Contribution of Ammonium Transporters to Growth, Nitrate Uptake, Metabolism and Development in *Arabidopsis thaliana*

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1 Summary / Zusammenfassung

1.1 Summary

Nitrogen (N) is the most abundant mineral nutrient in plant tissues and therefore, often a limiting factor for crop production. N is primarily taken up in the form of ammonium (NH_4^+) and nitrate (NO_3^-). High-affinity NH_4^+ uptake is mediated by AMT-type transporters, while NO_3^- is transported by proteins of the NRT1 and NRT2 family.

The observation that the NH_4^+ uptake-defective *AMT*-quadruple knock-out line *qko* develops larger shoots than the wild-type under certain growth conditions served as the starting point for the present study. This observation led to the question why the absence of AMT-type transporters promotes growth when NO_3^- is the predominant N source. Therefore, the aim of this study was i) to verify the growth phenotype of *qko* under NO_3^- supply and its dependence on the growth conditions; ii) to investigate the impact of the lacking expression of *AMTs* on NO_3^- uptake and iii) on primary metabolism and phytohormone homeostasis; and iv) to describe the long-term consequences of lacking *AMT* gene expression in plant development.

The present study showed that the *qko* mutant develops larger shoots than the wild-type under non-limiting NO_3^- or NH_4NO_3 availability. Under these conditions, *qko* produced larger shoots with less leaves that had a larger area. When plants were grown under N-sufficient conditions, the absence of AMTs in the *qko* mutant increased the capacity for high-affinity NO_3^- uptake by derepression of NRT2.1 at the transcript and protein level. Moreover, transcript levels of *NRT1.1* were also repressed in roots of *qko* plants. The NO_3^- transceptor NRT1.1 is known to mediate the induction of *NRT2.1* after short-term exposure to NO_3^- and its repression by high N provision. Therefore, AMT-mediated NH_4^+ transport is proposed to be involved in NRT1.1-dependent repression of *NRT2.1* by high N provision. Among the *AMTs* disrupted in the *qko* mutant AMT1;1 showed the largest contribution to modulation of high-affinity NO_3^- uptake. Modulation of the NO_3^- uptake capacity by AMT-mediated NH_4^+ transport may represent a mechanism to ensure equilibrium of the electrochemical gradient across the plasma membrane of root cells involved in ion uptake from the soil solution. Disruption of *AMTs* also affected primary metabolism.

The roots of *qko* plants exhibited decreased amino acid concentrations and elevated sucrose levels, which may have been responsible for the derepression of *NRT2.1* and high-affinity NO_3^- uptake. In addition, *qko* plants exhibited a shorter life cycle with an earlier flowering time and decreased leaf number. *NRT2.1* is developmentally regulated and was higher expressed in *qko* roots in the pre-flowering phase. Interestingly, NH_4^+ supplied as the sole N source suppressed floral induction and the expression of the flowering time genes *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *APETALA1* (*AP1*) in wild-type plants. As the expression of the flowering time genes was also not considerably affected by NH_4^+ supply in *qko*, *qko* plants initiated flowering irrespective of being supplied with NH_4^+ or NO_3^- as the sole N source. This indicated that NH_4^+ nutrition delays flowering time by affecting the photoperiod pathway in an AMT-dependent manner. The involvement of CO in N-dependent flowering control is consistent with recent findings proposing that the photoperiod pathway is a major target in N-signaling to modulate flowering time in dependence of the N availability. Taken together, the investigation of the AMT quadruple knock-out line *qko* allowed uncovering novel roles of AMT transporters that go beyond their function in NH_4^+ uptake.

1.2 Zusammenfassung

Stickstoff (N) ist der quantitativ bedeutendste mineralische Nährstoff in pflanzlichen Geweben und deshalb oft limitierender Faktor in der Pflanzenproduktion. N wird hauptsächlich in Form von Ammonium (NH_4^+) und Nitrat (NO_3^-) aufgenommen. Die hochaffine NH_4^+-Aufnahme wird durch Transporter des AMT-Typs vermittelt, während NO_3^- von Proteinen der NRT1- und NRT2-Familie transportiert wird.

Die Beobachtung, dass die NH_4^+-aufnahmedefekte *AMT*-vierfach Insertionslinie *qko* unter bestimmten Ernährungsbedingungen größere Sprosse als der Wildtyp entwickelt, diente als Startpunkt dieser Arbeit. Diese Beobachtung führte zur Frage, warum die Abwesenheit von Transportern des AMT-Typs das Wachstum begünstigt, wenn NO_3^- die hauptsächliche N-Quelle ist. Deshalb waren die Ziele dieser Arbeit, i) das Wachstum von *qko* unter NO_3^--Versorgung und dessen Abhängigkeit von der Kulturbedingung zu charakterisieren; ii) die Auswirkung fehlender *AMT*-Expression auf die NO_3^--Aufnahme und iii) den Stoffwechsel zu untersuchen; und iv) die Langzeitfolgen fehlender *AMT*-Expression auf die Pflanzenentwicklung zu beschreiben.

Die vorliegende Studie konnte zeigen, dass die *qko*-Mutante größere Sprosse entwickelt als der Wildtyp unter nicht-limitierender NO_3^-- oder NH_4NO_3-Verfügbarkeit. Unter diesen Bedingungen bildete *qko* größere Sprosse mit weniger Blättern, die eine größere Blattfläche aufwiesen. Die Abwesenheit von AMTs in der *qko*-Mutante erhöhte die Kapazität für die hochaffine NO_3^--Aufnahme durch Derepression von NRT2.1 auf der Transkript- und Proteinebene, wenn die Pflanzen unter repressiven N-hinreichenden Bedingungen angezogen worden waren. Unter dieser Bedingung waren die *NRT1.1* mRNA-Gehalte in Wurzeln von *qko*-Pflanzen zusätzlich reprimiert. Es ist bekannt, dass der NO_3^--Transzeptor NRT1.1 die Induktion von *NRT2.1* nach kurzzeitiger NO_3^--Exposition und dessen Repression durch hohe N-Gaben vermittelt. Es wird daher angenommen, dass AMT-vermittelter NH_4^+-Transport an der NRT1.1-abhängigen Repression von *NRT2.1* durch hohe N-Gaben beteiligt ist. Unter den *AMTs*, die in *qko* deletiert sind, zeigte AMT1;1 den größten Beitrag zur Modulierung der hochaffinen NO_3^--Aufnahme. Die Modulierung der NO_3^--Aufnahmekapazität durch AMT-vermittelten NH_4^+-Transport könnte einen Mechanismus darstellen, der die Stabilisierung des Kationen-Anionen-Verhältnisses bei der Aufnahme und des

elektrochemischen Gradienten an der Plasmamembran von Wurzelzellen, die an der Ionenaufnahme beteiligt sind, unterstützt. Insertionen in *AMTs* beeinträchtigten auch den Primärmetabolismus. Die Wurzeln von *qko*-Pflanzen wiesen verringerte Aminosäure- und erhöhte Saccharose-Konzentrationen auf. In Übereinstimmung mit früheren Studien, reprimieren reduzierte N-Metabolite die Expression von *NRT2.1* und die hochaffine NO_3^--Aufnahme, während Zucker diese induzieren. *qko*-Pflanzen wiesen zusätzlich einen kürzeren Lebenszyklus mit einem früheren Blühzeitpunkt und einer geringeren Blattanzahl auf. *NRT2.1* wird entwicklungsabhängig reguliert und war höher in *qko*-Wurzeln in der Phase vor der Blüte exprimiert. Interessanterweise hemmte die Zufuhr von NH_4^+ als einzige N-Quelle die Induktion der Blüte und die Expression der Blühzeitpunkt-Gene *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *APETALA1* (*AP1*) in Wildtyppflanzen. Wohingegen *qko*-Pflanzen, unabhängig davon ob sie mit NH_4^+ oder NO_3^- als einzige N-Quelle ernährt worden waren, zu blühen begannen. Die Expression der Blühzeitpunkt-Gene in der *qko*-Mutante war durch NH_4^+-Ernährung kaum beeinträchtigt. Dies deutet darauf hin, dass NH_4^+-Ernährung den Blühzeitpunkt durch Beeinflussung des Photoperiodenwegs in AMT-abhängiger Weise verzögert. Die Beteiligung von CO in der N-abhängigen Steuerung der Blüte deckt sich mit neueren Studien, die vorschlagen, dass der Photoperiodenweg ein Hauptziel für die N-Signaltransduktion sei, um den Blühzeitpunkt in Abhängigkeit der N-Verfügbarkeit zu modulieren. Zusammenfassend ermöglichte die Untersuchung der AMT-vierfach Insertionslinie *qko* die Entdeckung neuer Funktionen von AMT-Transportern, die über ihre Aufgabe in der NH_4^+-Aufnahme hinausgehen.

2 Introduction

2.1 Importance of N nutrition

Nitrogen (N) represents about 2 to 5% of the total dry matter of plants and is therefore quantitatively the most important essential mineral element for plants (Marschner, 2012). A central function of N is to provide amino groups for the synthesis of amino acids as well as of purine and pyrimidine bases, which are constituents of nucleotides. Besides being the building blocks for nucleic acids, nucleotides are involved in energy homeostasis, signaling and protein regulation. Another essential function of N is to be part of tetrapyrroles e.g. in chlorophylls and heme-groups. Heterocyclic N is present in compounds like auxins and cytokinins. N can also be found in many secondary metabolites like alkaloids and cyanogenic glycosides (Buchanan et al., 2000; Maathuis, 2009).

Because of the high plant demand, N is a major factor limiting crop yield in agricultural plant production. However, just a small portion of 0.00024% of planetary N is available for plants. Indeed, from the total of N that is not available to plants, approx. 2% is in the form of N_2 and 98% is immobilized in the geosphere (Miller and Cramer, 2004). As a result of its restricted availability worldwide, the annual N fertilizer consumption has been rising continuously. At the beginning of this millennium the annual demand for N in agricultural plant production was about 80 million metric tons and this number is predicted to increase above 192 million metric tons in 2070, when the human population is expected to reach 10 billion people (Frink et al., 1999). Problems resulting from the application of nitrogenous fertilizers are among others the leaching of nitrate (NO_3^-) and volatilization of ammonia (NH_3) and nitrous oxide, which lead to the eutrophication of surface and ground water, and to an enrichment of reactive N in the atmosphere, contributing to global warming. In addition to the enormous environmental costs, the use of N fertilizers becomes more expensive with rising energy costs. Improved uptake of N from the soil and more efficient utilization by crop plants could allow for a reduction of N fertilizer applications with environmental and economic advantages (Miller and Cramer, 2004).

In the biosphere plants are exposed to different forms of N, which include molecular N_2, volatile NH_3 or nitrogen oxides (NO_x), mineral nitrogen (ammonium (NH_4^+), NO_3^-) and organic N (amino acids, peptides, urea, etc.) (von Wirén et al., 1997). The majority of the N in soils is present in the form of complex organic molecules, which can be converted to NH_4^+ by soil microorganisms through mineralization. NH_4^+ may then be oxidized by *Nitrosomonas spp.* to nitrite (NO_2^-) and further by *Nitrobacter spp* to NO_3^- through a process known as nitrification (Miller and Cramer, 2004). NH_4^+ and NO_3^- are the predominant sources of N available to plants in most soils (von Wirén et al., 2000). In well-aerated agricultural soils, NH_4^+ concentrations usually do not exceed 50 µM and those of NO_3^- are typically 10-1000 times higher (Marschner, 2012). However, this difference in soil concentration does not reflect the uptake ratio of both N forms. In fact, in most plant species the uptake of NH_4^+ is preferred over NO_3^- (Xu et al., 1992; Gazzarrini et al., 1999). NH_4^+ requires less energy for uptake and assimilation than NO_3^-, mainly because NO_3^- has to be reduced prior to assimilation (Bloom et al., 1992). Furthermore, low pH, low temperature and poor oxygen supply (for example in waterlogged soils) inhibit many nitrifying microorganisms, resulting in higher rates of net mineralization than net nitrification. Under such conditions, NH_4^+ can significantly accumulate in the soil, with concentrations averaging 2 mM in some forest soils and up to 20 mM in some agricultural soils (Britto and Kronzucker, 2002). Such high NH_4^+ concentrations can cause toxicity symptoms, when plants are cultured on NH_4^+ as the exclusive nitrogen source, and result in severe yield depression, growth suppression or even death (Britto and Kronzucker, 2002). In order to avoid NH_4^+ toxicity, but still ensure proper growth, the uptake of NH_4^+ and NO_3^- must be tightly regulated. For this purpose, plants evolved sophisticated molecular, physiological and morphological mechanisms.

2.2 Uptake of inorganic N and regulation of involved transporters

2.2.1 NH_4^+ uptake and regulation of AMTs

NH_4^+ uptake into plant roots exhibits biphasic kinetics that can be divided in a high-affinity transport system (HATS) and a low-affinity transport system (LATS). The HATS for NH_4^+ shows Michalis-Menten kinetics, with influx saturating at external NH_4^+ concentrations below 1 mM. The LATS, in turn, plays a role at higher external NH_4^+ concentrations (above 1 mM), where uptake rates increase linearly with increasing NH_4^+ supply (Ullrich et al., 1984; Wang et al., 1993; Kronzucker et al., 1996; Rawat et al., 1999). In contrast to the HATS for NH_4^+, not very much is known about the molecular mechanisms underlying the LATS.

The first step in uncovering the molecular mechanism underlying high-affinity NH_4^+ uptake into Arabidopsis roots was the identification of AMT1;1 by screening a yeast mutant defective in high-affinity NH_4^+ uptake and transformed with a cDNA library derived from Arabidopsis seedlings on NH_4^+ as a sole nitrogen source (Ninnemann et al., 1994). Later on, three additional NH_4^+ transporter (*AMT*) genes, *AMT1;2*, *AMT1;3* and *AMT2;1*, were isolated and shown to functionally complement an NH_4^+ uptake-defective yeast mutant (Gazzarrini et al., 1999; Sohlenkamp et al., 2000). Besides being expressed in roots, *AMT1;1* and *AMT2;1* were shown to be also expressed in the shoot (Gazzarrini et al., 1999; Sohlenkamp et al., 2000). Additionally, high-affinity ^{14}C-methylammonium uptake in *AMT1;1*, *AMT1;2* or *AMT1;3*-expressing yeast cells provided functional evidence for the contribution of these transporters in NH_4^+ uptake from the growth medium.

Direct evidence for the contribution of AMTs to high-affinity NH_4^+ uptake was obtained by using single and multiple T-DNA knock-out lines (Kaiser et al., 2002; Loqué et al., 2006; Yuan et al., 2007a). The loss of *AMT1;1* expression in the *amt1;1-1* T-DNA insertion line resulted in a 30% decrease in the high-affinity NH_4^+ uptake by N-deficient roots. Disrupting *AMT1;3* expression in the T-DNA insertion line *amt1;3-1* also reduced high-affinity NH_4^+ influx by approximately 30% (Loqué et al., 2006). An additive contribution of AMT1;1 and AMT1;3 to the overall NH_4^+ uptake capacity in Arabidopsis roots under N deficiency was supported by the fact that the double insertion line *amt1;1-1 amt1;3-1* lost up to 70% of the high-affinity NH_4^+ influx relative

to wildtype plants. Additionally, the plasma membrane localization of AMT1;1 and AMT1;3 and their preferential expression in rhizodermal cells, including root hairs, further supported the notion that both proteins are involved in primary NH_4^+ uptake from the soil solution.

A deeper understanding of the physiological contribution of root-expressed AMTs to high-affinity NH_4^+ uptake and how they coordinate NH_4^+ transport was obtained by Yuan et al. (2007a). When quadruple knock-out line (*qko*) was generated with lacking expression of *AMT1;1*, *AMT1;2*, *AMT1;3*, and *AMT2;1*, it retained only 5 to 10% of the wildtype high-affinity NH_4^+ uptake capacity and showed severe growth depression under NH_4^+ supply. Transcriptional upregulation of *AMT1;5* in N-deficient rhizodermal cells including root hairs and the ability of AMT1;5 to transport NH_4^+ in yeast suggested that AMT1;5 accounts for the remaining 5 - 10% of uptake capacity in *qko*. Then, triple insertion lines expressing either *AMT1;1* (*qko11*), *AMT1;2* (*qko12*), *AMT1;3* (*qko13*), or *AMT2;1* (*qko21*) in the *qko* background were obtained by backcrossing to the wildtype. Influx of ^{15}N-labeled NH_4^+ into roots of *qko*, and triple insertion lines revealed the *in planta* NH_4^+ substrate affinities of 50, 234, 61, and 4.5 µM for AMT1;1, AMT1;2, AMT1;3, and AMT1;5, respectively, but no NH_4^+ influx activity for AMT2;1. Furthermore it was shown that AMT1;2 is expressed at the plasma membrane of the endodermis as well as of cortical root cells while *AMT1;5* promoter activity was found in rhizodermal and root hair cells (Yuan et al., 2007a). Taken altogether, it was proposed that AMT1;1, AMT1;3 and AMT1;5 are responsible for the primary NH_4^+ uptake from the soil solution while AMT1;2 engages in the uptake and retrieval of apoplastic NH_4^+ in inner root cells. These physiological functions of AMT1-type transporters are summarized in Figure 1 taken from Yuan et al. (2007a).

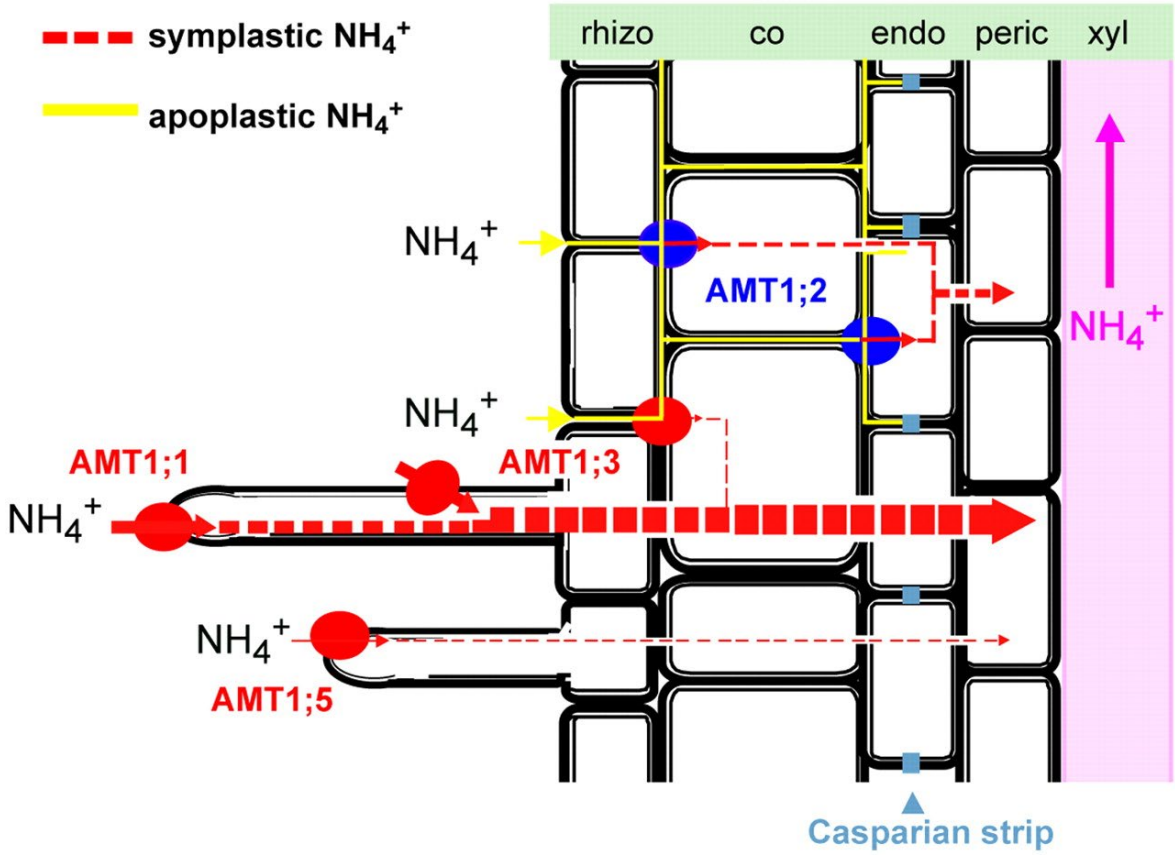


Figure 1. Model summarizing the functions of AMT1-type transporters in high-affinity NH_4^+ uptake in Arabidopsis roots from Yuan et al. (2007a).

Schematic representation of the contribution to NH_4^+ uptake and spatial expression in root tissues of AMT1;1, AMT1;3, AMT1;5 (all in red), and AMT1;2 (blue) under nitrogen deficiency. AMT-dependent NH_4^+ influx is proportionally represented by the size of their arrows. NH_4^+ can enter the symplastic route (dashed red line) for radial transport toward the root stele via AMT1;1, AMT1;3, and AMT1;5, which are localized at the plasma membrane of rhizodermis cells, including root hairs. NH_4^+ can also bypass outer root cells via the apoplastic transport route (yellow line) and subsequently enter the root symplast by AMT1;2-mediated transport across the plasma membrane of endodermal (in the root hair zone) and cortical (in more basal root zones) cells. In the symplast, NH_4^+ can either be assimilated into amino acids or loaded into the xylem by an as yet unidentified transport process. rhizo, rhizodermis; co, cortex; endo, endodermis; peric, pericycle; xyl, xylem. **(Yuan et al., 2007a)**

The localization of *AMT2;1* in the stele (Sohlenkamp et al., 2002; Laginha, 2010), reduced accumulation of ^{15}N in shoots after the supply of ^{15}N-labeled NH_4^+ and lower NH_4^+ concentrations in the xylem sap of *amt2;1* insertion mutants suggested that AMT2;1 might be involved in translocation of NH_4^+ from the root to the shoot under excessive NH_4^+ supply (Laginha, 2010).

Transcriptional Regulation of NH_4^+ transporters

NH_4^+ uptake into Arabidopsis roots increased continuously during the light period and dropped strongly after dusk (Gazzarrini et al., 1999). The mRNA levels of *AMT1;1*, *AMT1;2* and in particular of *AMT1;3* correlated with the diurnal changes in NH_4^+ uptake capacity. These diurnal changes were suggested to be regulated by photoassimilates, since the decline in *AMT1;1*, *AMT1;2* and *AMT1;3* transcript levels and in high-affinity NH_4^+ uptake in the dark period could be prevented by sucrose supply (Lejay et al., 2003). All five root expressed AMTs, *AMT1;1, AMT1;2, AMT1;3, AMT1;5* and *AMT2;1* are transcriptionally upregulated under N deficiency (Gazzarrini et al., 1999; Rawat et al., 1999; Sohlenkamp et al., 2000; Kaiser et al., 2002; Sohlenkamp et al., 2002; Loqué et al., 2006; Yuan et al., 2007a; Laginha, 2010). Resupplying NH_4NO_3 to N-starved plants decreased NH_4^+ influx and *AMT1;1* mRNA levels rapidly, but remained high when NH_4^+ assimilation was blocked with methionine sulfoximine (Rawat et al., 1999). Additionally, provision of glutamine repressed *AMT1;1* transcript levels, suggesting that the root concentration of NH_4^+ assimilation products rather than NH_4^+ was responsible for transcriptional down-regulation of *AMT1;1* (Rawat et al., 1999). Transferring plants precultured with NH_4NO_3 to NO_3^- as the sole N source induced NH_4^+ influx but not the expression of *AMT1*-type transporters, suggesting a derepression of NH_4^+ transport at the posttranscriptional level by the absence of NH_4^+ (Gazzarrini et al., 1999; Gansel et al., 2001). Split-root experiments suggested that NH_4^+ uptake is predominantly dependent on the local N status of the roots rather than on the N status of the whole plant as in the case of NO_3^- uptake, since NH_4^+ influx and *AMT1;1* expression were mainly induced in the portion of the root system directly experiencing N starvation (Gansel et al., 2001).

