changing environment.

6 References

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7 List of Figures and Tables

7.1 Figures

7.2 Tables

8 Abbreviations

%	percent
°C	degree Celsius
CLSM	confocal laser scanning microscope
CoA	coenzyme A
µ	micro
µg	microgram
µl	microliter
µM	micromolar
ABA	abscisic acid
AMT	ammonium transporter
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
cm	centimeter
Col-0	Columbia-0, ecotype of Arabidopsis thaliana
DNA	deoxyribonucleic acid
dpi	dots per inch
DW	dry weight
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FLD	fluorescence detector
DW	dry weight
g	gram
GC	gas chromatography
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
Gln	glutamine
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour
HATS	high-affinity transport system
HN	high nirtogen
HPLC	high performance liquid chromatography
i.e.	id est (from Latin: that is)
IAA	indole-3-acetic acid
IAA-ala	indole-3-acetyl-L-alanin
IAA-Me	indole-3-acetic acid methyl ester
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
LATS	low-affinity transport system
LN	low nitrogen
LR	lateral root
ln	natural logarithm
m	milli
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MS	mass spectrometry
MSX	methionine sulfoximine
N	nitrogen
n	nano
n	number of biological replicates
N	nitrogen

n.d.	not detected
NH_4^+	ammonium
nm	nanometer
NO_3^-	nitrate
NRT	nitrate transporter
ns	not significant
OxIAA	2-oxoindole-3-acetic acid
p	pico
P	p-value (‘probability’)
PCR	polymerase chain reaction
PR	primary root
pH	‘power of hydrogen’, measure of the acidity or basicity of an aqueous solution
qko	ammonium transporter quadruple knock-out line
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
SAIL	Syngenta *Arabidopsis* Insertion Library
SALK	Salk Institute for biological studies
SD	standard deviation
SE	standard error
sec	second
T-DNA	transfer-DNA
t-test	Student’s t distribution test
UPLC	ultra performance liquid chromatography
v/v	volume-to-volume ratio
WT	wild type
x *g*	gravitation force

9 Curriculum Vitae

Name:	Markus Meier
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Education

09/2012 - 09/2018	PhD study on “Ammonium-dependent changes in apoplastic pH control lateral root emergence via radial auxin diffusion**”** at the Molecular Plant Nutrition Group, Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), in Gaterleben, Germany
10/2006 - 01/2012	Studies in Plantbiotechnology at the University of Hannover (LUH), Germany (Master of Science) Main focus: plant nutrition, molecular biology, protein biochemistry *Master thesis:* Influence of an elevated manganese supply on the phenyl-propanoid metabolism and its interaction with apoplastic H_2O_2-consuming peroxidises in leafs of *Vigna unguiculata* *Bachelor thesis:* Isolation and characterization of H_2O_2-consuming peroxidises in the leaf apoplast of *Vigna unguiculata*
09/1999 - 06/2006	Secondary school (Albert Einstein Schule Laatzen, Abitur)

Work experience

since 09/2012	Research Scientist at the Molecular Plant Nutrition Group, Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), in Gaterleben, Germany
06/2011 - 07/2012	Research Assistant at the university of Hannover (LUH)

Presentations in scientific conferences during the PhD study

"Role of auxin in AMT1:3-dependent lateral root branching"
(Poster presentation)
Nitrogen2016, August 22nd – 26th 2016, Montpellier, France

"The role of auxin in ammonium-dependent higher-order lateral root branching"
(Oral presentation)
12th Plant Science Student Conference, June 2nd - 4th 2016, Gatersleben, Germany

"The role of auxin in AMT1:3-dependent lateral root branching"
(Oral presentation)
11th Plant Science Student Conference, June 2nd - 5th 2015, Halle, Germany

"Role of auxin in AMT1:3-dependent lateral root branching in Arabidopsis"
(Poster presentation)
Plant Nutrition 2014, September 10th - 12th 2014, Halle, Germany

"Role of auxin in AMT1:3-dependent lateral root branching in Arabidopsis"
(Oral presentation)
First Doctoral Ressearcher's Conference of GRK 1798, September 1st – 2nd 2014, Bad Bevensen, Germany

"The role of auxin in ammonium-dependent higher-order lateral root branching"
(Poster presentation)
10th Plant Science Student Conference, June 2nd - 4th 2014, Gatersleben, Germany

"Role of auxin in AMT1:3-dependent lateral root branching in Arabidopsis"
(Poster presentation)
Deutsche Botanikertagung, September 30th – Oktober 4th 2013, Tübingen.

"The role of AMT1:3 in higher-order lateral root branching"
(Poster presentation)
9th Plant Science Student Conference, May 28th - 31st 2013, Halle, Germany

Supervision activity

Supervision of an undergraduate student from the Anhalt University for applied sciences in Köthen for research in molecular plant nutrition on “Impact of H^+-ATPase activity on lateral root branching on local ammonium supply” at the IPK (11.02.2019 - 22.03.2019)

Supervision of different undergraduated students at the IPK for research in molecular plant nutrition with the focus on ammonium sensing and signaling(2014 and 2015).

Markus Meier

Halle (Saale), July 24th, 2019

10 Affirmation

I hereby declare that the submitted work has been completed by me, and I have not used any other than permitted reference sources or materials. All the references and the other sources used in the presented work have been appropriately acknowledged in this work. I further declare that the work has not been previously submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die den benutzten Hilfsmitteln wörtlich oder inhaltlich entnommenen Stellen habe ich unter Quellenangaben kenntlich gemacht. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Institution oder Prüfungsbehörde vorgelegt.

Declaration concerning Criminal Record and Pending Investigations

Hiermit erkläre ich, dass ich weder vorbestraft bin, noch dass gegen mich Ermittlungsverfahren anhängig sind. / *I hereby declare that I have no criminal record and that no preliminary investigations are pending against me.*

Markus Meier

Halle (Saale), July 24th, 2019

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