Posttranscriptional Regulation of NH_4^+ transporters

At the mRNA level, *AMT1;1* was proposed to be regulated in a N-dependent manner by adjusting mRNA turnover in response to the N nutritional status of the plant (Yuan et al., 2007b). High-affinity NH_4^+ influx into Arabidopsis roots is further regulated at the protein level (Rawat et al., 1999; Lanquar et al., 2009; Wang et al., 2013; Yuan et al., 2013). Rawat et al. (1999) suggested posttranslational downregulation of NH_4^+ uptake after observing that influx dropped before *AMT1;1* levels, when N-starved plants were resupplied with N. In plasma membranes AMT1;1 and AMT1;3 proteins

arrange in homo- and heterotrimers (Yuan et al., 2013). Elevated extracellular NH_4^+ triggers phosphorylation of AMT1;1 at the Thr residue T460 in the trans-activation domain of the cytosolic C terminus in Arabidopsis roots (Lanquar et al., 2009). C-terminal phosphorylation leads to inactivation of the whole trimeric AMT protein complex by closing the pore of the subunits (Loqué et al., 2007; Neuhäuser et al., 2007; Loqué et al., 2009). This allosteric mechanism regulates NH_4^+ uptake by externally supplied NH_4^+ in a feedback loop that is either sensed by AMT1;1 itself as a transceptor (transporter and receptor) or by a cell-surface receptor, like a receptor kinase (Lanquar et al., 2009). The authors proposed that this rapid shut-off mechanism repressing NH_4^+ transport capacities represents a feature to avoid the accumulation of toxic levels of NH_4^+ (Lanquar et al., 2009). Another posttranslational mechanism by which plant cells can prevent NH_4^+ overload is by eliminating active AMT1;3 from the plasma membrane by endocytosis (Wang et al., 2013). Wang et al. (2013) observed clustering and internalization of EGFP-tagged AMT1;3 into the cytoplasm under excess NH_4^+ conditions.

2.2.2 NO_3^- uptake and regulation of NRTs

Similar to NH_4^+, also concentration-dependent uptake of NO_3^- can be separated into two distinct phases, mediated by a HATS and a LATS (Crawford and Glass, 1998). Unlike the NH_4^+ LATS, transporters belonging to the NO_3^- LATS have been identified (Tsay et al., 1993; Huang et al., 1999). In Arabidopsis two gene families have been identified encoding predicted NO_3^- transporters: the NRT1 family with 53 members and the NRT2 family with 7 members (Tsay et al., 2007).

High-affinity NO_3^- uptake is mainly mediated by proteins of the NRT2 family, while low-affinity NO_3^- uptake is facilitated by NRT1-type transporters (Nacry et al., 2013). To date three NRT2-type transporters have been identified that contribute to high-affinity root NO_3^- uptake, namely NRT2.1, NRT2.2 and NRT2.4 (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007; Kiba et al., 2012). The major transporter for high-affinity NO_3^- uptake is NRT2.1 with an estimated contribution of 72% of the total HATS activity in Arabidopsis plants (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007). *NRT2.1* is mainly expressed in the epidermis and cortex of older root parts (Nazoa et al., 2003; Wirth et al., 2007; Kiba et al., 2012). NRT2.2 contributed with 19% to high-affinity NO_3^- influx (Li et al., 2007). NRT2.4 is a transporter with a very

high affinity that mainly conferred NO_3^- uptake at concentrations below 25 µM (Kiba et al., 2012). In contrast, NRT2.1 and NRT2.2 are important at concentrations above 50 µM (Cerezo et al., 2001). The localization of *NRT2.4* expression in Arabidopsis roots opposes that of *NRT2.1* as it was only found in lateral roots and the younger parts of the primary root in N-starved plants (Kiba et al., 2012). In order to mediate NO_3^- transport, NRT2.1 requires the protein NAR2.1 (NRT3.1) for both expression and activity (Okamoto et al., 2006; Orsel et al., 2006; Wirth et al., 2007). Yong et al. (2010) suggested that a tetramer consisting of two subunits, each of NRT2.1 and NAR2.1, is the functional unit responsible for high-affinity NO_3^- uptake. Recently, it was proposed that NAR2.1 is required for the functionality of most NRT2-type transporters (Okamoto et al., 2006; Kotur et al., 2012).

Transporters mediating low-affinity NO_3^- influx belong to the large NRT1 (PTR) family that also comprise nitrite, peptide or carboxylic acid transporters (Forde, 2000; Tsay et al., 2007; Nacry et al., 2013). So far, only NRT1.1 and NRT1.2 were shown to contribute to root NO_3^- uptake in the low-affinity range (Nacry et al., 2013). However, NRT1.1 is a highly specialized NO_3^- transporter. Initially, NRT1.1 was characterized as a low-affinity NO_3^- transporter (Tsay et al., 1993; Huang et al., 1996). Later, it was shown that *chl1* (*nrt1.1*) mutants were also defective in high-affinity NO_3^- uptake (Wang et al., 1998). Liu et al. (1999) observed a biphasic kinetic pattern for the uptake of NO_3^- into *NRT1.1*-injected Xenopus oocytes with a K_m-value of approximately 50 µM for the high-affinity phase and a K_m-value of approximately 4 mM for the low-affinity phase. The dual-affinity NO_3^- transporter NRT1.1 switches its transport mode by phosphorylation and dephosphorylation: The non-phosphorylated form exhibits low-affinity transport activity, while phosphorylation at the T101 residue transforms it into a high-affinity transporter (Liu and Tsay, 2003). Phosphorylation of NRT1.1 at this position is mediated by the calcineurin B-like-interacting protein kinase CIPK23 (Ho et al., 2009). In contrast to *NRT2.1*, *NRT1.1* is mainly expressed in younger parts of the root, namely the tips of primary and lateral roots and at the base of lateral roots. In more mature parts of the root *NRT1.1* expression is lower and confined to the stele (Huang et al., 1996; Guo et al., 2001; Remans et al., 2006). NRT1.2 is a strict low-affinity NO_3^- transporter that is rather constitutively expressed in the epidermis of young and mature parts of the root and has a K_m-value of approximately 6 mM, when expressed in Xenopus oocytes (Huang et al., 1999).

Transcriptional Regulation of NO_3^- transporters

The LATS for NO_3^- is generally not substantially affected by high or low N provision. Like for NH_4^+, also for NO_3^- the HATS appears to be the main target for regulating the uptake capacity (Huang et al., 1996; Lejay et al., 1999). High-affinity root NO_3^- uptake is regulated at the transcriptional level diurnally, by sucrose, by reduced N, by N-deficiency and by the NO_3^- ion itself (NO_3^- signaling) (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003; Scheible et al., 2004; Wang et al., 2004).

Induction of the HATS by NO_3^- supply, is a consequence of the so-called "primary NO_3^- response", which is characterized by a strong and rapid transcriptional induction of nitrate-responsive genes including those involved in NO_3^- uptake (e.g. *NRT1.1*, *NRT2.1*) and NO_3^- assimilation (e.g. *NIR*, or *NIA1*) (Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004; Wang et al., 2004; Ho et al., 2009; Hu et al., 2009; Krouk et al., 2010a). Stimulation of the NO_3^- HATS is dependent on NRT2.1, since loss of its expression in *nrt2.1* mutants reduced induction of high-affinity NO_3^- uptake by NO_3^- to a large extent (Cerezo et al., 2001; Li et al., 2007). In split-root experiments, the root part receiving NO_3^- increased high-affinity NO_3^- uptake and *NRT2.1* expression, but not the part deprived of N. This showed that induction of high-affinity NO_3^- uptake and *NRT2.1* by NO_3^- are local processes, but a systemic signal indicating N deficiency in other root parts is needed (Cerezo et al., 2001; Gansel et al., 2001). The extent to which *NRT2.1* is induced correlates positively with the external NO_3^- concentration and depends on the transport mode of NRT1.1 (Ho et al., 2009). Phosphorylated NRT1.1, which acts as a high-affinity NO_3^- transporter, mediates a low-level primary NO_3^- response at low external NO_3^- concentrations. While non-phosphorylated NRT1.1, acting as a low-affinity transporter, causes a high-level primary NO_3^- response under high NO_3^- conditions (Ho et al., 2009). By finding and characterizing an uptake- and signaling-decoupled *nrt1.1* mutant, Ho et al. (2009) showed that the NO_3^- transport activity is not required for the sensing function and that therefore NRT1.1 is a NO_3^- sensor/transceptor. Furthermore, the kinase CIPK8 (Hu et al., 2009) and the transcription factor NLP7 (Castaings et al., 2009) are positive regulators of the primary NO_3^- response.

In addition to induction by NO_3^-, *NRT2.1* and the capacity for high-affinity NO_3^- uptake are also temporarily up-regulated by N starvation to meet the plant demand for N (Lejay et al., 1999). Besides induction, *NRT2.1* expression and NO_3^- HATS activity,

are feedback repressed by reduced N metabolites, like NH_4^+ and amino acids (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003). This repression involves systemic signaling, providing control of root NO_3^- uptake by the N status of the whole plant (Gansel et al., 2001). NRT2.1 plays a crucial role in these processes, since repression of NO_3^- HATS by reduced N and its stimulation by N deprivation are suppressed in the *nrt2.1-1* mutant (Cerezo et al., 2001). Interestingly, *NRT2.1* repression by NH_4^+ or glutamine is relieved in mixed N medium, when NO_3^- concentrations decrease to a low level (< 0.5 mM), causing an induction of *NRT2.1* expression and high-affinity NO_3^- uptake (Muños et al., 2004; Krouk et al., 2006). This mechanism mediates modulation of *NRT2.1* by the external NO_3^- availability independent of that involving reduced N metabolites. In *nrt1.1* mutants repression of *NRT2.1* by high NO_3^- provision in the presence of reduced N was fully suppressed, showing that this repression is mediated by NRT1.1 (Muños et al., 2004; Krouk et al., 2006). This mechanism ensures that either NRT1.1 or NRT2.1 is active in taking up NO_3^- in the presence of NH_4^+. When the availability of NH_4^+ is high and of NO_3^- low, NRT1.1-mediated repression of *NRT2.1* is relieved, which allows reactivation of the NO_3^- HATS (Krouk et al., 2006). By analyzing *nrt2.1* mutants Krouk et al. (2006) showed that this constitutes a crucial adaptive response against NH_4^+ toxicity because NO_3^- taken up by the HATS in this situation prevented the detrimental effects of pure NH_4^+ nutrition. The mechanism by which NRT1.1 modulates *NRT2.1* expression in response to high N provision is unknown.

Posttranscriptional Regulation of NO_3^- transporters

Although NO_3^- transporters, especially *NRT2.1*, are regulated at the mRNA level, increasing evidence indicates that post-transcriptional regulation plays also an important role in modulating root NO_3^- uptake. As mentioned above, the dual-affinity NO_3^- transporter NRT1.1 can be phosphorylated to switch its transport mode from a low- to a high-affinity transporter, when the external NO_3^- concentrations decrease (Liu and Tsay, 2003; Ho et al., 2009). NRT2.1-mediated high-affinity NO_3^- uptake is subjected to post-translational regulation as well (Wirth et al., 2007; Laugier et al., 2012). Wirth et al. (2007) observed that in response to high N provision the NRT2.1 protein abundance decreases much later and to a lower extent than the *NRT2.1* mRNA level and the NO_3^- HATS activity, suggesting the occurrence of post-translational regulatory mechanisms. In a later study transgenic lines expressing

functional *NRT2.1* under the control of a *35S* promotor in a *nrt2.1* mutant background were used (Laugier et al., 2012). The authors observed a reduced capacity for high-affinity NO_3^- influx into roots of transgenic lines in response to N and dark treatments, that generally repress *NRT2.1* and NO_3^- HATS activity in the wild-type, although the *35S::NRT2.1* lines had a high and constitutive *NRT2.1* accumulation. In some treatments the repression of the NO_3^- HATS was associated with a declined NRT2.1 protein abundance, indicating the occurrence of multiple levels of NO_3^- HATS regulation, including post-translational control of NRT2.1 activity (Laugier et al., 2012). In addition, a phospho-proteomic study revealed that NRT2.1 is phosphorylated in response to NO_3^- supply (Engelsberger and Schulze, 2012). The physiological consequences of this post-translational modification is unknown. Recently, it was shown that root NO_3^- uptake can be also regulated at the epigenetic level by chromatin modification (Girin et al., 2010; Widiez et al., 2011). An Arabidopsis mutant *high nitrogen-insensitive 9-1* (*hni9*-1), impaired in systemic repression of *NRT2.1* by high N supply, was isolated (Girin et al., 2010). Subsequent cloning revealed that *HNI9* encodes INTERACT WITH SPT6 (IWS1), an evolutionary conserved component of the RNA polymerase II complex of transcription (Widiez et al., 2011). Repression of *NRT2.1* expression in response to high N provision was associated with an HNI9/AtIWS1-dependent increase in histone methylation at the *NRT2.1* locus (Widiez et al., 2011).

2.3 Influence of N nutrition on plant metabolism

2.3.1 N assimilation

To fulfill a function in metabolism, inorganic N must be first assimilated into organic compounds. Prior to assimilation, NO_3^- must be reduced to NO_2^- and then further to NH_4^+. NH_4^+ originated from NO_3^- reduction, primary NH_4^+ uptake, amino acid catabolism or photorespiration is then assimilated into organic N via the GS/GOGAT cycle (Marschner, 2012).

NO_3^- reduction is mediated by the two enzymes nitrate reductase (NR), which reduces NO_3^- to NO_2^- and nitrite reductase (NiR), which catalyzes the conversion of NO_2^- to NH_4^+ (Crawford and Forde, 2002). NR is a cytosolic enzyme that uses NAD(P)H as a reductant (Campbell, 1999). In Arabidopsis, it is encoded by the two

genes *NIA1* and *NIA2* which are expressed both in roots and shoots. *NIA2* accounts for 85-90% of the NR activity, whereas *NIA1* is only responsible for 10-15% of the NR activity (Wilkinson and Crawford, 1993). *NIA* genes are induced by NO_3^-, sucrose, light and cytokinins while the reduced N forms NH_4^+ and amino acids repress expression of *NIA* genes (Krapp et al., 1998; Crawford and Forde, 2002). In order to avoid the accumulation of the toxic NR product NO_2^-, plants additionally developed fast and reversible post-translational mechanisms to regulate NR activity at the protein level, involving phosphorylation and binding of 14-3-3 proteins (MacKintosh and Meek, 2001). NO_2^- generated by NR is transported to plastids and chloroplasts for reduction to NH_4^+ by NiR. In photoautotrophic tissues, NiR is localized in chloroplasts and uses reduced ferredoxin, generated by photosystem I during the light reaction, whereas in root plastids, reduced ferredoxin is generated using NADPH originating from the pentose phosphate pathway (Hawkesford et al., 2012). NiR is encoded by only one gene which is up-regulated by NO_3^- supply (Rastogi et al., 1997; Krapp et al., 1998).

NH_4^+ assimilation is mediated via the GS/GOGAT cycle by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT: glutamine-2-oxoglutarate aminotransferase). The products of the GS/GOGAT cycle, glutamine (Gln) and glutamate (Glu) serve as N donors for the biosynthesis of all amino acids, nucleic acids, and other nitrogen-containing compounds such as chlorophyll (Coruzzi, 2003). Together with aspartate (Asp) and asparagine (Asn), these four amino acids are used to translocate organic N from source organs to sink tissues (Coruzzi and Last, 2000). Asp and Asn are synthesized by aspartate aminotransferase (AAT) and asparagine synthetase (AS), respectively. In Arabidopsis and other plants GS, GOGAT, AAT and AS exist as multiple isoenzymes encoded by individual genes.

GS catalyzes the ATP-dependent synthesis of glutamine by incorporating one NH_4^+ molecule into glutamate, and GOGAT catalyzes the transfer of an amide group from glutamine to α-ketoglutarate (2-oxoglutarate) to yield two molecules of glutamate, one of which is cycled back to GS (Lam et al., 1996). Plants have two classes of GS isoenzymes, cytosolic GS1 and chloroplastic GS2. The chloroplast-localized GS2 is encoded by a single nuclear gene (*GLN2*), which is almost exclusively expressed in shoots (Peterman and Goodman, 1991; Ishiyama et al., 2004). The expression of *GLN2* in Arabidopsis is induced by light in part via phytochrome and in part via sugar

and repressed by amino acids (Oliveira and Coruzzi, 1999). The cytosolic form (GS1), in turn, is encoded by four genes (*GLN1;1-GLN1;4*) and is expressed in roots and shoots of Arabidopsis (Peterman and Goodman, 1991; Ishiyama et al., 2004). GS1 is also regulated by sugar and amino acids, although not as strong as GS2 (Oliveira and Coruzzi, 1999). Due to the organ-specific expression pattern, *GLN2* is proposed to be involved in primary N assimilation in leaves and in the reassimilation of photorespiratory NH_4^+ (Coruzzi, 2003), while in roots, glutamine is synthesized by GS1 (Coruzzi, 2003; Ishiyama et al., 2004). Ishiyama et al. (2004) demonstrated that the multiplicity of cytosolic GS1 isoforms in Arabidopsis roots does not simply provide functional redundancy, but rather confers specific roles to the individual isoenzymes since they show distinctive kinetic properties, NH_4^+ responsiveness and cell type-specific localization. Taking all these features into account, they were classified into two distinct groups: the high-affinity GS isoforms (GLN1;1, and GLN1;4) and the low-affinity enzymes (GLN1;2 and GLN1;3) (Ishiyama et al., 2004). The high-affinity GLN1;1 was shown to be up-regulated under N-deficient conditions and located in root tips and rhizodermal cells, including root hairs (Ishiyama et al., 2004). These results suggest the importance of GLN1;1 under N-limited conditions, since it might be responsible for the rapid conversion of NH_4^+ at the cell layers that are in contact with the soil solution. By contrast, the low-affinity GLN1;2, which is expressed in the pericycle and up-regulated by high NH_4^+ concentrations, was proposed to assimilate NH_4^+ in the root under excessive NH_4^+ uptake (Ishiyama et al., 2004).

The second enzyme from the GS/GOGAT cycle - glutamate synthase (GOGAT) - catalyzes the reductant-dependent conversion of glutamine and 2-oxoglutarate to two molecules of glutamate. With regard to the electron donor specificity, two different forms of plastid-localized GOGAT exist in higher plants: one is ferredoxin-dependent (Fd-GOGAT) while the other is NADH-dependent (NADH-GOGAT) (Coruzzi and Last, 2000). In Arabidopsis leaves, Fd-GOGAT accounts for 95% of the total GOGAT activity (Somerville and Ogren, 1980). Fd-GOGAT is encoded by two genes in Arabidopsis, namely *GLU1* and *GLU2* (Lam et al., 1995; Coschigano et al., 1998). *GLU1* is mainly expressed in the shoot, where its expression is stimulated by light or by sucrose (Coschigano et al., 1998). *GLU1* is involved in primary shoot N assimilation and is essential for the assimilation of photorespiratory NH_4^+ (Somerville and Ogren, 1980; Coschigano et al., 1998). *GLU2* expression is constitutive, relatively low in leaves, higher in roots, and not prone to sucrose induction

(Coschigano et al., 1998). Arabidopsis has a single gene for NADH-GOGAT, *GLT1*, which is expressed at low constitutive levels in leaves, and at high levels in roots. NADH-GOGAT is suggested to play an important role in primary N assimilation (Lancien et al., 2002).

Glutamate dehydrogenase (GDH) is an enzyme that can catalyze forward and reverse biochemical reactions: the amination of 2-oxoglutarate into glutamate (anabolic) or the deamination of glutamate into NH_4^+ and 2-oxoglutarate (catabolic) (Coruzzi, 2003). GDH plays neither a role in primary N assimilation nor in the assimilation of photorespiratory NH_4^+. The main function of GDH is most likely the catabolism of glutamate under carbon-limiting conditions (e.g. at night) and the assimilation of NH_4^+ under excessive supply (Melo-Oliveira et al., 1996).

2.3.2 Influence of N nutrition on primary metabolism

N nutrition strongly affects N and carbon (C) metabolism. On the one hand, N deficiency causes a decrease in amino acids, particularly Gln, protein content and other N-containing compounds like chlorophyll. On the other hand, carbon-containing compounds like starch, specific flavonoids (e.g. rutin and ferulic acid) and phenylpropanoids are increased (Scheible et al., 2004). These metabolic alterations are reflected in the down-regulation of genes involved in amino acid synthesis and photosynthesis and up-regulation of genes involved in amino acid breakdown. NO_3^- resupply leads to an increase in the concentration of various amino acids (Gln, Glu, Asp, Ala, Asn, Arg, and His). This increase is also reflected in the induction of genes involved in amino acid biosynthesis and in the repression of genes involved in amino acid catabolism. In contrast to most biosynthetic pathways for amino acids, the shikimate pathway is repressed by NO_3^- as is phenylpropanoid and flavonoid metabolism (Scheible et al., 2004). The accumulation of amino acids in Arabidopsis shoots is stronger, when NH_4^+ is supplied solely or in combination with NO_3^- as compared to exclusive NO_3^- nutrition (Hachiya et al., 2012).

N metabolism interacts with C metabolism in a complex manner, amongst others for the provision of 2-oxoglutarate as the primary acceptor of NH_4^+ in the GS/GOGAT pathway and for the supply of malate as a counter-anion to prevent alkalization during NO_3^- reduction (Stitt et al., 2002). The supply of NO_3^- as sole N source induces genes involved in the synthesis of these organic acids, in the activity of the

corresponding proteins (phosphoenolpyruvate carboxylase, cytosolic pyruvate kinase, citrate synthase and NADP-isocitrate dehydrogenase) and increases the accumulation of malate and 2-oxoglutarate (Scheible et al., 1997b; Scheible et al., 2004). The increased synthesis of organic acids demands a diversion of C from carbohydrate production, leading to a repression of *AGPS*, a gene encoding a subunit of ADP-glucose pyrophosphorylase, and to decreased starch accumulation (Scheible et al., 1997b; Stitt, 1999). Scheible et al. (1997b) did not observe a NO_3^--mediated repression of sucrose phosphate synthase (SPS) in tobacco and assumed that sucrose synthesis is not affected by NO_3^-. Furthermore, various amino acid pathways need several other organic acids and phosphorylated intermediates as C precursors (Stitt et al., 2002). In contrast, addition of NH_4^+ to the medium results in organic acid depletion in Arabidopsis shoots. This depletion is thought to be a consequence of the necessity for NH_4^+ assimilation after excessive NH_4^+ supply (Hachiya et al., 2012). N assimilation requires also carbohydrate breakdown to fuel the oxidative pentose phosphate (OPP) cycle, in order to generate reducing equivalents in heterotrophic tissues or during the night (Stitt et al., 2002). These and other findings demonstrate that N interacts at numerous sites with C metabolism, pH regulation, and ion and assimilate fluxes at the cell and whole plant level (Stitt et al., 2002).

2.4 The role of N nutrition in plant growth and development

Plants have evolved sophisticated adaptive mechanisms to cope with temporal and spatial variations in N availability. These mechanisms consist of physiological and morphological responses to balance the amount of N acquired from the soil with what is needed for growth and development (Vidal and Gutiérrez, 2008; Kiba et al., 2011). Responses to a variable N availability include regulation of the uptake capacity (section 2.2), adaptation of shoot-root allocation (shoot:root ratio) (Scheible et al., 1997a; Walch-Liu et al., 2005), changes in root architecture (Walch-Liu et al., 2006; Lima et al., 2010) and leaf expansion (Walch-Liu et al., 2000; Rahayu et al., 2005; Helali et al., 2010), altered flowering time (Marín et al., 2011) and changes in seed dormancy (Alboresi et al., 2005) and senescence (Stitt, 1999). In order to coordinate these responses of multiple organs with different functions and nutritional requirements at the whole-plant level, both local and long-distance signaling are

required. NO_3^-, amino acids, sugars, and phytohormones have been implicated in this signaling (Kiba et al., 2011).

2.4.1 Influence of N on plant growth

N nutrition has a strong influence on plant growth. In any plant species, N deficiency decrease biomass formation and yield (Marschner, 2012). In Arabidopsis, sustained N limitation caused a 20% decrease in relative growth rate (RGR) and resulted in a two- to threefold reduction in shoot biomass at the end of the experiment (Tschoep et al., 2009). In tomato, N deprivation reduced the rate of leaf growth and was already detectable 6 h after N withdrawal (Rahayu et al., 2005).

For optimal growth, sufficient N must be available to plants. However, the form of N supplied to plants plays an important role. Many plants exhibit growth suppression when NH_4^+ is supplied as the sole N source (Britto and Kronzucker, 2002). In tobacco, tomato and Arabidopsis shoot biomass and/or the rate of leaf expansion were significantly reduced, when NH_4^+ was the exclusive N form (Walch-Liu et al., 2000; Rahayu et al., 2005; Helali et al., 2010; Hachiya et al., 2012). The inhibition of leaf expansion in tobacco was related to a decrease in cell size and particularly to a decrease in cell number (Walch-Liu et al., 2000). Growth inhibition by NH_4^+ is also referred to as NH_4^+ toxicity. NH_4^+ toxicity additionally displays in other symptoms such as chlorosis, decline in mycorrhizal association and inhibition of seed germination and seedling establishment (Britto and Kronzucker, 2002). NH_4^+ toxicity has been attributed to various factors, such as a reduced accumulation of the essential cations K^+, Ca^{2+} and Mg^{2+} (Salsac et al., 1987; Britto and Kronzucker, 2002; Roosta and Schjoerring, 2007), depletion of carbohydrates for NH_4^+ assimilation (Hawkesford et al., 2012), acidification of the rhizosphere (Britto and Kronzucker, 2002) and the energy-intensive futile transmembrane cycling of NH_4^+ (Britto et al., 2001b). Interestingly, plants receiving a mixed NH_4^+ and NO_3^- nutrition generally do not exhibit NH_4^+-mediated growth suppression (Britto and Kronzucker, 2002; Rahayu et al., 2005; Helali et al., 2010; Hachiya et al., 2012). The addition of only 10 µM NO_3^- to a nutrient solution containing 2 mM NH_4^+ was already sufficient to alleviate NH_4^+-induced inhibition of the leaf growth rate in tomato plants (Rahayu et al., 2005). NO_3^- is a signaling molecule that can elicit changes in gene expression of about 10 % of the transcriptome and thereby modify plant metabolism and development (Wang et

al., 2003; Scheible et al., 2004; Krouk et al., 2010a). There is increasing evidence that the N form affects phytohormone homeostasis and thereby plays a role in modulating plant growth and development (Walch-Liu et al., 2000; Rahayu et al., 2005; Sakakibara et al., 2006; Kiba et al., 2011).

2.4.2 Interaction of N nutrition with phytohormones

Phytohormones are a group of naturally occurring, organic substances, which influence physiological processes at low concentrations (Davies, 2010). The processes influenced consist mainly of growth, differentiation and development. Besides their known functions in growth and development, phytohormones have been linked to diverse types of environmental responses, such as light, temperature, salt, drought, pathogen, and nutrient responses (Kiba et al., 2011). Several studies indicate that abscisic acid (ABA), auxin and cytokinins are involved in coordinating the demand and acquisition of N (reviewed in (Kiba et al., 2011).

Abscisic acid (ABA), often called a stress hormone, is synthesized from glyceraldehyde-3-phosphate via isopentenyl diphosphate and carotenoids in roots and mature leaves. Seeds are also rich in ABA, which may be imported from the leaves or synthesized *in situ*. ABA is transported from roots in the xylem and from leaves in the phloem (Davies, 2010). It is involved in abiotic and biotic stress responses. The most prominent function of ABA is stomatal closure under water stress. Water shortage brings about an increase in ABA, which leads to stomatal closure. ABA affects the induction and maintenance of some aspects of dormancy in seeds. It inhibits shoot growth, induces storage protein synthesis in seeds and counteracts the effect of gibberellin on α-amylase synthesis in germinating cereal grains. Wounding increases ABA and induces gene transcription of proteinase inhibitors, indicating that ABA may be involved in defense against insect attack (Davies, 2010). There are some indications linking N nutrition and ABA. Under N deficiency various plant species exhibit increased concentrations of ABA (Wilkinson and Davies, 2002; Engels et al., 2012). Interestingly, besides an increased ABA concentration, water stress and N deficiency share additional common symptoms such as stomatal closure, slower leaf growth, maintained root growth and often greater lateral root proliferation (Wilkinson and Davies, 2002). Furthermore, N-deficient plants respond to a shortage in water availability with a faster stomatal

closure than N-sufficient plants (Radin and Ackerson, 1981). Increased ABA levels are, however, not ubiquitously observed in all plant species (Wilkinson and Davies, 2002), amongst them *Arabidopsis thaliana* (Kiba et al., 2011). Nevertheless, there is some genetic evidence for the involvement of ABA in N signaling. In *Arabidopsis thaliana* and *Medicago truncatula*, ABA signaling mutants have been described, in which root development is insensitive to NO_3^- supply (Signora et al., 2001; Zhang et al., 2007; Yendrek et al., 2010).

Auxins are compounds derived from tryptophan or indole, with indole-3-acetic acid (IAA) as the major physiologically active auxin form in most plants. Auxin is synthesized primarily in leaf primordia, young leaves and in developing seeds (Davies, 2010). The action of auxin is determined by its levels in the tissue, its concentration gradient and the differential sensitivity of various cell types. Auxin homeostasis is regulated and maintained by *de novo* synthesis, degradation, transport and by inactivation and activation through synthesis and hydrolysis of various auxin conjugates (Normanly et al., 2010). Biosynthesis of IAA is complex and can involve various routes leading to different precursors, amongst them indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) (Ljung et al., 2002; Pollmann et al., 2003). Auxin catabolism in plants occurs mainly by oxidation of the ring structure of IAA, generating the catabolite 2-oxoindole-3-acetic acid (OxIAA) (Normanly et al., 2010). Auxin can be transported over long distances via the phloem or can be redistributed locally from cell to cell, resulting in auxin concentration gradients and localized areas with a high auxin concentration. The directional, intercellular auxin transport is mediated by the polar localization of the AUX1/LAX auxin influx carriers, the PIN auxin efflux carriers and the PGP auxin efflux transporters (Robert and Friml, 2009). Most IAA found in plants is conjugated to various sugars, sugar alcohols, amino acids and proteins. Conjugated auxins are thought to be either storage or long-distance mobile forms of the active hormone (Normanly et al., 2010). Two examples are the conjugates indole-3-acetic acid methyl ester (IAA-Me) and indole-3-acetyl-L-alanin (IAA-Ala) (Bartel and Fink, 1995; Yang et al., 2008). Auxin stimulates cell expansion and division, is implicated in apical dominance and tropistic bending responses to light and gravity, it promotes root initiation and vascular tissue differentiation (Normanly et al., 2010). Auxin is also involved in mediating responses to N supply. The concentration of auxin in the phloem sap and roots of maize plants supplied with high doses of NO_3^- are lower and this reduction is correlated with

reduced root growth (Tian et al., 2008). In Arabidopsis root auxin levels also respond to NO_3^- supply (Walch-Liu et al., 2006; Kiba et al., 2011). Furthermore, auxin is involved in promoting lateral root growth as a response to NO_3^- supply. Krouk et al. (2010b) showed that the NO_3^- transceptor NRT1.1 can facilitate cell-to-cell auxin transport. The *nrt1.1* (*chl1*) mutant accumulated auxin in lateral root primordia and showed increased lateral root growth. The authors hypothesized that under low NO_3^- availability, NRT1.1 prevents auxin accumulation in the root tip, resulting in inhibition of lateral root growth. In contrast, when NO_3^- availability and the NO_3^- transport activity of NRT1.1 are high, the auxin transport activity of NRT1.1 is attenuated, leading to an accumulation of auxin within the root tip and to promotion of lateral root growth (Krouk et al., 2010b).

Cytokinins are a group of mobile phytohormones that play an important role in plant growth and development by regulating cell division and differentiation, leaf senescence, apical dominance, meristem function and macronutrient acquisition (Mok and Mok, 2001; Hirose et al., 2008; Argueso et al., 2009). The naturally occurring cytokinins trans-zeatin (tZ), isopentenyl-adenine (iP), cis-zeatin (cZ), and dihydro-zeatin (DZ) are widely found in higher plant species, with tZ and iP being the most common ones (Hirose et al., 2008). The major initial product of cytokinin biosynthesis is iP that is catalyzed by adenosine phosphate-isopentenyltransferase (IPT) (Sakakibara, 2006). The formation of tZ-type cytokinins from iP-type cytokinins is then catalyzed by the cytochrome P450 mono-oxygenases CYP735A1 and CYP735A2 (Takei et al., 2004b). The Arabidopsis genome encodes seven *IPT* genes (Sakakibara et al., 2006). Various studies have shown that the supply of NO_3^- can increase cytokinin levels in many plant species, which were previously grown under N-limiting conditions or on NH_4^+ as N source (Sattelmacher and Marschner, 1978; Samuelson et al., 1992; Walch-Liu et al., 2000; Takei et al., 2001; Takei et al., 2002; Rahayu et al., 2005; Sakakibara et al., 2006). Molecular biological analyzes revealed that *IPT3* and *IPT5* are both regulated by the availability of inorganic N (Takei et al., 2004a). The transcript level of *IPT5*, which is expressed in the lateral root primordium and pericycle (Miyawaki et al., 2004; Takei et al., 2004a), was positively correlated with concentration of NH_4^+ and NO_3^- in the medium during long-term treatments (Takei et al., 2004a). Whereas, phloem-expressed *IPT3*, was rapidly up-regulated as a response to the resupply of NO_3^- to N-deprived plants (Miyawaki et al., 2004; Takei et al., 2004a). Furthermore, *IPT3* levels correlated well with the NO_3^- induced

accumulation of cytokinins in roots of wildtype Arabidopsis plants, but in an *ipt3* mutant the NO_3^--dependent accumulation of cytokinins was strongly reduced (Takei et al., 2004a). According to transcriptome data, the level of *CYP735A2* expression is also up-regulated by NO_3^- in the roots of Arabidopsis plants (Wang et al., 2004). The above-mentioned inhibition of leaf expansion in tobacco and tomato grown with NH_4^+ as the sole N source was associated with a sharp decline in the concentration of cytokinins in the xylem sap (Walch-Liu et al., 2000; Rahayu et al., 2005). This observation indicates that cytokinins play a major role as long-distance signals mediating the shoot response to NO_3^- perception in roots. Another interesting link between N nutrition and cytokinins is that *NRTs* are repressed by them (Brenner et al., 2005; Kiba et al., 2011). This observation and the fact that *IPT3* is expressed in the phloem (Miyawaki et al., 2004; Takei et al., 2004a) and iP-type cytokinins are translocated from the shoot to roots (Matsumoto-Kitano et al., 2008) led Kiba et al. (2011) to propose that cytokinins may act as a N satiety signal. As a shoot-to-root signal, cytokinins may report the NO_3^- status of the shoot to repress N uptake by the roots under N-sufficient conditions (Kiba et al., 2011).

Salicylic acid is synthesized from the amino acid phenylalanine and plays a major role in the resistance to pathogens by inducing the production of pathogenesis-related proteins. It is involved in the systemic acquired resistance response (SAR) in which a pathogenic attack on older leaves causes the development of resistance in younger leaves. It has also been reported to enhance flower longevity, inhibit ethylene synthesis and seed germination, block the wound response, and reverse the effects of ABA (Davies, 2010). The involvement of salicylic acid in the response to N availability or to the N status of the plant has not been reported yet.

Despite increasing evidence that N nutrition influences the homeostasis of phytohormones, in particular of cytokinins and auxins, the consequences for plant growth and development are not yet fully elucidated. To what extent salicylic acid and further phytohormones are involved in mediating responses to N nutrition is still poorly understood.

2.4.3 Influence of N on flowering

Flowering is a prerequisite for successful sexual reproduction and is of great importance for agricultural food production since the major source of staple diet of

humans and livestock is formed by fruits of angiosperm flowers. The timing of floral induction is tightly controlled by environmental and endogenous cues, ensuring synchronous flowering of individuals of a species, under favorable external conditions (Bernier et al., 1993; Fornara et al., 2010; Srikanth and Schmid, 2011). In *Arabidopsis thaliana* many genes involved in flowering-time control have been identified. These genes are active in leaves or the shoot apical meristem and occur in a network of six major pathways: the photoperiod, the vernalization, the ambient temperature, the age, the autonomous and the gibberellic acid (GA) pathway (Fornara et al., 2010). The six pathways converge to regulate a common set of "floral integrator genes," including *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which both rapidly promote floral development by activation of floral meristem identity genes such as *APETALA 1* (*AP1*) (Moon et al., 2003; Wigge et al., 2005; Turck et al., 2008). Arabidopsis is a facultative long day plant, in which flowering is induced by exposure to long summer days and is repressed by short winter days. The photoperiod pathway integrates inputs from the circadian clock and light receptors, thereby promoting flowering in long days through transcriptional regulators such as CONSTANS (CO) (Suárez-López et al., 2001; Valverde et al., 2004; Fornara et al., 2010). CO promotes floral initiation by inducing expression of the integrators *FT* and *TWIN SISTER OF FT* (*TSF*) genes (Turck et al., 2008). In several Arabidopsis accessions vernalization is a prerequisite for flowering (Srikanth and Schmid, 2011). The MADS-box transcription factor FLOWERING LOCUS C (FLC) is a repressor of flowering (Srikanth and Schmid, 2011). The vernalization pathway initiates flowering by silencing *FLC* in response to prolonged exposure to low temperatures (Fornara et al., 2010). The control of flowering by the ambient temperature, the age, the autonomous and the gibberellic acid (GA) pathway were summarized by Fornara et al. (2010) and reviewed by Srikanth and Schmid (2011). In addition, flowering time is also influenced by other environmental stimuli, such as the balance of different wavelengths of light or nutrient availability, but how these processes interact with these six pathways mentioned above is not fully understood (Fornara et al., 2010).

Nevertheless, the implication of nutrients in influencing flowering time has been reported (Marschner, 2012). It has been observed some time ago that the reduction in supply of mineral nutrients to Arabidopsis plants promotes flowering (Lang, 1965). More recent studies confirmed the observation that increased nutrient supply delays

flowering in several Arabidopsis lines (Zhang and Lechowicz, 1994; Pigliucci and Schlichting, 1995; Tienderen et al., 1996; Kolář and Seňková, 2008). In the case of N it has been known for over a century that N nutrition can modify flowering time (Klebs, 1913). N limitation often promotes early flowering (Dickens and Van Staden, 1988; Loeppky and Coulman, 2001). In apple trees, flower formation is greatly affected by the form of N supplied (Grasmanis and Edwards, 1974; Rohozinski et al., 1986). Short-term NH_4^+ supply more than doubled both the percentage of buds developing inflorescences and the arginine concentration in the stem as compared to continuous NO_3^- supply (Rohozinski et al., 1986). The supply of polyamines also promoted inflorescence formation (Rohozinski et al., 1986). Since arginine is a precursor for the synthesis of polyamines and increased concentrations of polyamines accumulated in the leaves of plants amply supplied with NH_4^+ (Gerendás and Sattelmacher, 1990), it was suggested that polyamines might be involved in NH_4^+-induced enhancement of inflorescence development in apple trees (Rohozinski et al., 1986; Marschner, 2012). The involvement of polyamines in the biochemical control of the development of reproductive structures has been shown in apricot, kiwi and maize (Engels et al., 2012). In Arabidopsis, the supply of low NO_3^- concentrations accelerates flowering as compared to the supply of high NO_3^- concentrations (Kant et al., 2011; Marín et al., 2011; Liu et al., 2013). Marín et al. (2011) observed that low NO_3^- still promoted flowering in late-flowering mutants impaired in the photoperiod, temperature, GA and autonomous flowering pathways as well as in mutants of floral integrators. The authors therefore proposed that NO_3^- modulates flowering time via a novel signaling pathway acting in parallel with the photoperiod, GA and autonomous pathways and entering downstream of the known floral integrators. In contrast, Liu et al. (2013) found that plants grown under low NO_3^- conditions induced the expression of the GA biosynthesis gene *GA1* and exhibited higher concentrations of the GA GA_3. In addition, they observed an increased expression of *CO*, a gene closely associated with the photoperiod pathway, and of *SOC1* under low NO_3^- conditions. In another study, the expression of the flowering repressor *FLC* was repressed and of the positive regulators of flowering *FT*, *AP1* and *LEAFY* (*LFY*) was induced under the supply of low NO_3^- (Kant et al., 2011).

These observations strongly support the involvement of N in modulating flowering time in plants, including Arabidopsis. However, how the form and amount of N

influences the transition from vegetative to reproductive growth and how N signaling interacts with flowering time pathways is not yet understood.

2.5 Aim of the study

Yuan et al. (2007a) showed that the *AMT*-quadruple knock-out line *qko*, which has a reduced capacity for high-affinity NH_4^+ uptake, has a lower shoot biomass as compared to wild-type plants when grown under low N conditions (500 µM N as NH_4^+ or NH_4NO_3). Interestingly, it was later observed, that when *qko* plants were cultivated under standard conditions, where N was not limiting, their shoots appeared to be visually larger, than wild-type shoots (own observation and personal communication with several colleagues). This observation led to the question why the absence of AMT-type transporters or a reduced capacity for NH_4^+ uptake promotes growth when NO_3^- is the predominant N source? Therefore, the aim of this study was i) to verify the growth phenotype of *qko* under NO_3^- supply and its dependence on the growth conditions; ii) to investigate the impact of the lacking expression of *AMTs* on NO_3^- uptake and iii) metabolism; and iv) to describe the long-term consequences of lacking *AMT* gene expression in plant development.

Accordingly, the present thesis has been structured into four chapters, in which chapter 4.1 describes the growth phenotype of *qko* under supply of different N forms, chapter 4.2 characterizes the uptake of ^{15}N-labelled NO_3^- in *qko* and the expression of NO_3^- transporter genes, while chapter 4.3 describes the metabolic consequences of varied N nutrition. Finally, chapter 4.4 deals with the phenological development of Arabidopsis lines lacking *AMT* gene expression. Taken together, these approaches allow uncovering novel roles of AMT transporters that go beyond their function in NH_4^+ uptake.

3 Materials and Methods

3.1 Plant material

In the present study, the *Arabidopsis thaliana* accession line Columbia-0 (Col-0) was used as wild-type. In order to investigate the effect of AMT-dependent NH_4^+ transport, the NH_4^+ uptake defective *AMT*-quadruple knock-out line called *qko* was used (Yuan et al., 2007a). The *qko* mutant lacks the expression of the NH_4^+ transporter genes *AMT1;1*, *AMT1;2*, *AMT1;3*, and *AMT2;1*. Furthermore, triple insertion lines expressing either functional *AMT1;1* (*qko11*), *AMT1;2* (*qko12*), *AMT1;3* (*qko13*), or *AMT2;1* (*qko21*) in the *qko* background were used in this study. *qko11*, *qko12*, *qko13* and *qko21* were obtained by backcrossing *qko* to the wild-type (Col-0) (Yuan et al., 2007a).

3.2 Growth conditions for hydroponic culture

For hydroponic cultivation of Arabidopsis, aerated and unbuffered nutrient solutions containing the following salts were used: 2 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 100 µM Na-Fe-EDTA, 50 µM KCl, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$ and 1 µM Na_2MoO_4. The pH was adjusted to 5.8 with KOH. For N deficiency treatments N was omitted. N forms and concentrations other than 2 mM KNO_3 are indicated. The plants were cultivated for the indicated time in a climate-controlled growth chamber under the following conditions: a 10 h light/14 h dark cycle, a light intensity of 240 µmoles m^{-2} s^{-1}, temperature of 22/18°C at day/night and 70% relative humidity. Seedlings were acclimatized in half-strength nutrient solution and lower light intensity beforehand.

3.3 Growth conditions on agar plates and soil substrate

Arabidopsis seeds were surface sterilized by using a solution of 70% ethanol and 0.05% Triton X100. To 20-30 µl of seeds in a 1.5 ml tube 1 ml of the solution was added and then shaken at room temperature (22°C) for 23 min at 1400 rpm. Seeds were then washed twice with 1 ml of 100% ethanol.

Surface-sterilized seeds were sown on sterile half-strength MS medium (Murashige and Skoog, 1962) without N (PhytoTechnology Laboratories®), supplemented with 0.5 % sucrose, solidified with 1 % (w/v) granulated Difco agar (Becton Dickinson) and buffered with 5 mM MES-KOH pH 5.7. N was supplemented at the indicated form and concentration. The agar plates containing the seeds were then incubated at 4°C for 2 days in the dark for stratification. Afterwards, they were placed horizontally inside a controlled-environment growth cabinet (Percival Scientific, Inc., Perry, IA, USA) for the indicated time under the following conditions: 10/14 h light/dark, with a light intensity of 120 µmole photons m^{-2} s^{-1}; and temperature of 22°C/19°C at day/night. For long-day treatments 16/8 h light/dark cycles were used.

For flowering time experiments conducted on soil substrate a standard substrate (Substrat 1; Klasmann-Deilmann GmbH, Geeste, Germany) was used. These plants were also grown inside a controlled-environment growth cabinet under the same conditions as the agar plates with the exception that for short-day treatments 8/16 h light/dark cycles and for long-day treatments 16/8 h light/dark cycles were used.

3.4 Determination of leaf area and rosette diameter

Leaf area and rosette diameter were determined by image analysis. Arabidopsis leaves were separated and scanned with an Epson Expression 10000XL scanner at a resolution of 360 dpi for leaf area determination. Top view photographs for rosette diameter determination and other plant photographs were taken with a Canon EOS 7D. Image analysis was conducted using the open-source software Fiji (Schindelin et al., 2012). Fiji is an image processing package distribution of ImageJ, bundling several plugins focused on biological-image analysis.

3.5 Determination of flowering time and leaf number

Plants were visually scored every day during the course of the experiments to investigate the influence of lacking *AMT* gene expression on the transition to flowering. The time of flowering was determined when approximately 30% of the plants of a given genotype had started to flower, i.e. had formed at least one visible petal. The number of rosette leaves at the transition to flowering was counted when 30% of the plants had at least one visible petal.

3.6 Histological analysis of cotyledons

For histological characterization of Arabidopsis cotyledons, samples were fixed in 2% formaldehyde, 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2), dehydrated in an ethanol series (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%), and embedded in Spurr's resin. 5 µm cross sections of cotyledons were obtained by cutting the fixed and resin-embedded samples using a microtome (Ultracut; Leica, Nussloch, Germany). Cotyledon cross sections were observed under a light microscope (Axioskop; Carl Zeiss, Jena, Germany). Image analysis was conducted with the open-source software Fiji (Schindelin et al., 2012).

3.7 Phenotyping shoot growth with a LemnaTec system

For non-destructive assessment of the leaf area on a daily basis and for calculation of relative growth rates (RGR) wild-type (Col-0), *qko*, *qko11*, *qko12*, *qko13* and *qko21* plants were grown at 20/18°C, 60/75% relative humidity, 130-150 µmoles m^{-2} s^{-1} and a 16/8h day/night regime in a climate-controlled growth chamber equipped with a LemnaTec phenotyping system (Junker et al., 2015). Seeds were sown in pots filled with a mixture of 85% (v) substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany and 15% (v) sand. After 2-3 days of stratification at 5°C in constant darkness, the pots were inserted into the carriers of the LemnaTec system. Seeds were germinated and seedlings cultivated under a 16/8 h day/night regime with 16/14°C, 75% relative humidity, and 130-150 µmoles m^{-2} s^{-1} light intensity until 3 days after appearance of both cotyledons (usually reached at 4 days after sowing). To obtain suitable images for analysis, blue rubber mats were placed on the pots covering the soil. Soil moisture was adjusted daily to 70% field capacity. In the LemnaTec Scanalyzer system top and side view images were taken of the visible range of the light spectrum, of fluorescence signals, and of a broader band of the near infrared spectrum as described in Junker et al. (2015). The imaging-based phenotyping started 8 days after sowing and ended 30 days after sowing. The IAP (Integrated Analysis Platform) open-source software for high-throughput plant image analyses (Klukas et al., 2014) was used for image-based plant feature extraction. During the experiment the transition to flowering was additionally measured by determining the day at which the first petal was visible. Relative growth rate (RGR) was computed according to Poorter and Lewis (1986).

3.8 Gene expression analysis

Extraction of RNA was conducted with the TRIzol® Reagent (Invitrogen) which is based on the 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 of the genes analyzed in this study was assessed using the specific primers listed in table 1. Relative expression was calculated according to Pfaffl (2001) and normalized to *UBIQUITIN2* (*UBQ2*) as constitutively expressed housekeeping gene.

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

	oligonucleotides in 5'-3' orientation	
	Forward	Reverse
AP1	CGGACTCAGGTGCAATAAGC	CTCTGTGATGCTGAAGTTGCTC
CIPK23	GCACCTATGGGGTTTGA	GTCCCGTGGTAAGGTT
CLE3	CCTGCTTCTAGTACTCGAATTGAC	ATTTCCAAGGATCGTCTCTTCGCC
CO	TAAGGATGCCAAGGAGGTTG	CCCTGAGGAGCCATATTTGA
FT	CGAGTAACGAACGGTGATGA	CGCATCACACACTATATAAGTAAAACA
GDH2	CACTGGAAAGCCCATTGATCT	TGTCGCTTACTGCAACCACT
GLN1;2	TGTTAACCTTGACATCTCAGACAACAGT	ACTTCAGCAATAACATCAGGGTTAGCA
NRT1.1	ACGACATTATCAGTCGC	CTGTCCTGTGTAGATTAACG
NRT1.2	TCGTGCGATAGTCCCACATG	GGTACAACCCCACGAATAGCA
NRT2.1	AACAAGGGCTAACGTGGATG	CTGCTTCTCCTGCTCATTCC
NRT2.2	CGGAGCACTATTATGTTGGC	GGTTGCGTTCCCTTTGT
NRT2.4	CCGTCTTCTCCATGTCTTTC	CTGACCATTGAACATTGTGC
NRT3.1	GGCCATGAAGTTGCCTATG	TCTTGGCCTTCCTCTTCTCA
TSF	CTCGGGAATTCATCGTATTG	CCCTCTGGCAGTTGAAGTAA
UBQ2	CCAAGATCCAGGACAAAGAAGGA	TGGAGACGAGCATAACACTTGC
UMAMIT14	AAATTCGCACTGAACCAAGG	TCTTCGGCCGTATTTTTCTATC

3.9 Protein gel blot analysis

Fresh root tissues were used for the preparation of microsomal membrane fractions. Microsomal membrane fractions were isolated as previously described by Loqué et al. (2006). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) with BSA as standard. Proteins were separated on SDS-PAGE followed by an electrotransfer onto a nitrocellulose membrane. NRT2.1 was detected using an anti-NRT2.1 antiserum raised against an oligopeptide on the C-terminus of NRT2.1 (c-KNMHQGSLR-FAENAK-n). The polyclonal antibody anti-NRT2.1 20 was kindly provided by Dr. Alain Gojon and is described in Wirth et al. (2007). The immunodetection was performed with the chemiluminescent detection kit Amersham™ ECL™ Select Western Blotting Detection Reagent (GE Healthcare). Amido Black-stained membrane served as loading control.

3.10 NH_4^+ analysis

For the detection of NH_4^+ in plant tissues a customized method according to Goyal et al. (1988) and Husted et al. (2000) was used. 50 mg grinded and frozen plant material was extracted with 1 ml ice-cold 10 mM formic acid. After freezing the tissue extracts at -20°C overnight, 30 µl of the extract were used for derivatization with 1 ml OPA-buffer (100 mM KH_2PO_4/K_2HPO_4 phosphate buffer pH 6.8, 3 mM *o*-phthalaldehyde, 10 mM β-mercaptoethanol) for 15 min in a water bath at 80°C. After chilling for at least 1 minute on ice, 200 µl of the derivative were transferred into a black 96-well plate. NH_4^+ was detected in a fluorescence spectrometer (Multimode microplate reader *Infinite® M200*, TECAN) at an excitation wavelength of 410 nm and an emission wavelength of 470 nm.

3.11 NO_3^- analysis

For the detection of NO_3^- 50 mg grinded and frozen plant material was used. Tissue samples were extracted with 1 ml water by thoroughly vortexing and subsequent centrifugation (13,000 rpm, 5 min). 40 µl supernatant was mixed with 160 µl 1 % salicylic acid in concentrated H_2SO_4. After 20 min incubation on ice, 1.8 ml cold 4M NaOH was added and gently mixed. NO_3^- concentrations were measured with a spectrophotometer at 410 nm.

3.12 ^{15}N uptake and total N analysis

Influx of ^{15}N-labeled NO_3^- into plant roots was measured by rinsing the roots in a 1 mM $CaSO_4$ solution for 1 min and then transferring the plants to a nutrient solution containing different concentrations of ^{15}N-labeled NO_3^- (95 atom% ^{15}N) as the sole N source for 6 min. Subsequently, the roots were washed with 1 mM $CaSO_4$ for 1 min to remove the ^{15}N-containing uptake solution from the apoplast. Roots and shoots were separated and stored at -80°C before freeze-drying. Grinded samples were used for ^{15}N and total N determination by isotope ratio mass spectrometry (Horizon, NU Instruments).

3.13 Amino acid analysis

Free amino acids were extracted from 50 mg frozen and grinded plant tissues with 0.75 ml of 80% ethanol by incubation at 80°C for 60 min and subsequent centrifugation at 14,000 rpm at 4ºC for 5 min. The extract was concentrated in a speed vacuum concentrator and resuspended in 250 µl water. For the detection of primary and secondary amino acids the fluorescent reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) was used. 3 mg ACQ was dissolved in 1 ml acetonitrile and incubated at 55°C for 10 min. 20 µl of concentrated extract was derivatized in a solution containing 20 µl of dissolved ACQ and 160 µl of 0.2 M boric acid buffer (pH 8.8). The solution was derivatized at 55°C for 10 min. The separation of derivatized samples was carried out with a reversed phase HPLC system (Waters, Germany) consisting of a gradient pump (Alliance 2795 HT, Waters, Germany), a degassing module, an autosampler and a fluorescence detector (Waters 2475, Germany). A reversed phase column (XBridge; 150 mm, 5 µm) was used for separation and detection of amino acids at an excitation wavelength of 300 nm and an emission wavelength of 400 nm. The gradient was accomplished with buffer A containing 140 mM sodium acetate, pH 5.8 (Suprapur, Merck) and 7 mM triethanolamine (Sigma, Germany). Acetonitrile (Roti C Solv HPLC, Roth) and purest HPLC water (Geyer, Germany) were used as eluents B and C. Chromatograms were recorded using the software program Empower Pro.

3.14 Sugar analysis

Soluble sugars were determined in 50 mg frozen and grinded plant tissues. Plant material was dissolved in 0.75 ml 80% ethanol and incubated at 80°C for 60 min. Crude extracts were centrifuged at 14,000 rpm at 4ºC for 5 min and the supernatant was concentrated in a speed vacuum concentrator. The pellet was resuspended in 250 µl water and shaken for 15 min at 4°C. A buffer containing 100 mM imidazole-HCl (pH 6.9), 5 mM $MgCl_2$, 2.25 mM NAD, 1 mM ATP was used to measure soluble sugars. The sequential addition of auxiliary enzymes allowed the detection of glucose (Glc), fructose (Fru) and sucrose (Suc). Glucose-6-phosphate dehydrogenase (G6PDH) was first added to remove endogenous hexose-phosphates. Subsequently, hexokinase (HK), phosphoglucoisomerase (PGI) and β-fructosidase were added successively to measure Glc, Fru and Suc. Soluble sugars were detected with an EL808 ultramicroplate reader (BioTeK Inc., Germany) at 340 nm.

3.15 Phytohormone analysis

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 and is described in detail in Eggert and von Wirén (2017). Cytokinins, auxins, salicylic acid and abscisic acid (ABA) were extracted twice from 100 mg freeze-dried, grinded plant material in 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. Subsequently, 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. For the elution of cytokinins, 1 x 600 µl 60% MeOH containing 5% ammonium and 1 x 600 µl 60% acetonitrile

containing 5% ammonium were used. Samples were evaporated in a vacuum centrifuge to dryness, resolved in 10 µl 50% MeOH containing 0.5% FA, vortexed for 30 s and sonicated for 2 min, filled up to 50 µl with H_2O and transferred to a glass vial for analysis.

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 was set to 40°C and 4°C, respectively.

4 Results

4.1 Growth phenotype of wild-type and *qko* plants

In order to quantify growth differences between wild-type and *qko*, plants were cultivated hydroponically with 2 mM NO_3^- as the sole N source. Since the *qko* mutant has a reduced capacity for NH_4^+ uptake (Yuan et al., 2007a), NO_3^- was chosen as the sole N source to provide growth conditions in which N supply to plants is not limiting. After five weeks of growth, phenotypic differences became clearly visible. The shoots of *qko* plants appeared larger, leaf petioles more expanded and the leaf area appeared bigger (Figure 2A). In quantitative terms, the rosette diameter of *qko* shoots was 25% larger than that of wild-type shoots (Figure 2B).

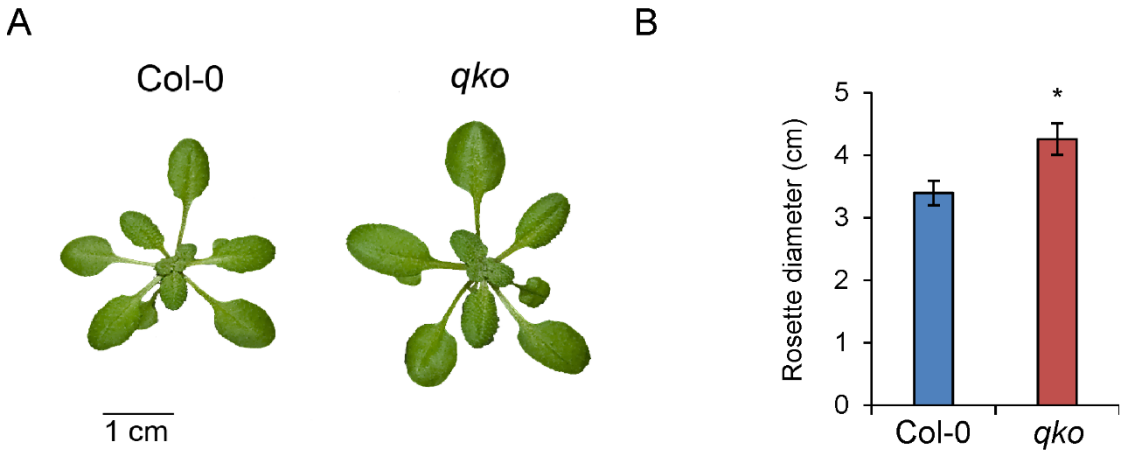


Figure 2. Phenotype of hydroponically-grown wild-type and *qko* plants. Phenotype (A) and rosette diameter (B) of wild-type (Col-0) and *qko* plants that were cultured for five weeks in nutrient solution containing 2 mM KNO_3 as the sole N source. Bars represent means ± SD (n = 6). Asterisk indicates a significant difference according to Student's t-test ($P < 0.05$).

In order to verify this phenotype also under different growth conditions, plants were grown on agar medium, which contained 2 mM NO_3^- as the sole N source. After 16 days of growth, the plants were phenotyped. In this growth system the shoots of *qko* plants appeared to be larger too, however differences were smaller (Figure 3A). The rosette diameter of *qko* shoots was 13% larger than that of wild-type shoots (Figure 3B) and confirmed the previous observation made in hydroponics.

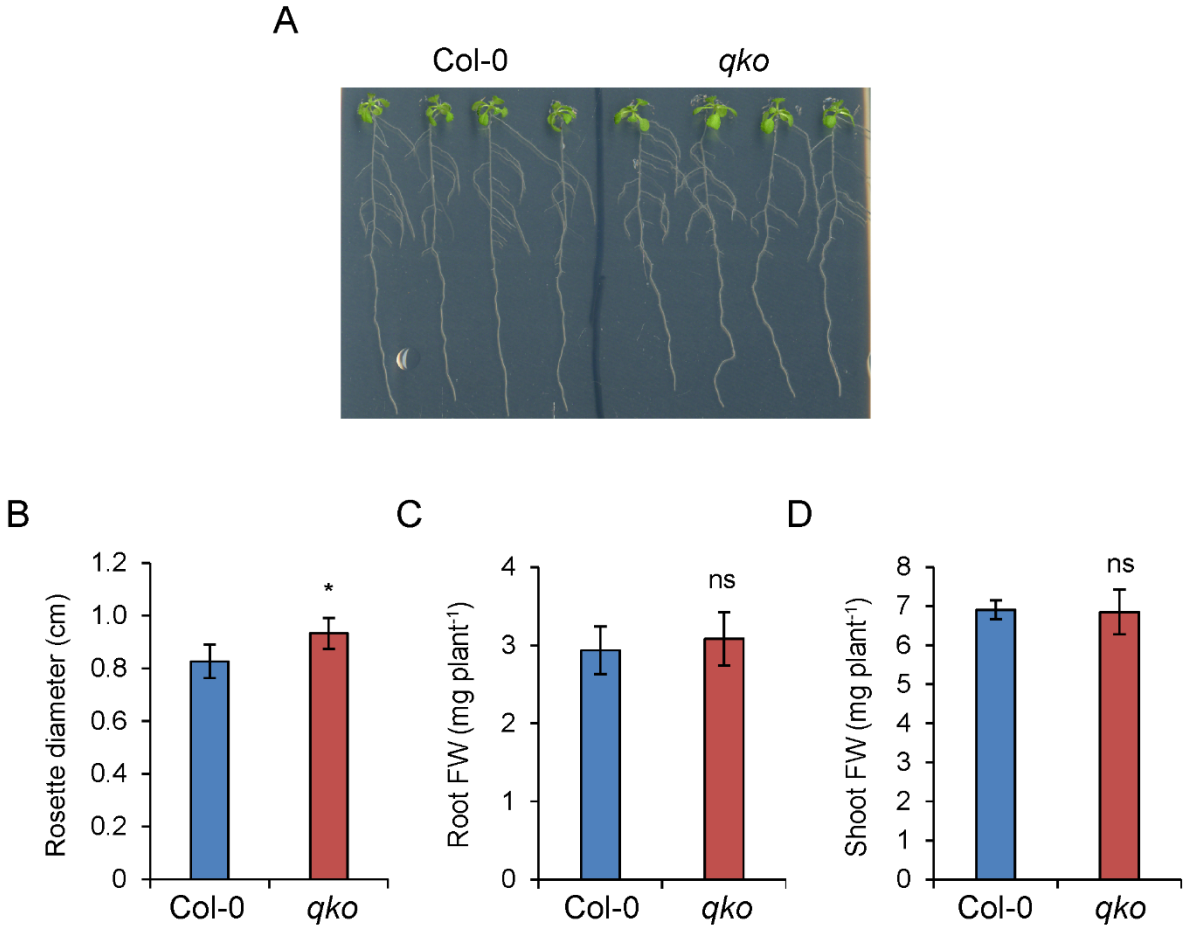


Figure 3. Phenotype and biomass of wild-type and *qko* plants grown on agar medium. Growth phenotype (A), rosette diameter (B), root (C) and shoot fresh matter (D) of wild-type (Col-0) and *qko* plants that were cultivated on 0.5 MS agar medium for 16 days containing 2 mM KNO_3 as the sole N source. Bars represent means ± SD (n = 4-5). Asterisk indicates a significant difference according to Student's t-test ($P < 0.05$). ns refers to not significant and FW to fresh weight.

Although the shoots of *qko* plants were bigger, the fresh weight of *qko* shoots was not significantly higher than of wild-type shoots (Figure 3D). Also the comparison of the root fresh weight of both genotypes showed no significant difference (Figure 3C). The phenotypic differences between wild-type and *qko* plants were small in young plants (Figure 3) and became more prominent at later developmental stages (Figure 2).

In order to understand how *qko* plants can grow larger shoots than wild-type plants without accumulating more biomass, several shoot and leaf traits were examined (Figure 4). In addition to the standard growth condition with 2 mM NO_3^- as N source, two further N treatments were included. Plants were provided with 0.3 mM NO_3^-, which is known to induce high-affinity NO_3^- uptake systems and NRT2-type nitrate

transporters (Crawford and Glass, 1998; Filleur et al., 2001; Li et al., 2007). Alternatively, adequate N supply was provided by 1 mM NH_4NO_3, a condition in which the high-affinity NO_3^- transport system is known to be repressed (Lejay et al., 1999). Again, the shoot fresh weights of wild-type and *qko* plants grown on 2 mM NO_3^- did not differ significantly from each other, and also in the other two N treatments the shoot biomass of wild-type and *qko* plants were not significantly different from each other (Figure 4A). Although under adequate NO_3^- supply (2 mM NO_3^-) the rosettes of *qko* plants showed a larger diameter (Figure 3A and B), the total leaf area of *qko* shoots was not bigger as compared to the wild-type (Figure 4B). However, the shoots of *qko* plants grown on 0.3 mM NO_3^- or 1 mM NH_4NO_3 as N source had a larger leaf area than wild-type shoots (Figure 4B). When comparing the specific leaf area of NO_3^--fed wild-type and *qko* plants, significant differences could only be observed when *qko* plants were cultivated on 1 mM NH_4NO_3 (Figure 4C).

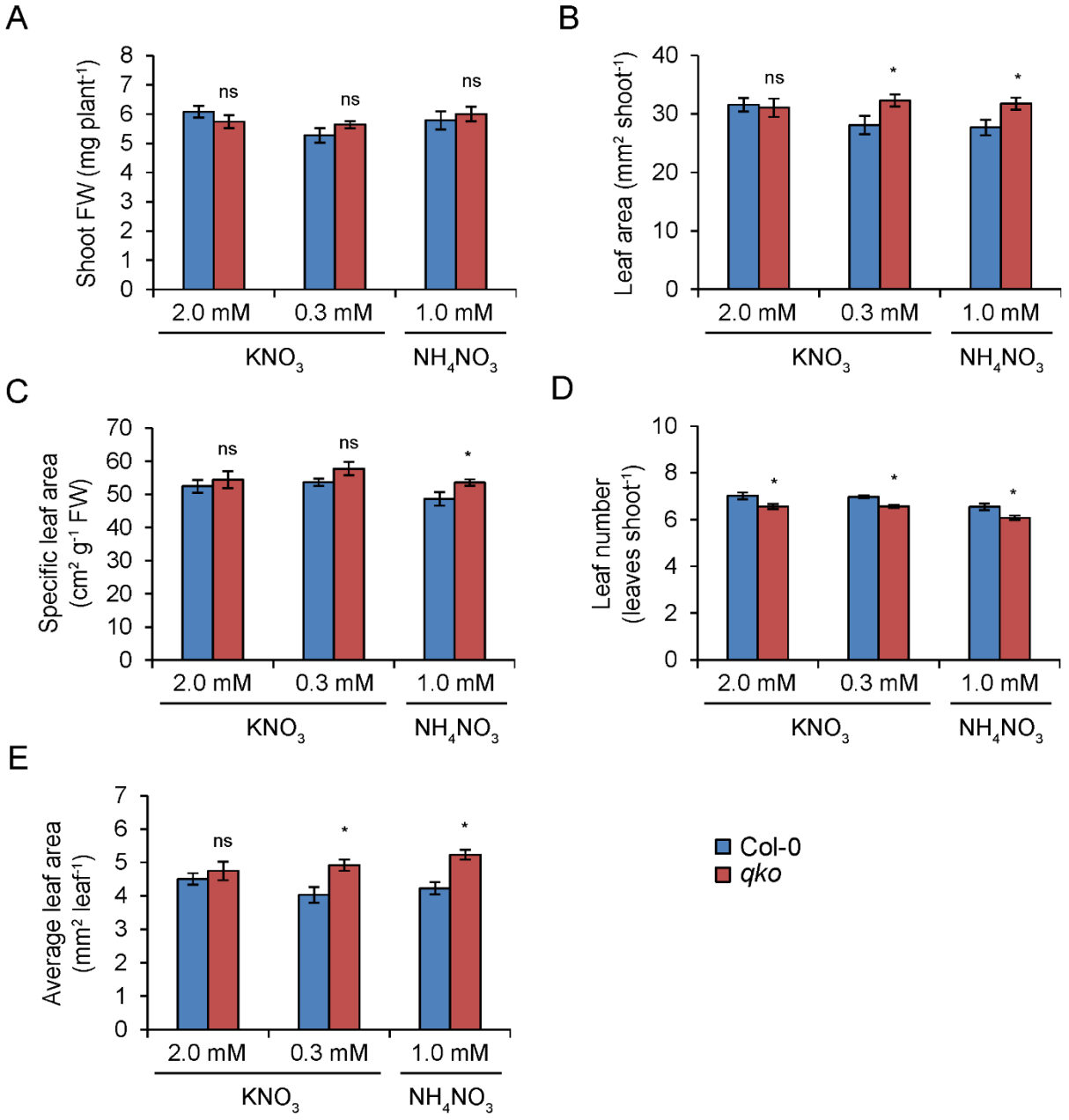


Figure 4. Shoot and leaf traits of wild-type and *qko* plants grown on agar medium. Shoot biomass (A), leaf area (B), specific leaf area (C), leaf number (D) and average leaf area (E) of wild-type (Col-0) and *qko* plants that were precultured on 0.5 MS agar medium for 9 days containing 2 mM KNO_3 as the sole N source and then transferred to either 2 mM KNO_3, 0.3 mM KNO_3 or 1 mM NH_4NO_3 for 7 days. Bars represents means ± SE (n = 6 – 7). Significant differences to the wild-type, Col-0, at $P < 0.05$ as determined by t-test are indicated by asterisks. ns refers to not significant. FW, fresh weight.

Interestingly, in all three N treatments, *qko* plants developed less leaves than wild-type plants (Figure 4D). Dividing the total leaf area by the number of leaves showed that *qko* plants had a larger area per leaf than wild-type plants, except for the 2 mM NO_3^- treatment (Figure 4E). These results indicated that the shoots of *qko* plants

appeared to be bigger, because *qko* plants were producing less leaves with a larger area (Figure 3 and Figure 4).

For further phenotypic and histological analyses, the cotyledons of wild-type and *qko* plants were sampled (Figure 5).

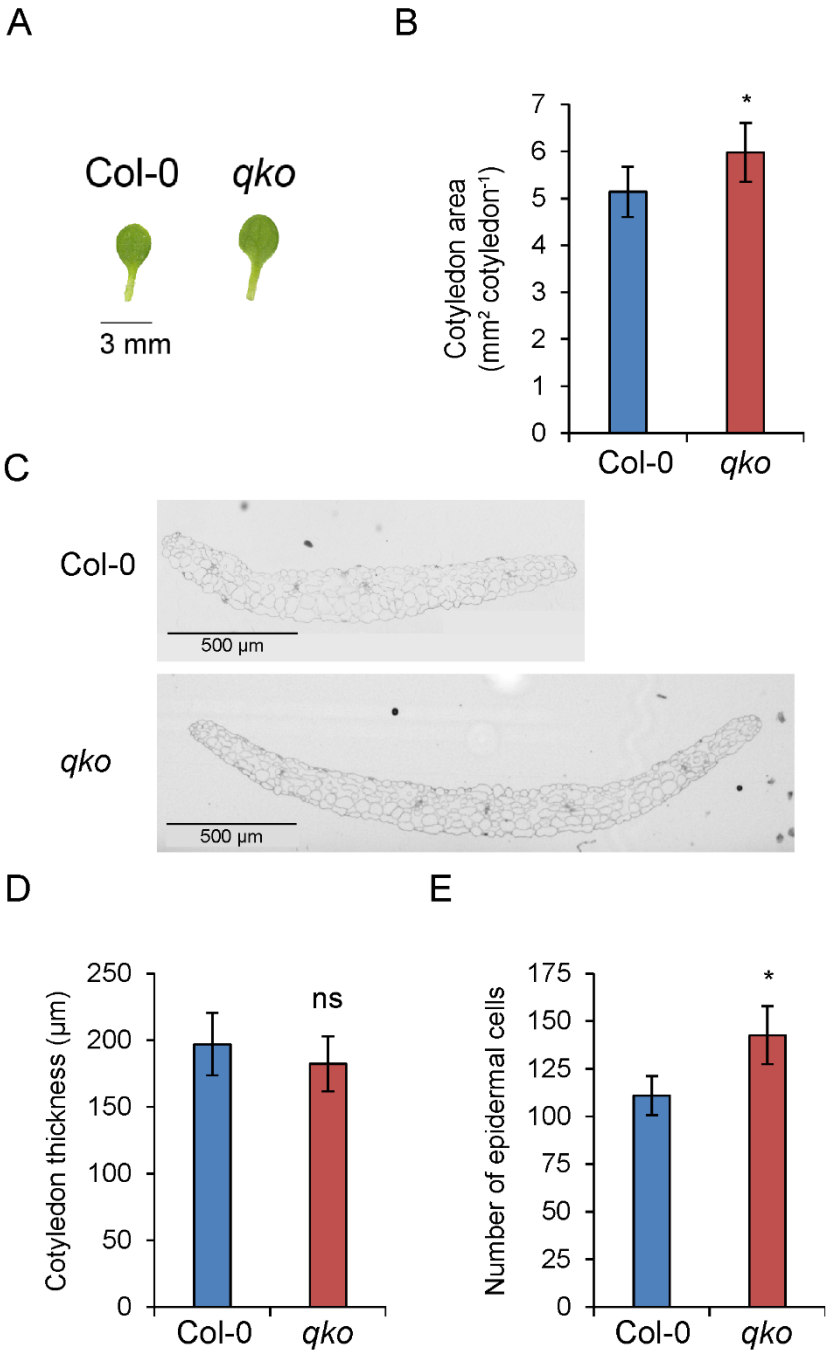


Figure 5. Phenotype and histological characterization of cotyledons from wild-type and *qko* plants. Leaf shape (A), area (B) cross section (C) and thickness of cotyledons (D) as well as number of epidermal cells (E) from cotyledons of wild-type (Col-0) and *qko* plants cultured for 16 days on 0.5 MS agar medium containing 2 mM KNO_3 as the sole N source. Bars represent means ± SD (n = 7). Asterisks indicate

significant differences according to Student's t-test ($P < 0.05$). ns refers to not significant.

The size of a cotyledon of a *qko* plant was visibly larger compared to the size of a cotyledon of a wild-type plant (Figure 5A). This observation was confirmed by image analysis. The area of *qko* cotyledons was significantly larger when compared to the wild-type, representing an increase of 16% (Figure 5B). The size difference became very clear in the cross sections of the cotyledons (Figure 5C). The cross sections were taken from the middle of the cotyledon, where the leaf was widest. Examination of the cross sections revealed that wild-type and *qko* cotyledons did not differ in thickness (Figure 5D). This observation is in accordance with the previous analysis of the specific leaf area of NO_3^--fed plants (Figure 4C). There could be two reasons at the histological level why *qko* leaves were larger than wild-type leaves. One possibility is that the leaves of *qko* plants have larger cells. Alternatively, *qko* leaves may have more cells. Since the size of the cells in the cross section of wild-type and *qko* cotyledons did not appear to be larger (Figure 5C), the number of epidermal cells in wild-type and *qko* cotyledons was counted. The cotyledons of *qko* plants produced in average 143 epidermal cells, whereas the cotyledons of wild-type plants just had 111 epidermal cells, corresponding to an increase in the number of epidermal cells by 29% in *qko* plants (Figure 5E).

These results showed that the shoots of *qko* plants appear bigger than wild-type shoots, because they have a larger diameter and produce less leaves with a larger area. In the case of cotyledons, the larger area was associated with the production of more cells.

4.2 NO_3^- influx studies in wild-type and *qko* plants

Previous studies have shown that the shoot growth of NO_3^--fed plants is stronger than of NH_4^+-fed plants (Walch-Liu et al., 2000; Helali et al., 2010). Since *qko* plants have a reduced capacity for NH_4^+ uptake (Yuan et al., 2007a), a putatively increased capacity for NO_3^- uptake might compensate for this and be linked to the increased shoot growth of *qko* plants. To test this hypothesis, the N nutritional status in wild-type and *qko* plants was determined and NO_3^- influx studies were carried out.

4.2.1 N nutritional status of wild-type and *qko* plants

In order to investigate the N nutritional status of wild-type and *qko* plants, plants were precultured hydroponically under continuous supply of 2 mM NO_3^- as the sole N source, before half of the plants were subjected to N-deficient conditions for three days. The root biomass of wild-type plants was significantly higher when N was omitted for three days as compared to the root biomass of wild-type plants grown under continuous NO_3^- supply (Figure 6A). At that time, the shoot biomass of wild-type plants was not yet affected by N supply (Figure 6B). This increase in root-to-shoot biomass ratio is a common response to N deficiency and has been reported in many plant species (Marschner, 2012). Also, *qko* plants showed an increase in root biomass when grown under N-deficient conditions (Figure 6A). Interestingly, the shoot biomass of *qko* plants, that had been subjected to N-deficient conditions, also increased compared to the shoot biomass of *qko* plants under continuous NO_3^- supply (Figure 6B). Wild-type plants showed an increased root-to-shoot biomass ratio by 53%, whereas the root-to-shoot biomass ratio of *qko* plants increased only by 28% in response to N deficiency. The root (Figure 6A) and the shoot dry weights (Figure 6B) of *qko* plants grown under N-deficient conditions were significantly higher than of those of wild-type plants. For plants grown under N-sufficient conditions no changes in biomass could be observed between the genotypes (Figure 6A and B).

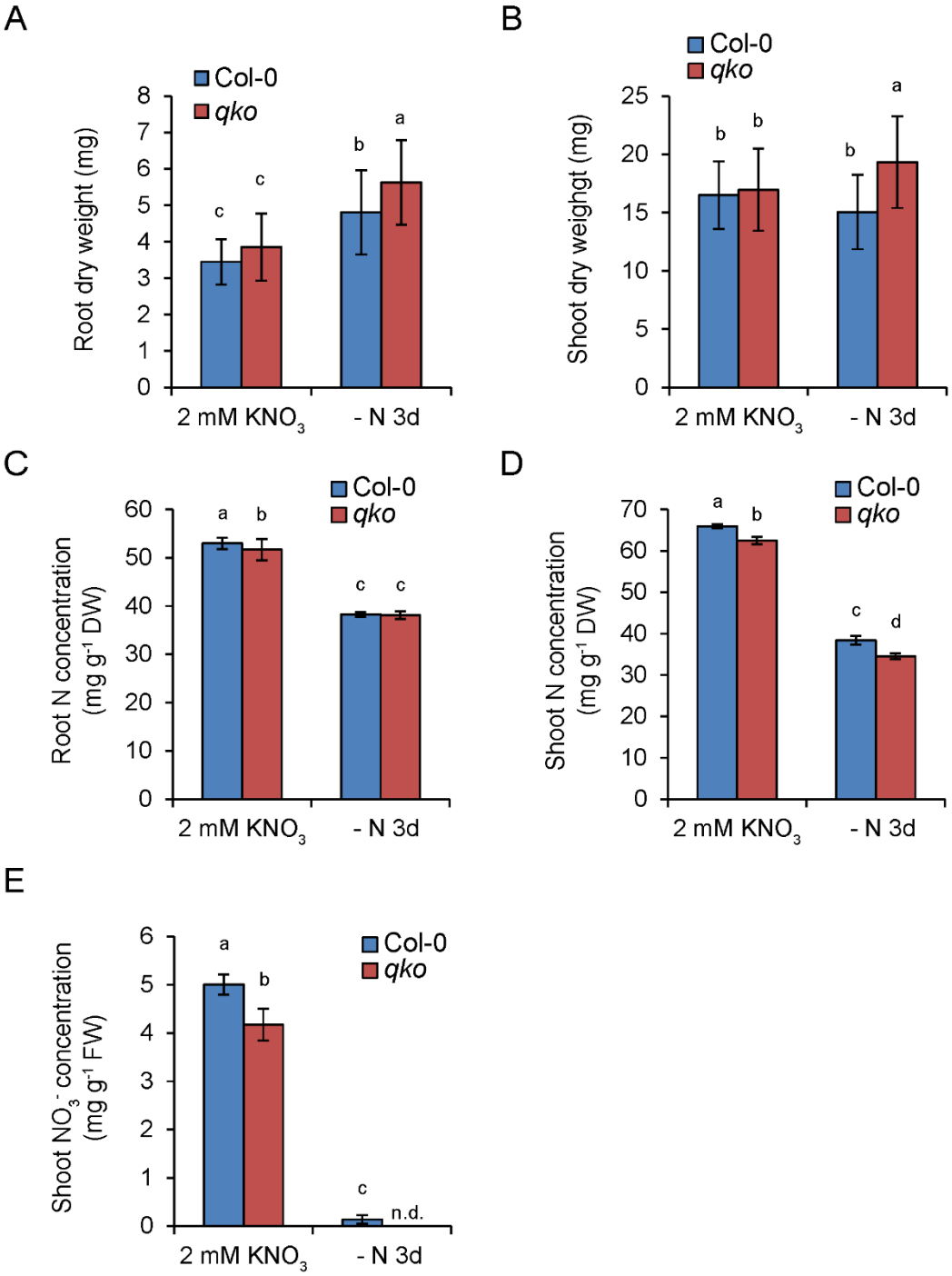


Figure 6. Nitrogen nutritional status and biomass of wild-type and *qko* plants. Root (A) and shoot (B) dry weights, total N concentrations in roots (C) and shoots (D) and shoot NO_3^- concentrations (E) of wild-type (Col-0) and *qko* plants precultured for six weeks under continuous supply of 2 mM KNO_3 as the sole N source or subjected to N deficiency for three days (- N 3d). Bars in A to D represent means ± SD (n = 9 - 10). Bars in E represent means ± SE (n = 3 - 4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight, n.d. to not detectable. FW, fresh weight.

The concentrations of total N in roots (Figure 6C) and shoots (Figure 6D) of *qko* plants grown under the continuous supply of NO_3^- were slightly lower compared to the wild-type. In both genotypes, when cultivated in nutrient solution containing 2 mM NO_3^-, the concentration of total N in roots of were between five and six percent (Figure 6C) and between six and seven percent in shoots (Figure 6D). These N values indicated a sufficient or even "luxury" N status of the plants. Shifting wild-type and *qko* plants to N-free nutrient solution for three days resulted in a significant reduction in the concentration of total N in roots (Figure 6C) and shoots (Figure 6D). The shoots of N-deprived *qko* plants had a slightly lower concentration of N as compared to wild-type shoots (Figure 6D). Between roots of wild-type and *qko* plants, that had been cultivated in N-free medium, no significant differences were observed (Figure 6C). The shoots of *qko* plants grown under NO_3^- supply accumulated lower amounts of NO_3^- than wild-type shoots (Figure 6E). Omitting N from the medium led to a 36 times lower NO_3^- concentration in wild-type shoots. The concentration of NO_3^- in *qko* shoots decreased to non-detectable levels upon N withdrawal (Figure 6E). Although *qko* plants had slightly lower total N and NO_3^- concentrations (Figure 6C, D and E) than the wild-type, the biomass was not lower under N-sufficient conditions; under N-deficient conditions it was even higher (Figure 6A and B). Considering the lower N and NO_3^- levels but higher shoot biomass in N-deficient *qko* plants indicated that the presence of NO_3^- itself in the shoot tissues was not the cause for the formation of larger leaves in *qko*.

4.2.2 *qko* has a higher capacity for high-affinity NO_3^- influx

Short-term influx of ^{15}N-labeled NO_3^- into roots of wild-type and *qko* plants at 2 mM and 0.2 mM external NO_3^- supply was examined as a measure for the combined low-affinity and high-affinity transport capacity (2 mM $^{15}NO_3^-$) and for the high-affinity transport capacity (0.2 mM $^{15}NO_3^-$), respectively. At 2 mM $^{15}NO_3^-$ supply the capacity for NO_3^- transport of *qko* plants was neither under N-sufficient nor under N-deficient conditions significantly different from that of Col-0 (Figure 7A). Wild-type and *qko* plants that had been precultured in nutrient solution in the absence of N for three days showed a decreased capacity for NO_3^- uptake at 2 mM $^{15}NO_3^-$ supply, as compared to plants that received a continuous supply of NO_3^- (Figure 7A). The high-affinity NO_3^- transport system responded in a different way to the absence of AMTs in the *qko* mutant. In plants, precultured with sufficient N, high-affinity NO_3^- influx into

the roots of the *qko* mutant was 49% higher than in Col-0 (Figure 7B). Under N-deficient conditions the capacity for high-affinity NO_3^- uptake of *qko* plants was still 20% larger compared to that of wild-type plants. In wild-type plants the N nutritional status did not affect the capacity for high-affinity NO_3^- influx (Figure 7B). This is in accordance to a previous study that showed that the capacity for high-affinity NO_3^- influx increases up to 48 hours of N-withdrawal but dropped to the capacity of N-sufficient plants after 72 hours of N deficiency (Lejay et al., 1999). By contrast, the high-affinity NO_3^- uptake capacity in *qko* plants was significantly higher under N-sufficient conditions than under N-deficient conditions (Figure 7B). This differential response of the high-affinity transport capacity to the N preculture condition in wild-type and *qko* plants suggested that components regulating high-affinity NO_3^- uptake were affected by the absence of AMTs in *qko* plants.

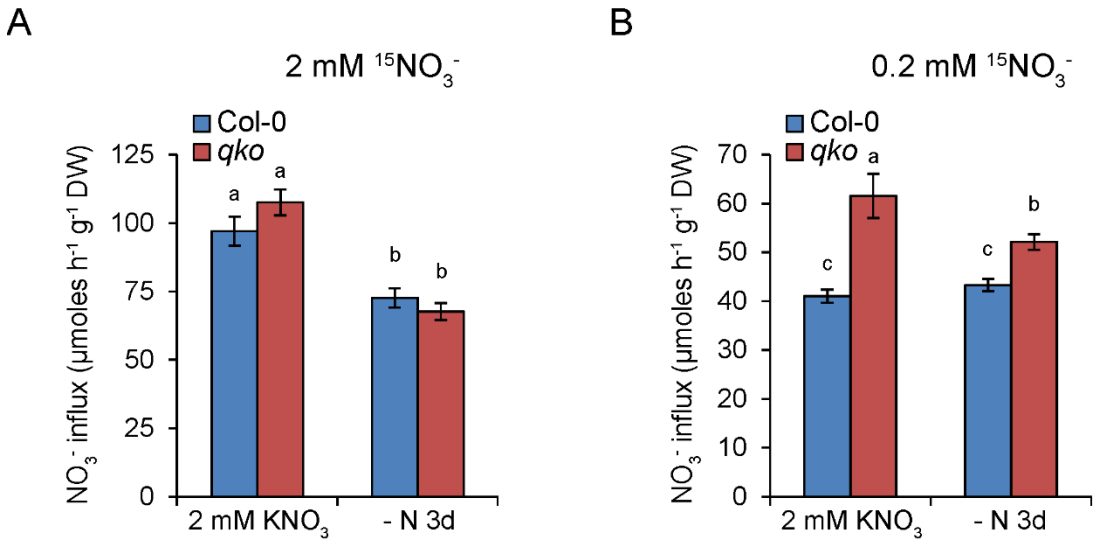


Figure 7. NO_3^- influx into wild-type and *qko* roots. Influx of ^{15}N-labeled NO_3^- supplied at a concentration of 2 mM being indicative for the combined low-affinity and high-affinity transport system (A) or at 0.2 mM being indicative for the high-affinity transport system (B) into roots of wild-type (Col-0) and *qko* plants that had been precultured for six weeks under continuous supply of 2 mM KNO_3 as the sole N source or subjected to N deficiency for three days (- N 3d). Bars represent means ± SE (n = 8 – 10). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight.

4.2.3 Gene expression analysis of the major NO_3^- influx transporters in roots of wild-type and *qko* plants

In order to investigate if the differential capacity for NO_3^- uptake in wild-type and *qko* plants is related to differences in the regulation of NO_3^- transporter genes, the transcript levels of the major NO_3^- influx transporters in roots of wild-type and *qko* plants were determined by qRT-PCR. Compared to N-sufficient wild-type plants, transcript levels of the dual-affinity NO_3^- transporter *NRT1.1* in *qko* roots were decreased (Figure 8A). In both lines, N deficiency decreased *NRT1.1* expression levels by > 10-fold. This reduced expression was in agreement with the reduced capacity for NO_3^- uptake at 2 mM $^{15}NO_3^-$ in plants pre-cultured under N deficiency (Figure 7A). Comparing the expression of the major high-affinity NO_3^- transporter *NRT2.1* between N-sufficient wild-type and *qko* plants revealed 72% higher transcript levels in *qko* roots (Figure 8B). These elevated transcript levels were reflected by a higher capacity for high-affinity NO_3^- uptake (Figure 7B). Omitting N from the nutrient solution for three days lead to a strong decrease in *NRT2.1* transcript levels in *qko* but did not change *NRT2.1* expression levels remarkably in wild-type roots (Figure 8B). Thus, gene expression analysis reinforced the observation that differences in NO_3^- uptake between the two lines are most prominent in plants adequately supplied with NO_3^-, while these differences disappear when plants become N deficient.

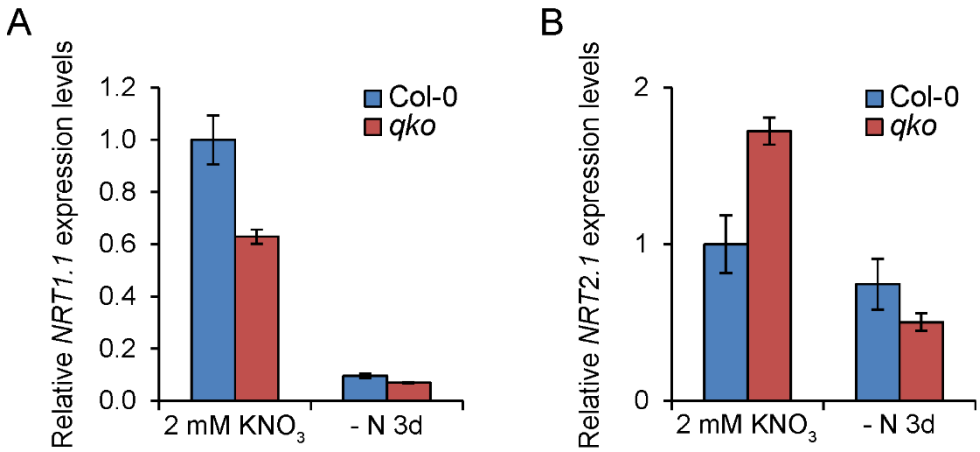


Figure 8. Gene expression analysis of the major NO_3^- uptake transporters in roots of wild-type and *qko* plants. Transcript levels of *NRT1.1* (A) and *NRT2.1* (B) in roots of wild-type (Col-0) and *qko* plants that had been precultured for six weeks under continuous supply of 2 mM KNO_3 as the sole N source or subjected to N deficiency for three days (- N 3d). Bars represent means ± SE (n = 3).

Relative to wild-type plants, elevated *NRT2.1* expression levels (Figure 8B) might contribute to the increased capacity for high-affinity NO_3^- influx in adult vegetatively growing *qko* roots (Figure 7B). To reinforce this hypothesis, expression analysis of NRT2.1 were conducted at the protein level. Since differences in high-affinity NO_3^- uptake and *NRT2.1* gene expression levels were mainly observed under N-sufficient conditions, another N-sufficiency treatment replaced the N-deficiency treatment in this experiment. As before, one treatment comprised of a continuous supply of 2 mM KNO_3. For the second N-sufficiency treatment plants were shifted to nutrient solution with 1 mM NH_4NO_3 as N source during the last seven days. The supply of 1 mM NH_4NO_3 is known to repress high-affinity NO_3^- uptake and *NRT2.1* gene expression levels (Lejay et al., 1999).

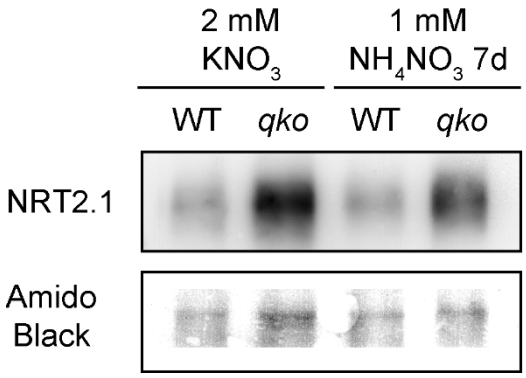


Figure 9. NRT2.1 protein levels in roots of wild-type and *qko* plants. Protein gel blot analysis of microsomal membrane fractions from roots of 6-week-old wild-type (Col-0) and *qko* plants using an antibody raised against NRT2.1. Plants were cultured hydroponically under continuous supply of 2 mM KNO_3 as the sole N source or were pre-cultured with 1 mM NH_4NO_3 for 7 d. Amido Black-staining served as loading control.

Even though protein loading of NO_3^--precultured *qko* roots was slightly higher than in wild-plants the expression levels of NRT2.1 were dramatically increased in the roots of *qko* (Figure 9). The NRT2.1 protein levels in the roots of *qko* plants that had been precultured on NH_4NO_3 were also higher in comparison to the wild-type. Under both N-sufficient conditions NRT2.1 levels were low in wild-type roots and derepressed in the roots of *qko* plants.

4.2.4 NO_3^- influx studies and expression analysis of genes involved in NO_3^- acquisition in roots of wild-type and *qko* plants

In order to investigate if the enhanced capacity for high-affinity NO_3^- uptake in *qko* is robust and holds true also in different growth systems, a short-term influx study with ^{15}N-labeled NO_3^- was conducted with plants that were precultured on agar medium. Seven days before the influx experiment, plants were transferred to plates containing different concentrations of NO_3^- to investigate the influence of the nutritional status on high-affinity NO_3^- uptake in wild-type and *qko* plants.

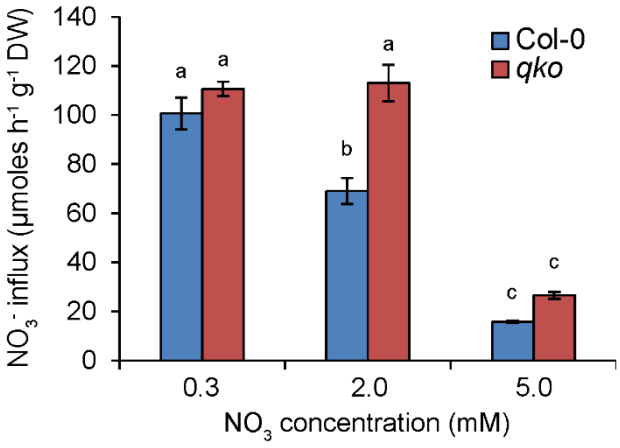


Figure 10. High-affinity NO_3^- influx in roots of wild-type and *qko* plants in dependence of NO_3^- concentrations. Influx of ^{15}N-labeled NO_3^- supplied at a concentration 0.2 mM in roots of wild-type (Col-0) and *qko* plants that had been precultured on 0.5 MS agar medium for 9 days and then transferred to either 0.3, 2.0 or 5.0 mM KNO_3 as the sole N source for 7 days. Bars represent means ± SE (n = 7 – 8). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight.

With increasing N supply during preculture roots of wild-type plants showed a gradual decrease in the capacity for high-affinity NO_3^- uptake (Figure 10). In contrast, the capacity for high-affinity NO_3^- uptake into *qko* roots was not repressed by increasing the NO_3^- pre-supply from 0.3 to 2 mM (Figure 10). However, a further elevation to 5 mM NO_3^- also repressed NO_3^- influx into roots of *qko* plants. When comparing the capacity for high-affinity NO_3^- uptake between wild-type and *qko* plants at a pre-supply level of 2 mM NO_3^-, the capacity of Col-0 was significantly lower than of *qko* (Figure 10). At the other two NO_3^- pre-treatments no significant differences between wild-type and *qko* plants were observed.

To investigate if the altered capacity for NO_3^- uptake in *qko* plants is also caused by a change in *NRT2.1* expression, relative transcript levels of genes involved in NO_3^- uptake, including *NRT2.1*, were determined. For this purpose, gene expression levels were determined in wild-type and *qko* plants grown at 0.3 mM or 2 mM NO_3^-. While mRNA levels of *NRT1.1* showed the expected response, i.e. higher levels in plants being supplied with 2 mM, there was no difference between wild-type and *qko* plants as previously observed (Figure 11A). Furthermore, no difference in *NRT2.1* mRNA levels were observed between wild-type and *qko* roots in either N treatment (Figure 11C). Transferring plants to plates with a low NO_3^- concentration for seven days derepressed *NRT2.1* in both lines. Expression analyses of the other two members of the *NRT2*-family, namely *NRT2.2* and *NRT2.4*, and of *NRT3.1* (*NAR2.1*) showed that they were also induced by low NO_3^- supply in both genotypes (Figure 11D, E and F). For *NRT2.2* and *NRT3.1*, no differences between wild-type and *qko* were observed under the tested conditions (Figure 11D and F). Interestingly, the relative transcript levels of the high-affinity NO_3^- transporter *NRT2.4* were 1.9-fold increased in *qko* roots compared to the wild-type under standard NO_3^- supply (Figure 11E). Under low NO_3^- supply no difference in *NRT2.4* expression was visible between the two lines. Similar to hydroponically-grown plants (Figure 8A), mRNA levels of *NRT1.1* in agar-grown plants were lower under low N supply compared to standard N supply (Figure 11A). In plate-grown plants, other than in hydroponically-cultivated plants, no differences in *NRT1.1* levels could be observed between wild-type and *qko* plants. Expression analyses of the NO_3^- transporter *NRT1.2* (Figure 11B) and *CIPK23* (Figure 11G), a kinase involved in NO_3^- signaling, revealed no major changes between the two genotypes under the two N conditions tested.

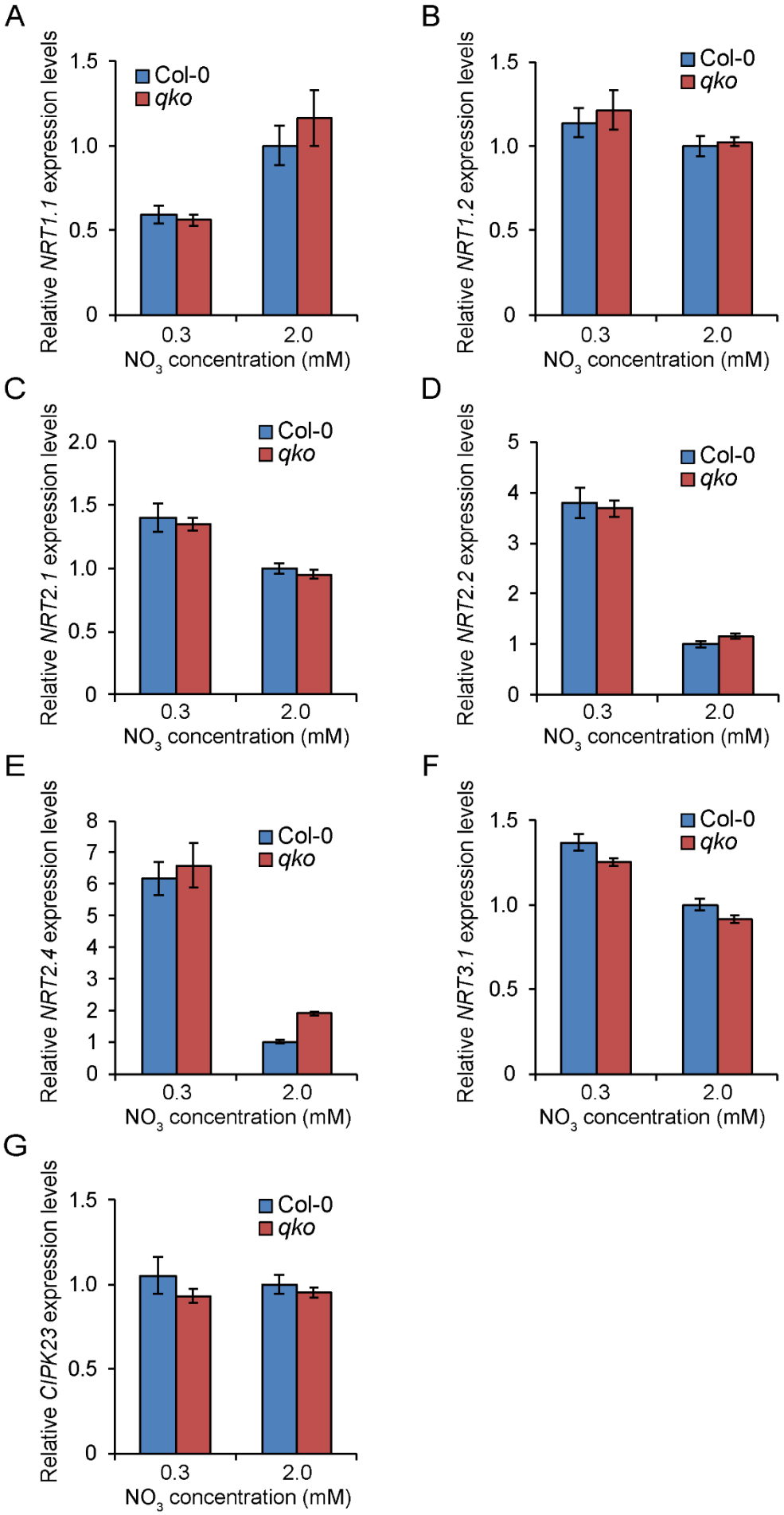

A
Relative NRT1.1 expression levels
1.5
1.0
0.5
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
B
Relative NRT1.2 expression levels
1.5
1.0
0.5
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
C
Relative NRT2.1 expression levels
2.0
1.5
1.0
0.5
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
D
Relative NRT2.2 expression levels
5
4
3
2
1
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
E
Relative NRT2.4 expression levels
8
7
6
5
4
3
2
1
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
F
Relative NRT3.1 expression levels
1.5
1.0
0.5
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)
G
Relative CIPK23 expression levels
1.5
1.0
0.5
0
Col-0
qko
0.3
2.0
NO3 concentration (mM)

Figure 11. Transcript levels of NO_3^- transporter genes in dependence of NO_3^- supply during preculture. Transcript levels of NO_3^- transporter genes were determined in roots of wild-type (Col-0) and *qko* plants that had been precultured on 0.5 MS agar medium for 9 days and then transferred to either 0.3 or 2.0 mM KNO_3 as the sole N source for 7 days. Bars represents means ± SE (n = 4).

In contrast to hydroponically-grown *qko* plants (Figure 7B and Figure 8B) that were older and further developed than agar-cultured plants, the higher capacity for high-affinity NO_3^- uptake in *qko* was not a result of higher *NRT2.1* transcript levels (Figure 10 and Figure 11C). Instead, higher *NRT2.4* mRNA levels in those plants might have contributed to the higher uptake capacity. Also, buffering the agar medium could have caused a different result compared to cultivation in unbuffered nutrient solution.

4.2.5 NO_3^- uptake and *NRT2.1* gene expression in wild-type, *qko* and triple *amt* insertion lines

In hydroponically-grown N-sufficient *qko* plants, the absence of the four ammonium transporters, *AMT1;1*, *AMT1;2*, *AMT1;3* and *AMT2;1* led to increased *NRT2.1* transcript levels and subsequently to an increased capacity for high-affinity NO_3^- uptake. To investigate if one specific AMT could be responsible for the deregulation of high-affinity NO_3^- influx, short-term uptake studies with ^{15}N-labeled NO_3^- were conducted in *amt* triple knock-out lines. Wild-type, *qko* and triple insertion lines encoding either functional *AMT1;1* (*qko11*), *AMT1;2* (*qko12*), *AMT1;3* (*qko13*), or *AMT2;1* (*qko21*) in the *qko* background were cultivated under continuous supply of 2 mM NO_3^- or treated with 1 mM NH_4NO_3 for seven days prior to the experiment.

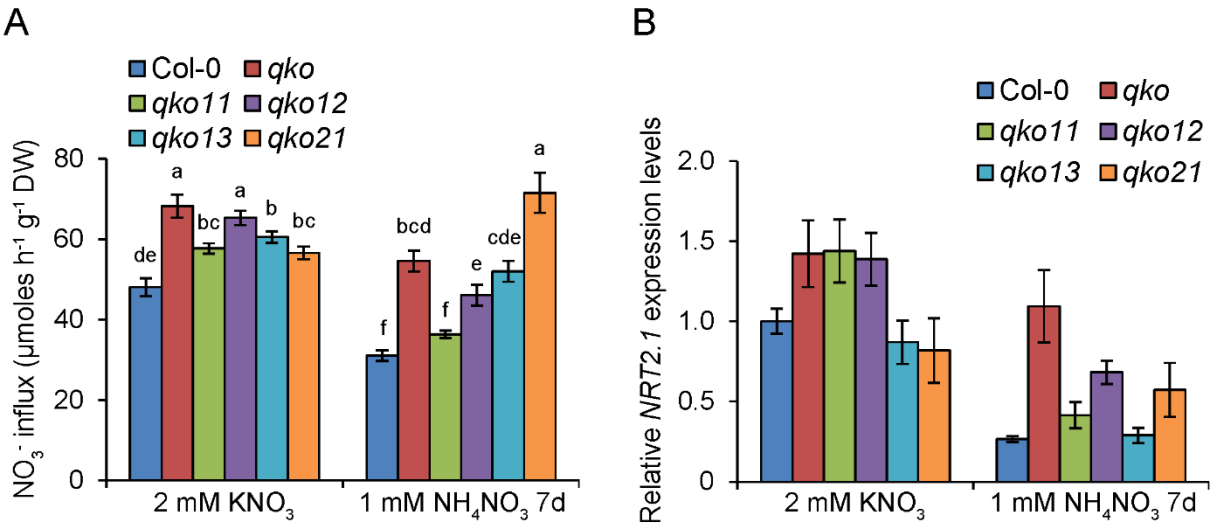


Figure 12. NO_3^- influx and *NRT2.1* gene expression in roots of wild-type, *qko* and triple insertion lines. (A) Influx of ^{15}N-labeled NO_3^- supplied at a concentration of 0.2 mM and *NRT2.1* transcript levels (B) in roots of wild-type (Col-0), the quadruple insertion line *qko*, and triple insertion lines encoding *AMT1;1* (*qko11*), *AMT1;2* (*qko12*), *AMT1;3* (*qko13*), or *AMT2;1* (*qko21*) in the *qko* background. Plants had been precultured for six weeks under continuous supply of 2 mM KNO_3 as the sole N source or shifted to nutrient solution containing 1 mM NH_4NO_3 for seven days. Bars represent means ± SE (n = 9 – 10 in (A) and n = 4 in (B)). Different letters indicate significant differences according to Fisher's LSD test (P < 0.05). DW refers to dry weight.

Under the pre-supply of NO_3^- as the sole N source, *qko* plants showed again a significantly increased capacity for high-affinity NO_3^- influx as compared to the wild-type (Figure 12A). In the triple insertion line *qko12*, NO_3^- uptake was as high as in *qko* plants. The capacity for high-affinity NO_3^- uptake in *qko11*, *qko13* and *qko21* was not significantly different from each other and reached an intermediate uptake capacity, between wild-type and *qko* levels. Pre-cultivating plants with NH_4NO_3 led to a reduction in the capacity for high-affinity NO_3^- uptake in all lines, but *qko21*. Also under this condition, the NO_3^- uptake capacity remained higher in *qko* than in wild-type plants. Surprisingly, *qko21* showed an even higher capacity for NO_3^- influx than *qko*. The pre-supply with NH_4NO_3 reduced NO_3^- influx in *qko11* roots to wild-type levels. NO_3^- uptake was significantly higher in *qko13* than in the wild-type and reached *qko* levels. The *qko12* line showed an intermediate capacity for high-affinity NO_3^- uptake, between wild-type and *qko* levels. The transcript levels of *NRT2.1* in the roots of NO_3^--grown wild-type, *qko* and *qko12* plants (Figure 12B) correlated well with the influx data (Figure 12A) with *qko* and *qko12* showing similar transcript levels that were higher than in the wild-type. Like the capacity for NO_3^- uptake, *NRT2.1* mRNA levels were higher in *qko11* than in Col-0, however not lower than in *qko* and *qko12* plants (Figure 12B). The relative *NRT2.1* mRNA levels in *qko13* and *qko21* were lower than in the wild-type. For these two triple insertion lines the transcript data did not correlate with the uptake data. Treating the plants with NH_4NO_3 reduced *NRT2.1* transcript levels in all lines (Figure 12B). Under this condition the expression levels of *NRT2.1* in Col-0, *qko*, *qko11* and *qko12* (Figure 12B) correlated well with the uptake data (Figure 12A). *NRT2.1* transcript levels were higher in *qko* than wild-type roots and intermediate in *qko12*. In *qko11 NRT2.1* levels were similar to the wild-type. In *qko13* and *qko21* the *NRT2.1* expression levels were also under this condition lower than the uptake data allowed to expect (Figure 12).

4.2.6 Influence of methionine sulfoximine on high-affinity NO_3^- influx in wild-type and *qko* plants

The absence of AMTs in NO_3^--fed *qko* plants led to increased NRT2.1 expression levels (Figure 8B, Figure 9 and Figure 12B) and a higher capacity for high-affinity NO_3^- uptake (Figure 7B and Figure 12A). To investigate if AMTs could play a regulatory role in repressing the high-affinity NO_3^- transport other than by merely increasing NH_4^+ concentrations in the cells of plant roots, short-term influx studies with ^{15}N-labeled NO_3^- were conducted after treatment with methionine sulfoximine (MSX). MSX is a glutamine synthetase inhibitor that constrains the incorporation of NH_4^+ into glutamate to build glutamine (Rhodes et al., 1986; Jackson et al., 1993). When plants take up NO_3^-, it is first reduced to NO_2^- and then to NH_4^+. In the presence of MSX, NH_4^+ cannot be assimilated and intracellular NH_4^+ concentrations increase (Rhodes et al., 1986). In order to increase intracellular NH_4^+ levels in the NH_4^+ uptake-defective mutant *qko*, plants were fed with 2mM KNO_3 and then treated with MSX for two or four hours. Treating NO_3^--fed plants with MSX for already two hours can increase root NH_4^+ concentrations substantially (Lanquar et al., 2009).

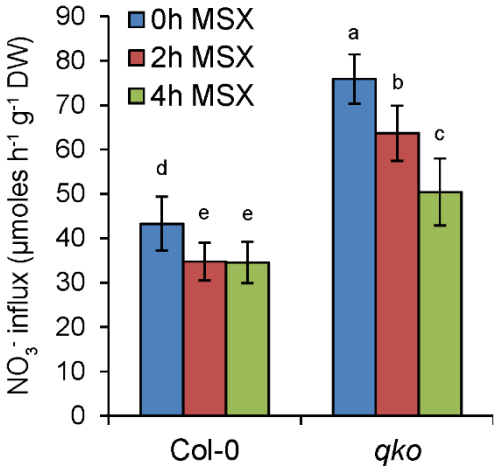


Figure 13. Influence of the glutamine synthetase inhibitor MSX on high-affinity NO_3^- uptake in wild-type and *qko* plants. Influx of ^{15}N-labeled NO_3^- supplied at a concentration 0.2 mM into roots of wild-type (Col-0) and *qko* plants that had been precultured for six weeks under continuous supply of 2 mM KNO_3 as the sole N source. One set of plants was treated with the glutamine synthetase inhibitor methionine sulfoximine (MSX) for 2 or 4 h. Bars represent means ± SE (n = 8 – 10). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight.

Also in this experiment, six weeks-old NO_3^--cultured *qko* plants exhibited a significantly higher capacity for high-affinity NO_3^- uptake than wild-type plants (Figure 13). The uptake capacity of untreated *qko* plants was 175% higher than that of Col-0 plants. Treating wild-type plants with MSX for two or four hours decreased the NO_3^- influx capacity by 20% compared to non-treated wild-type plants. Treating *qko* plants with MSX for two or four hours decreased the NO_3^- influx capacity to 84% or to 66%, respectively, of the capacity of non-treated *qko* plants. Thus, prolonged MSX treatment had a stronger effect on the capacity for high-affinity NO_3^- uptake in *qko* plants than in wild-type plants. These results suggested that intracellular NH_4^+ concentrations were involved in the repression of high-affinity NO_3^- uptake in *qko* plants.

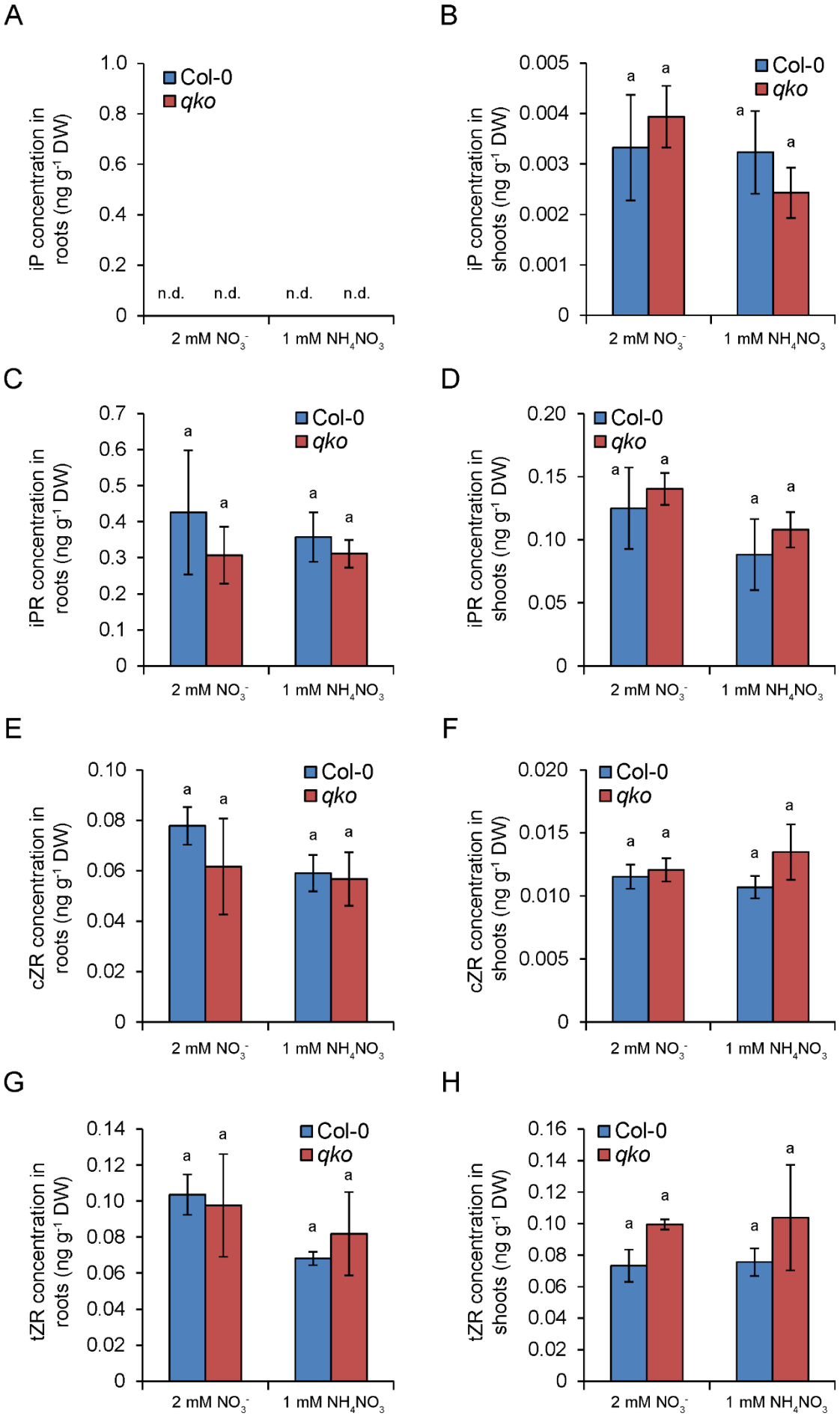
4.3 Biochemical characterization of wild-type and *qko* plants

The previous experiments have shown that N-sufficient *qko* plants had a larger shoot (Figure 2 - Figure 4) and a higher capacity for high-affinity NO_3^- uptake than wild-type plants (Figure 7, Figure 10 and Figure 12). Earlier studies have shown that phytohormones, especially cytokinins, are involved in regulating N acquisition (reviewed by Kiba et al. (2011) and that NO_3^- supply promotes cytokinin synthesis, distribution and leaf morphogenesis (Walch-Liu et al., 2000; Takei et al., 2001; Takei et al., 2004a; Rahayu et al., 2005; Lu et al., 2009; Kiba et al., 2011). It was also reported that amino acids (Quesada et al., 1997; Krapp et al., 1998; Vidmar et al., 2000; Nazoa et al., 2003) and carbohydrates (Lejay et al., 1999) play a role in regulating nitrate uptake. In order to determine if metabolic changes can be linked to altered capacities for NO_3^- uptake between wild-type and *qko* plants, biochemical analyses were conducted.

4.3.1 Phytohormone analyses in wild-type and *qko* plants

In order to see if the differences in plant growth and high-affinity NO_3^- uptake between wild-type and *qko* plants can be linked to altered phytohormone levels, the concentrations of cytokinins, auxins, abscisic acid (ABA) and salicylic acid were measured in roots and shoots of wild-type and *qko* plants that had been grown hydroponically under the continuous supply of 2 mM NO_3^- or had received 1 mM NH_4NO_3 for one week.

The concentrations of the biologically active cytokinin forms cis-zeatin and trans-zeatin were below the detection limit in all samples (data not shown). Isopentenyladenine (iP), another biologically active cytokinin, was detected only in shoots (Figure 14A and B). Its concentration did not differ significantly between wild-type and *qko* shoots of plants grown under continuous supply of NO_3^- or pre-supplied with NH_4NO_3 (Figure 14A and B). The concentration of the precursors and transport forms isopentenyladenine-riboside (iPR), cis-zeatin-riboside (cZR) and trans-zeatin-riboside (tZR) were slightly higher than that of iP and were detectable in shoots as well as in roots (Figure 14C to H). Comparing the concentrations of these precursors in roots and shoots of Col-0 and *qko* plants that were supplied with NO_3^- or NH_4NO_3 did not show significant differences (Figure 14C to H). The conjugated cytokinin-glucosides isopentenyladenin-9-glucoside (iP9G), trans-zeatin-O-glucoside (tZOG), trans-zeatin-9-glucoside (tZ9G) and trans-zeatin-O-glucoside riboside (tZOGR) were found in shoots, but not in roots of hydroponically cultivated plants (Figure 14I to P). The concentrations of iP9G and tZOG in shoots of wild-type and *qko* plants grown under either N treatment were not significantly different (Figure 14J and L). The concentration of tZ9G was significantly lower in wild-type shoots, when plants were supplied with NH_4NO_3 instead of KNO_3 (Figure 14N). In the shoots of NO_3^--supplied *qko* plants the concentration was slightly lower than in the wild-type and did not change significantly in the presence of NH_4^+ in the nutrient solution (Figure 14N). The shoots of wild-type plants showed an opposite behavior for the accumulation of the conjugate tZOGR (Figure 14P). Its concentration was significantly higher, when plants were supplied with NH_4NO_3 instead of KNO_3. In the shoots of NO_3^--supplied *qko* plants the tZOGR concentration was slightly higher than in the wild-type and did not differ significantly between N treatments (Figure 14P). Unfortunately, the detection of cis-zeatin and trans-zeatin was not possible. These minor differences in cytokinin conjugates made it impossible to draw any conclusion on differences in cytokinin action between wild-type and *qko* plants, that could have led to altered leaf growth or NO_3^- uptake capacities.


A
iP concentration in roots (ng g-1 DW)
Col-0
qko
n.d.
2 mM NO3-
1 mM NH4NO3
B
iP concentration in shoots (ng g-1 DW)
C
iPR concentration in roots (ng g-1 DW)
D
iPR concentration in shoots (ng g-1 DW)
E
cZR concentration in roots (ng g-1 DW)
F
cZR concentration in shoots (ng g-1 DW)
G
tZR concentration in roots (ng g-1 DW)
H
tZR concentration in shoots (ng g-1 DW)

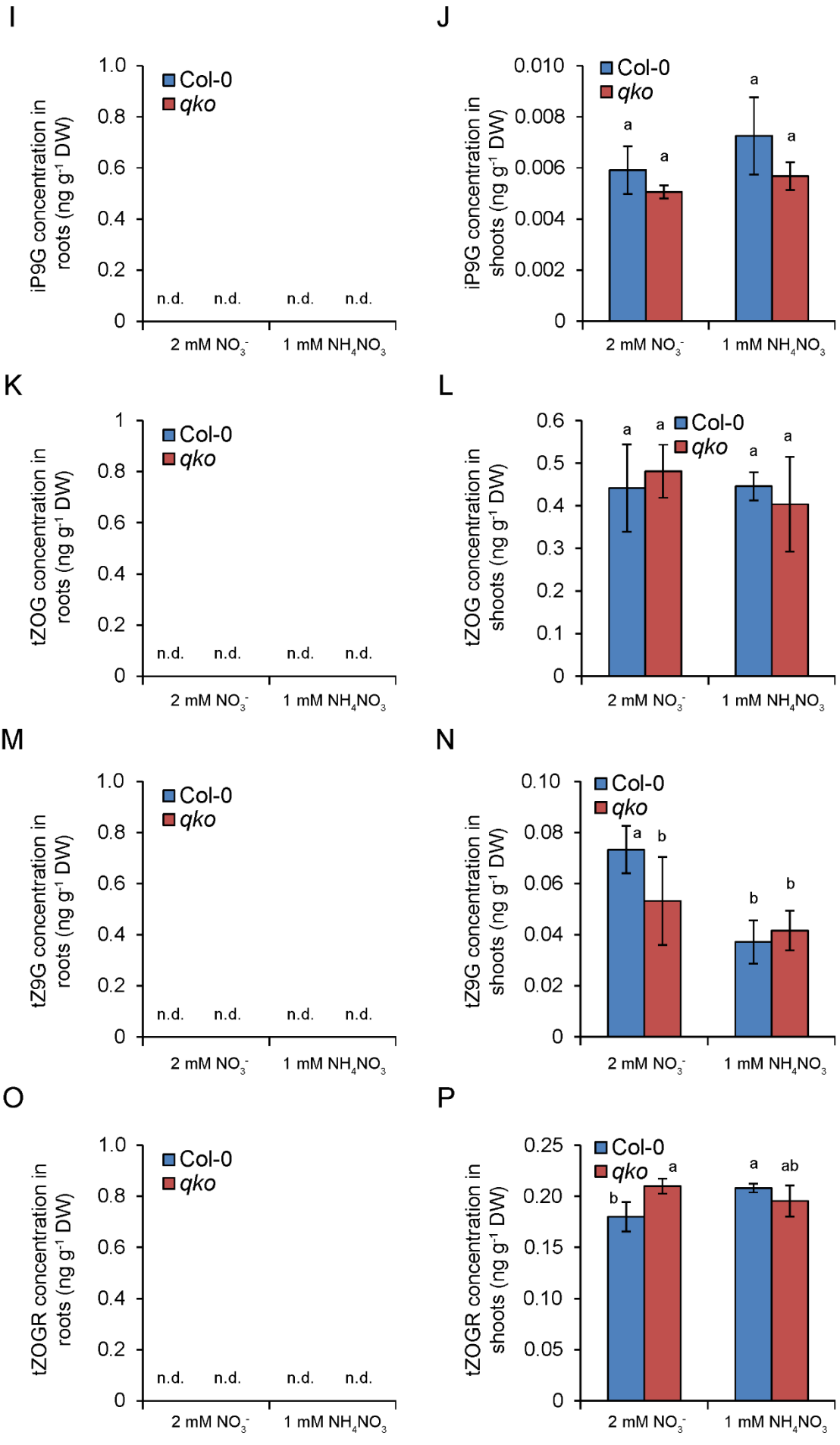
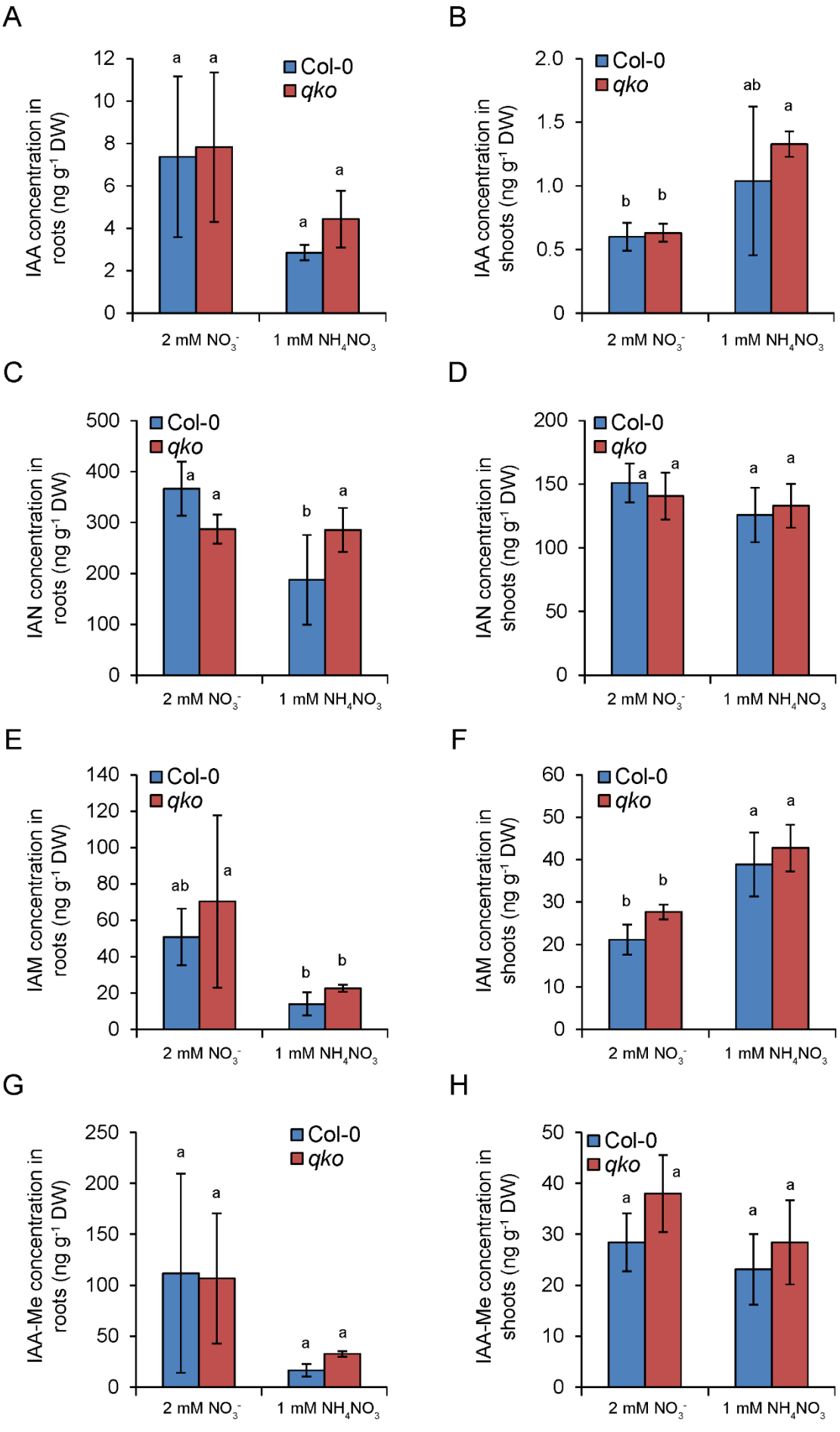

I
iP9G concentration in roots (ng g-1 DW)
Col-0
qko
n.d.
2 mM NO3-
1 mM NH4NO3
J
iP9G concentration in shoots (ng g-1 DW)
K
tZOG concentration in roots (ng g-1 DW)
L
tZOG concentration in shoots (ng g-1 DW)
M
tZ9G concentration in roots (ng g-1 DW)
N
tZ9G concentration in shoots (ng g-1 DW)
O
tZOGR concentration in roots (ng g-1 DW)
P
tZOGR concentration in shoots (ng g-1 DW)

Figure 14. Concentrations of cytokinins in wild-type and *qko* plants. Wild-type (Col-0) and *qko* plants were grown for six weeks in nutrient solution containing 2 mM NO_3^- as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. The concentrations of cytokinins were measured in roots (A, C, E, G, I, K, M, O) and shoots (B, D, F, H, J, L, N, P). Bars represent means ± SD (n = 3 - 4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight. iP, isopentenyladenine; iPR, isopentenyladenine-riboside; cZR, cis-zeatin-riboside; tZR, trans-zeatin-riboside; iP9G, isopentenyladenin-9-glucoside; tZOG, trans-zeatin-O-glucoside; tZ9G, trans-zeatin-9-glucoside; tZOGR, trans-zeatin-O-glucoside riboside.

When comparing the concentration of the active auxin indole-3-acetic acid (IAA) in shoots between wild-type and *qko* plants, no genotypic difference was observed under the tested conditions (Figure 15B). Incubating *qko* plants with NH_4NO_3 resulted in a significant elevation of IAA in the shoot as compared to NO_3^--fed *qko* plants. The IAA levels in the shoot of NH_4NO_3-pre-supplied wild-type plants were also elevated, but due to a high variance not statistically significant (Figure 15B). The concentrations of the auxin precursor indole-3-acetamide (IAM) and of the auxin catabolite 2-oxoindole-3-acetic acid (OxIAA) were also significantly increased in the shoot of wild-type and *qko* plants that had been pre-supplied with NH_4NO_3 instead of KNO_3 (Figure 15F and L). The OxIAA levels in shoots of NH_4NO_3-pre-supplied plants were even higher in *qko* than in the wild-type (Figure 15L). For the other auxin precursor indole-3-acetonitrile (IAN) (Figure 15D) and the conjugates indole-3-acetic acid methyl ester (IAA-Me) (Figure 15H) and indole-3-acetyl-L-alanin (IAA-ala) (Figure 15J), no genotypic or N supply-dependent differences were observed in the shoot. In roots, the only genotypic difference observed was for IAN (Figure 15C). Its concentration in NH_4NO_3-pre-supplied plants was significantly lower in wild-type roots than in *qko* roots. The IAN concentration in wild-type and *qko* roots was significantly higher in NO_3^--fed plants than in NH_4NO_3-pre-supplied wild-type roots. The roots of NH_4NO_3-pre-supplied *qko* plants did not accumulate IAN to significantly different levels than the roots of NO_3^--fed plants (Figure 15C). For IAA and the other auxin-derivatives a tendency was visible: In general, their concentrations were lower in roots of NH_4NO_3-pre-supplied plants than in roots of NO_3^--fed plants. This was significant for the IAA concentration in *qko* roots. IAA-ala could not be detected in the root samples (Figure 15I). The general trend of the auxin measurements was that their concentrations were higher in NH_4NO_3-pre-supplied shoots but lower in

NH_4NO_3-pre-supplied roots relative to NO_3^--fed plants, irrespective of the expression of AMTs.


A
IAA concentration in roots (ng g-1 DW)
Col-0
qko
2 mM NO3-
1 mM NH4NO3
B
IAA concentration in shoots (ng g-1 DW)
C
IAN concentration in roots (ng g-1 DW)
D
IAN concentration in shoots (ng g-1 DW)
E
IAM concentration in roots (ng g-1 DW)
F
IAM concentration in shoots (ng g-1 DW)
G
IAA-Me concentration in roots (ng g-1 DW)
H
IAA-Me concentration in shoots (ng g-1 DW)

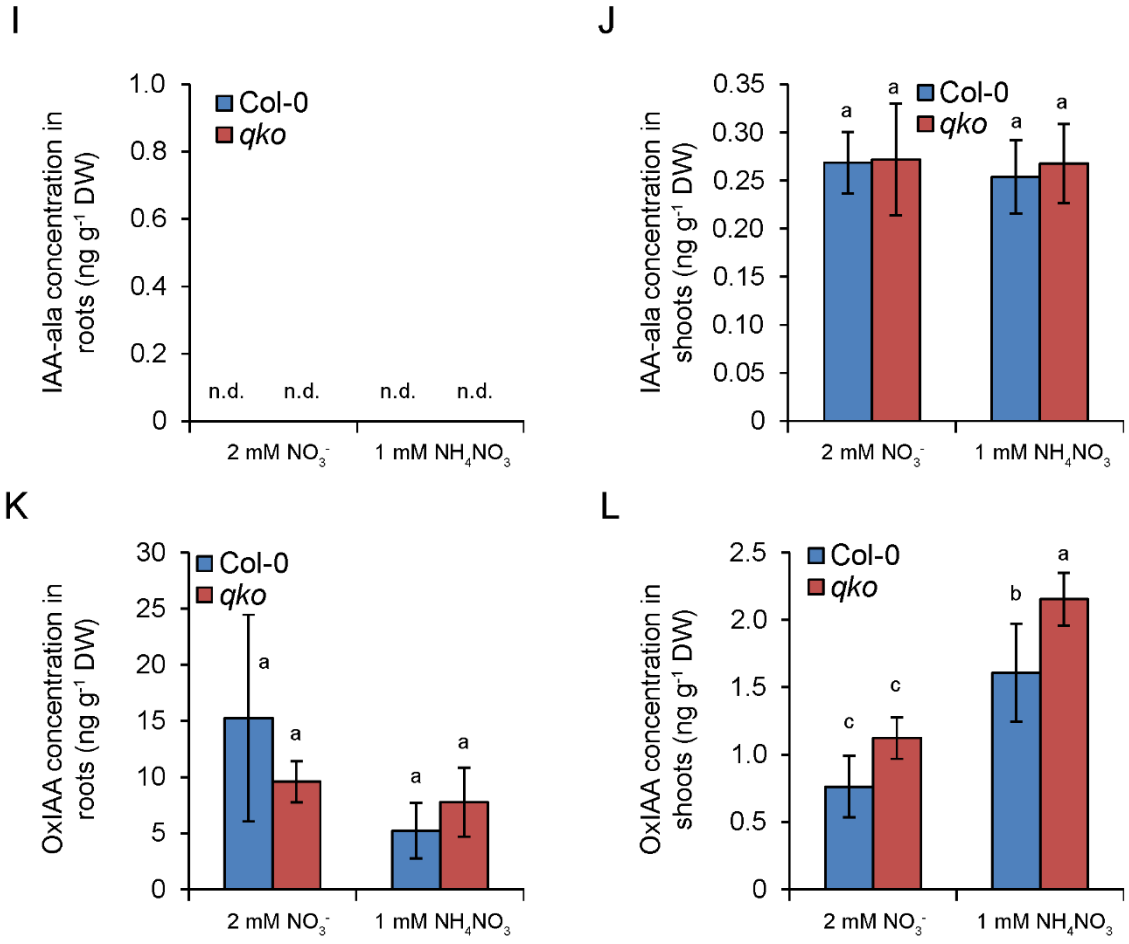


Figure 15. Concentrations of auxins in wild-type and *qko* plants. Wild-type (Col-0) and *qko* plants were grown for six weeks in nutrient solution containing 2 mM NO_3^- as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. The concentrations of auxins were measured in roots (A, C, E, G, I, K) and shoots (B, D, F, H, J, L). Bars represent means ± SD (n = 3 - 4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight. IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; IAM, indole-3-acetamide; IAA-Me, indole-3-acetic acid methyl ester; IAA-ala, indole-3-acetyl-L-alanin; OxIAA, 2-oxoindole-3-acetic acid.

The concentrations of salicylic acid in roots of wild-type and *qko* plants that had been grown hydroponically under the continuous supply of NO_3^- or had been pre-supplied with NH_4NO_3, were not significantly different from each other (Figure 16A). However, the shoots of *qko* plants accumulated salicylic acid at significantly higher concentrations than wild-type shoots, independent of the form of N supply (Figure 16B).

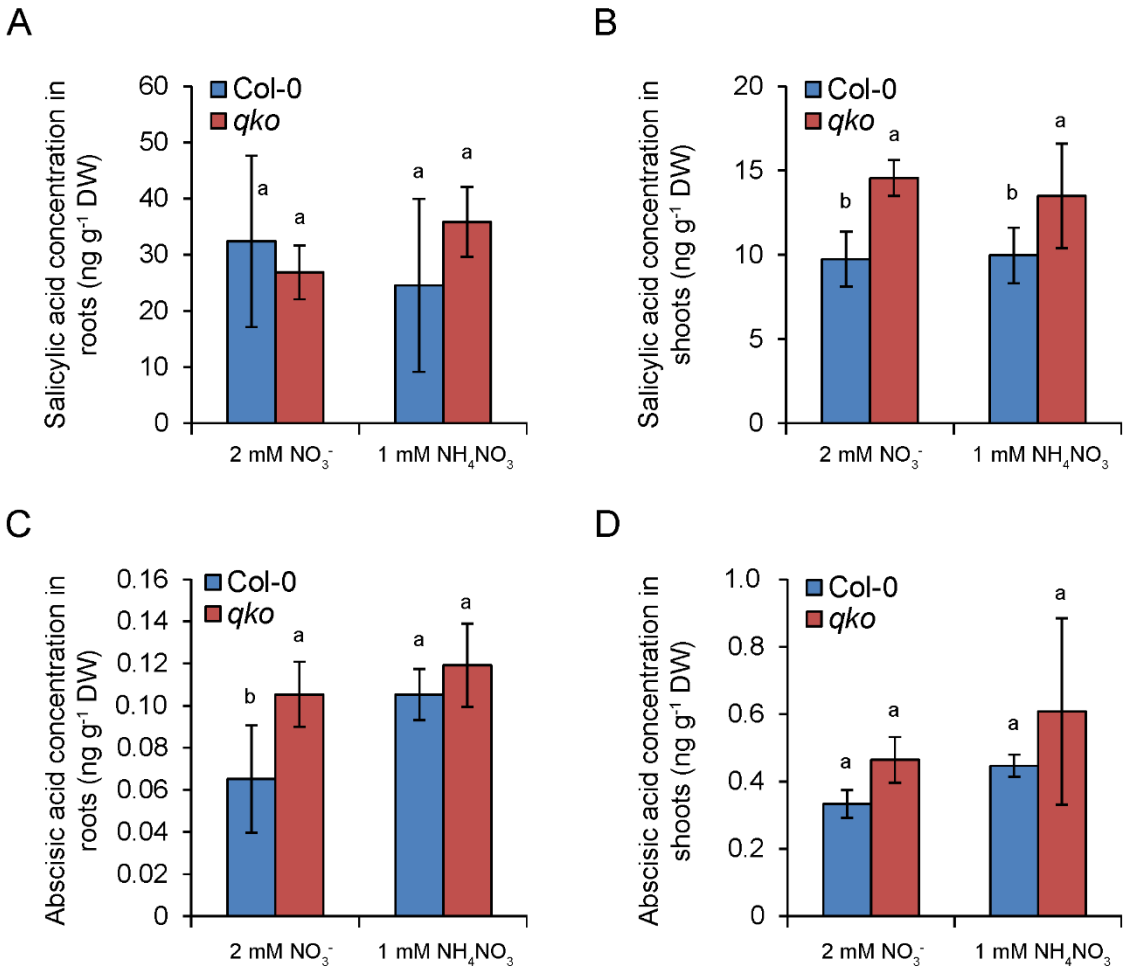


Figure 16. Concentrations of salicylic acid and abscisic acid in wild-type and *qko* plants. Wild-type (Col-0) and *qko* plants were grown for six weeks in nutrient solution containing 2 mM NO_3^- as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. The concentrations of salicylic acid (A, B) and abscisic acid (C, D) were measured in roots (A, C) and shoots (B, D). Bars represent means ± SD (n = 3 - 4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). DW refers to dry weight.

The concentration of abscisic acid (ABA) was significantly higher in the roots of NO_3^--fed *qko* plants than in roots of NO_3^--fed wild-type plants (Figure 16C). In the roots of wild-type plants, the concentration of abscisic acid was significantly higher, when plants were pre-supplied with NH_4NO_3 as compared to continuous NO_3^--supply and reached *qko* levels. In *qko* roots the ABA levels were unaffected by the form of N supply (Figure 16C). The concentrations of ABA in the shoot of wild-type and *qko* plants, that had been grown under continuous supply of NO_3^- or had been pre-supplied with NH_4NO_3, were not significantly different from each other (Figure 16D). Thus, elevated leaf concentrations of salicylic acid in *qko* relative to wild-type plants

was the only consistent and significant difference found in the phytohormone analysis of the two genotypes.

4.3.2 Influence of N supply on NH_4^+ accumulation and response in roots of wild-type and *qko* plants

In order to investigate how NH_4^+ accumulation and gene expression are affected by the presence of AMTs, wild-type and *qko* plants were cultivated hydroponically for six weeks under the supply of NO_3^- as the sole N source and then shifted to nutrient solution containing NO_3^-, NH_4NO_3 or different concentrations of NH_4^+ for two days. Roots and shoots were collected for the determination of NH_4^+ concentrations in the tissue and of gene expression of NH_4^+-responsive genes. In the roots of both, wild-type and *qko* plants that had been cultivated under continuous supply of NO_3^- ammonium accumulated up to 0.4 µmoles per g fresh weight (Figure 17A). Suppling plants with 1 mM NH_4NO_3 led to a significant increase in root NH_4^+ concentrations in wild-type plants. In *qko* plants the supply of NH_4NO_3 did not alter the NH_4^+ concentration in the root. The difference in NH_4^+ concentration between NH_4NO_3-supplied wild-type and *qko* roots was significant. In wild-type roots, the NH_4^+ concentrations of plants that received NH_4^+ as the sole N source even exceeded 2 µmoles per g FW. This corresponds to approximately 2 mM NH_4^+ in the cell sap. In contrast, in *qko* roots the concentration of NH_4^+ remained below 0.7 µmoles per g FW, even when 2 mM external NH_4^+ was supplied (Figure 17A).

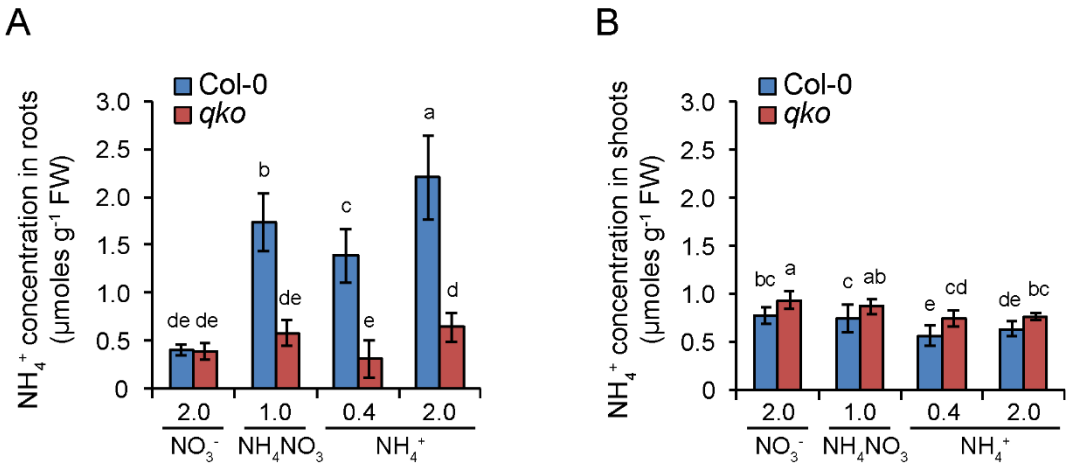


Figure 17. NH_4^+ concentrations in wild-type and *qko* plants. Wild-type (Col-0) and *qko* plants were grown for six weeks in nutrient solution containing 2 mM NO_3^- as the sole N source. Two days before sampling, the plants were shifted to nutrient solution containing the indicated N forms (in mM). The NH_4^+ concentration in roots (A) and

shoots (B) was determined by fluorescence spectrometry. Bars represent means ± SD (n = 5-6). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). FW refers to fresh weight.

Interestingly, the shoots of *qko* plants that had been supplied with either N form accumulated slightly more NH_4^+ than wild-type shoots (Figure 17B). Supplying plants with moderate concentrations of NH_4^+ (up to 2 mM) did not increase shoot NH_4^+ concentrations compared to NO_3^--fed plants. This is not surprising, because NH_4^+ is mainly assimilated in the root when supplied at moderate levels and NO_3^- is mainly reduced in the shoot, when supplied at millimolar external concentrations (Smirnoff and Stewart, 1985; Marschner, 2012).

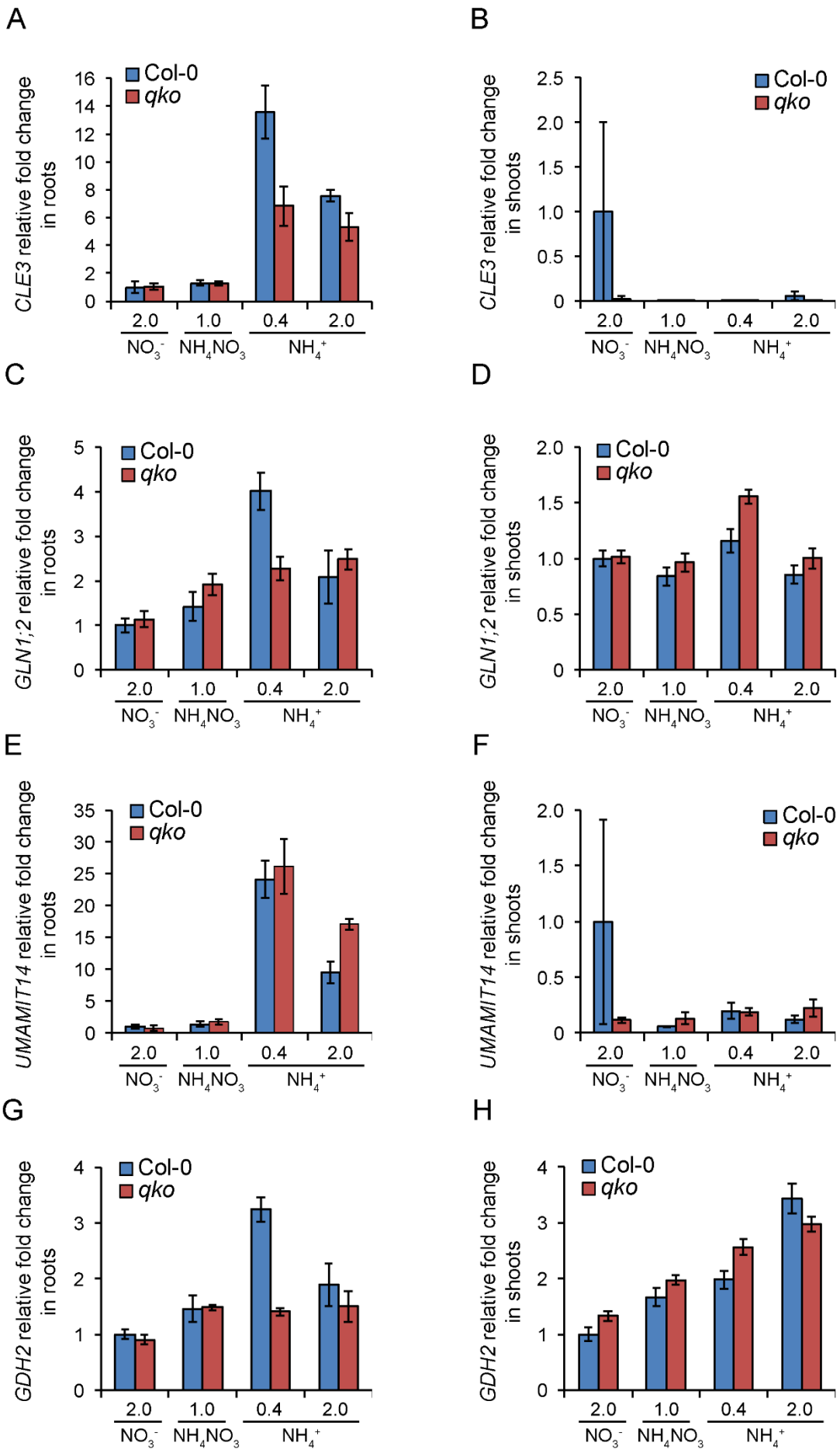

A
B
C
D
E
F
G
H
Col-0
qko
CLE3 relative fold change in roots
CLE3 relative fold change in shoots
GLN1;2 relative fold change in roots
GLN1;2 relative fold change in shoots
UMAMIT14 relative fold change in roots
UMAMIT14 relative fold change in shoots
GDH2 relative fold change in roots
GDH2 relative fold change in shoots
2.0
1.0
0.4
2.0
NO3-
NH4NO3
NH4+

Figure 18. Expression analysis of NH_4^+-inducible genes in wild-type and *qko* plants. Transcript levels of the NH_4^+-inducible genes *CLAVATA/ESR-RELATED 3* (*CLE3*, *At1g06225*) (A, B), *GLUTAMINE SYNTHETASE 1;2* (*GLN1;2*, *At1g66200*) (C, D), *USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 14* (*UMAMIT14*, *At2g39510*) (E, F) and *GLUTAMATE DEHYDROGENASE 2* (*GDH2*, *At5g07440*) (G, H) in roots (A, C, E, G) and shoots (B, D, F, H) of wild-type (Col-0) and *qko* plants, that had been grown for six weeks in nutrient solution containing 2 mM KNO_3 as the sole N source. Two days before sampling, the plants were shifted to nutrient solutions containing the indicated N forms (in mM). Bars represent means ± SE (n = 3-4).

The expression levels of the NH_4^+-responsive genes *CLE3*, *GLN1;2*, *UMAMIT14* and *GDH2* (Patterson et al., 2010) did not vary considerably in the roots of wild-type and *qko* plants that had been cultured under the supply of NO_3^- or NH_4NO_3 (Figure 18A, C, E and G). When NH_4^+ was supplied as the sole N source at a concentration of 0.4 mM and the high-affinity transport system is usually induced (Wang et al., 1993; Gazzarrini et al., 1999), the relative transcript levels of all four genes strongly increased (between 3.2 and 24.1-fold) in the roots of wild-type plants (Figure 18A, C, E and G). In the roots of *qko* plants, the levels *CLE3* and *GLN1;2* also increased, however, not to wild-type levels (Figure 18A and C). The expression of *GDH2* in the roots of *qko* plants was not considerably affected by the supply of 0.4 mM NH_4^+ (Figure 18G). Interestingly, *UMAMIT14* mRNA levels in *qko* roots increased as much as in wild-type roots under this condition (Figure 18E). At a concentration of 2 mM external NH_4^+, *UMAMIT14* transcript levels were even higher in the roots of *qko* than of wild-type plants (Figure 18E). In wild-type roots, supplying 2 mM NH_4^+ increased the expression levels of the four genes compared to NO_3^--fed roots, however to a lower extent than the supply of 0.4 mM NH_4^+ (Figure 18A, C, E and G). In the roots of *qko* plants the levels of *CLE3*, *GLN1;2* and *UMAMIT14* also increased to a lower extent than at the lower NH_4^+-supply level (Figure 18A, C and E). At 2 mM NH_4^+ the expression of *GDH2* was not considerably affected (< 2-fold) in *qko* roots and was slightly lower than in wild-type roots (Figure 18G). The relative *CLE3* mRNA levels were slightly lower in the roots of *qko* plants than in the wild-type at 2 mM NH_4^+ (Figure 18A). In shoots, transcript levels of *CLE3* and *UMAMIT14* were very low (high Ct-values) (Figure 18B and F). For the expression levels of *CLE3*, *GLN1;2*, and *UMAMIT14* in shoots, no AMT- or N treatment-dependent pattern was observed (Figure 18B, D and F). By contrast, transcript levels of *GDH2* in shoots of both wild-type and *qko* plants increased steadily with increasing external NH_4^+ supplies (Figure

18H). Except for the 2 mM NH_4^+ treatment, *GDH2* mRNA levels were slightly higher in *qko* shoots than in wild-type shoots.

As expected, the NH_4^+ uptake-deficient mutant *qko* (Yuan et al., 2007a) accumulated less NH_4^+ in the roots than the wild-type, when NH_4^+ was present in the nutrient solution (Figure 17A). This went along with a lower expression of NH_4^+-inducible genes in *qko* roots (except *UMAMIT14*) (Figure 18).

4.3.3 Influence of N supply on the concentrations of amino acids in wild-type and *qko* plants

Table 2 shows the concentrations of individual amino acids in roots and shoots of wild-type and *qko* plants that had been grown hydroponically under the continuous supply of NO_3^- or had been pre-supplied with NH_4NO_3. The most prominent change in response to the presence of NH_4^+ was that the concentration of some major amino acids increased in roots and/or in shoots of both genotypes. This was the case for the concentrations of arginine, asparagine, glutamine and glycine in roots and shoots and to a lower extent also for tyrosine in shoots. A second important observation was that *qko* plants had lower concentrations in particular of some major amino acids than wild-type plants, independent of the form of N pre-supply. This was most obvious for arginine, asparagine and lysine in roots and for asparagine and glycine in shoots. When plants were shifted from NO_3^- to NH_4NO_3, the concentration of some amino acids increased only in the wild-type but not in *qko* plants. This difference occurred for alanine, glutamine, glycine and serine in roots and for histidine in shoots. In turn, aspartic and glutamic acid levels were higher in wild-type and *qko* plants, when NO_3^- was supplied as the sole N source. Under this growth condition, also the serine concentrations in shoots were higher than in shoots of NH_4NO_3-supplied plants.

Table 2. Concentrations of amino acids in wild-type and *qko* plants. Wild-type (Col-0) and *qko* plants were grown for six weeks in nutrient solution containing 2 mM KNO_3 as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. Values represent means ± SD (n = 3-4) of the amino acid concentrations (nmoles g^{-1} FW). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$).

	Root				Shoot			
	2 mM KNO_3		1 mM NH_4NO_3 7d		2 mM KNO_3		1 mM NH_4NO_3 7d	
	Col-0	*qko*	Col-0	*qko*	Col-0	*qko*	Col-0	*qko*
Alanine	161.4 ± 13.0 b	127.9 ± 13.2 b	220.4 ± 46.5 a	150.3 ± 8.8 b	503.4 ± 50.3 a	476.7 ± 43.4 a	495.4 ± 32.5 a	488.0 ± 53.5 a
Arginine	64.7 ± 5.7 b	38.8 ± 5.9 c	129.3 ± 12.6 a	65.0 ± 4.7 b	41.6 ± 6.9 b	40.3 ± 4.7 b	80.4 ± 18.5 a	64.6 ± 4.9 a
Asparagine	292.5 ± 30.1 b	184.5 ± 19.1 c	441.1 ± 36.9 a	281.3 ± 32.0 b	224.8 ± 21.0 c	185.8 ± 10.0 d	341.4 ± 39.1 a	265.7 ± 16.5 b
Aspartic acid	397.0 ± 38.5 a	337.6 ± 31.3 b	262.0 ± 16.5 c	258.8 ± 15.8 c	435.3 ± 39.7 ab	459.2 ± 30.4 a	364.9 ± 17.0 c	395.5 ± 16.6 bc
Glutamic acid	796.2 ± 62.7 a	740.6 ± 53.9 a	534.4 ± 64.7 b	604.6 ± 52.8 b	1152.7 ± 48.7 a	1064.2 ± 47.6 b	909.6 ± 38.2 c	848.2 ± 60.6 c
Glutamine	451.8 ± 42.5 c	357.1 ± 20.7 c	905.6 ± 123.9 a	626.5 ± 86.1 b	645.5 ± 77.3 b	674.0 ± 38.3 b	893.4 ± 44.7 a	836.1 ± 36.9 a
Glycine	62.8 ± 5.3 c	48.3 ± 8.1 c	167.7 ± 27.2 a	89.3 ± 4.2 b	118.2 ± 11.9 b	84.3 ± 14.8 c	238.2 ± 13.9 a	132.4 ± 18.2 b
Histidine	23.2 ± 3.6 a	17.6 ± 2.0 b	21.5 ± 2.5 ab	17.1 ± 1.3 b	3.1 ± 0.7 b	3.0 ± 0.3 b	5.4 ± 0.7 a	3.5 ± 0.4 b
Isoleucine	21.7 ± 1.8 a	17.2 ± 4.0 a	22.5 ± 2.8 a	20.2 ± 4.3 a	12.8 ± 0.7 a	12.3 ± 1.2 a	11.9 ± 1.1 a	12.3 ± 1.4 a
Leucine	28.9 ± 2.1 a	25.7 ± 4.5 a	28.1 ± 4.1 a	27.0 ± 4.8 a	10.2 ± 0.5 a	10.6 ± 1.0 a	10.4 ± 1.5 a	11.4 ± 1.5 a
Lysine	27.6 ± 1.5 a	18.1 ± 1.8 b	32.2 ± 6.5 a	20.0 ± 3.7 b	10.9 ± 0.9 a	11.2 ± 1.7 a	10.8 ± 2.0 a	12.1 ± 1.8 a
Phenylalanine	32.6 ± 3.0 a	37.0 ± 4.0 a	30.1 ± 2.9 a	32.3 ± 6.2 a	28.0 ± 1.4 a	30.8 ± 1.0 a	28.6 ± 3.0 a	27.7 ± 1.4 a
Proline	32.6 ± 1.1 a	42.4 ± 15.0 a	40.9 ± 5.2 a	39.5 ± 3.0 a	141.6 ± 15.4 a	185.0 ± 20.0 a	176.7 ± 23.7 a	192.2 ± 40.6 a
Serine	223.5 ± 25.5 a	195.2 ± 27.0 ab	223.2 ± 27.3 a	171.5 ± 6.4 b	610.0 ± 14.9 a	593.0 ± 51.9 a	521.7 ± 24.3 b	528.7 ± 36.2 b
Threonine	188.4 ± 21.7 a	176.2 ± 31.3 a	178.5 ± 23.4 a	148.5 ± 5.1 a	235.8 ± 21.7 a	249.3 ± 15.9 a	215.2 ± 9.2 a	227.0 ± 16.5 a
Tyrosine	124.4 ± 7.3 a	88.4 ± 10.6 a	144.1 ± 44.4 a	123.6 ± 32.4 a	42.2 ± 4.2 c	64.3 ± 20.7 bc	77.4 ± 1.1 ab	89.2 ± 13.5 a
Valine	58.1 ± 4.5 a	55.1 ± 10.8 a	59.3 ± 5.9 a	59.6 ± 12.1 a	43.1 ± 4.4 a	48.0 ± 4.1 a	39.8 ± 2.1 a	44.5 ± 4.1 a

Figure 19 compares the concentrations of total amino acids in roots (A) and shoots (B) between wild-type and *qko* plants. In wild-type roots the amino acid concentration increased significantly when NH_4^+ was present in the nutrient solution (Figure 19A). However, in roots of *qko* plants, the amino acid concentration remained unchanged under NH_4^+ supply. In both N treatments, *qko* roots accumulated 16-21% less amino acids than the roots of wild-type plants (Figure 19A). In the shoots no significant differences in the concentration of total amino acids were observed between the two lines (Figure 19B).

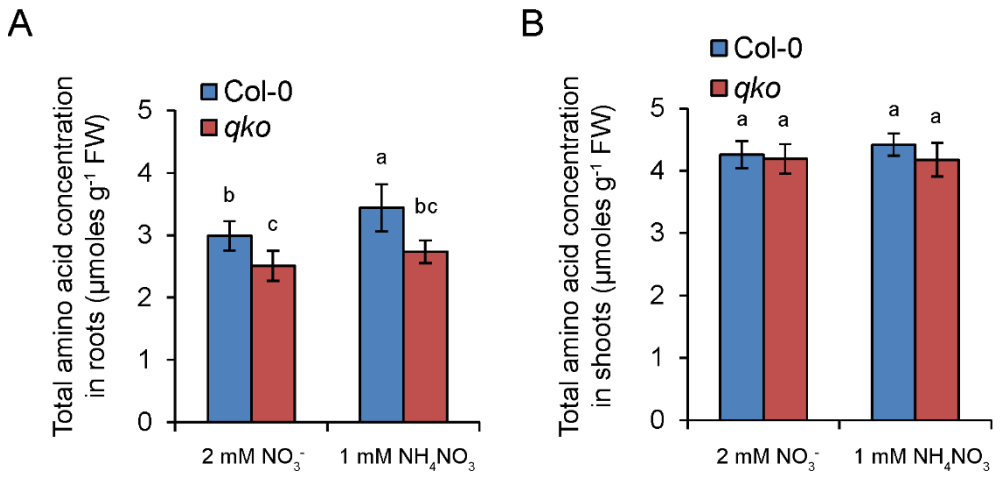


Figure 19. Total amino acid concentration in wild-type and *qko* plants. Total amino acid concentration in roots (A) and shoots (B) of wild-type (Col-0) and *qko* plants, that were grown for six weeks in nutrient solution containing 2 mM KNO_3 as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. Bars represent means ± SD (n = 3-4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). FW refers to fresh weight.

4.3.4 Influence of N supply on the concentrations of sugars in wild-type and *qko* plants

To monitor changes in sugar metabolism when AMTs are lacking, the concentrations of glucose, fructose and sucrose in roots and shoots of wild-type and *qko* plants were measured (Figure 20). For this purpose, hydroponically-grown plants were used that were supplied continuously with NO_3^- or for one week with NH_4NO_3.

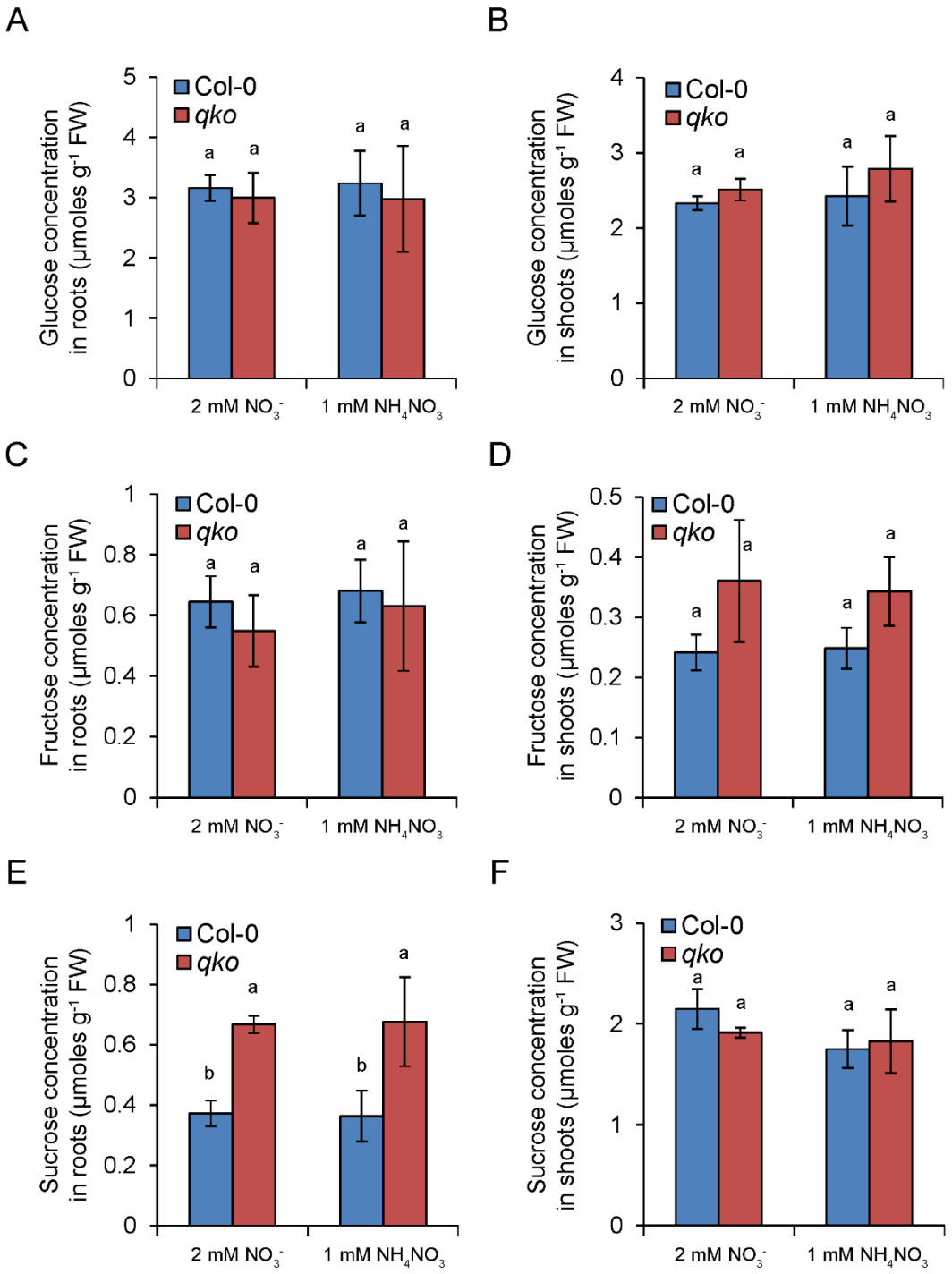


Figure 20. Concentration of sugars in wild-type and *qko* plants. Glucose (A, B), fructose (C, D) and sucrose (E, F) concentrations in roots (A, C, E) and shoots (B, D, F) of wild-type (Col-0) and *qko* plants, that were grown for six weeks in nutrient solution containing 2 mM KNO_3 as the sole N source. One set of plants was shifted to nutrient solution containing 1 mM NH_4NO_3 as the sole N source 7 days before sampling. Bars represent means ± SD (n = 3 - 4). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$). FW refers to fresh weight.

The concentration of glucose (Figure 20A and B) and fructose (Figure 20C and D) in roots and shoots of wild-type and *qko* plants were not significantly different from each other under either N treatment. Also, sucrose concentrations in shoots remained unaffected by the N treatment in both lines (Figure 20F). However, in the roots of *qko* plants significantly higher concentrations of sucrose were determined than in wild-type roots (Figure 20E). *qko* roots accumulated 79% and 86% more sucrose than wild-type roots, when grown under the supply of NO_3^- and NH_4NO_3, respectively.

Taken together, compared to wild-type roots a lower accumulation of NH_4^+ and amino acids in *qko* roots was observed (Figure 17, Table 2 and Figure 19). In turn, the roots of *qko* plants exhibited higher sucrose concentrations than the roots of wild-type plants (Figure 20).

4.4 Investigations on the transition to flowering and on the growth of wild-type and *qko* plants

When wild-type and *qko* plants were cultivated for a longer period, growth differences between the two lines were not perceivable anymore (data not shown). This observation led to the assumption that *qko* plants might develop faster and therefore appear in an early stage larger than wild-type plants. In order to test this hypothesis, the time of flowering and the growth phenotype of wild-type and *qko* plants were assessed.

4.4.1 Flowering time and shoot phenotype of wild-type and *qko* plants at flowering

Figure 21 summarizes the flowering behavior of wild-type and *qko* plants grown on a peat-based substrate under full nutrient provision. The plants were visually scored for the presence of the first visible petal. At the time when approximately 30% of the plants of a given genotype had started to flower, i.e. had formed at least one visible petal, plants were harvested for destructive analysis (Figure 22). The remaining plants were visually scored for flowering for the rest of the experiment (Figure 21C and D). When plants were grown under long day conditions, in a 16 h / 8 h light/dark regime, *qko* was bolting about 3 days earlier than Col-0 (Figure 21A and C). The determination of the number of rosette leaves at flowering confirmed this phenotype (Figure 21B). Wild-type plants started to flower after the formation of 15.9 rosette leaves, whereas *qko* plants were flowering after the formation of 12.1 rosette leaves (Figure 21B). This represents a significant earlier transition from the vegetative to the reproductive phase with a difference of 3.8 leaves. Moreover, when the plants were grown under short-day conditions, in an 8 h / 16 h light/dark rhythm, *qko* plants were flowering earlier (Figure 21C). Under this condition, about 30% of the wild-type plants were flowering 110 days after sowing while *qko* plants flowered already 85 days after sowing. Because the early flowering phenotype of *qko* was occurring under long- (Figure 21C) and short-day conditions (Figure 21D), further experiments were carried out under long-day conditions to save time and resources.

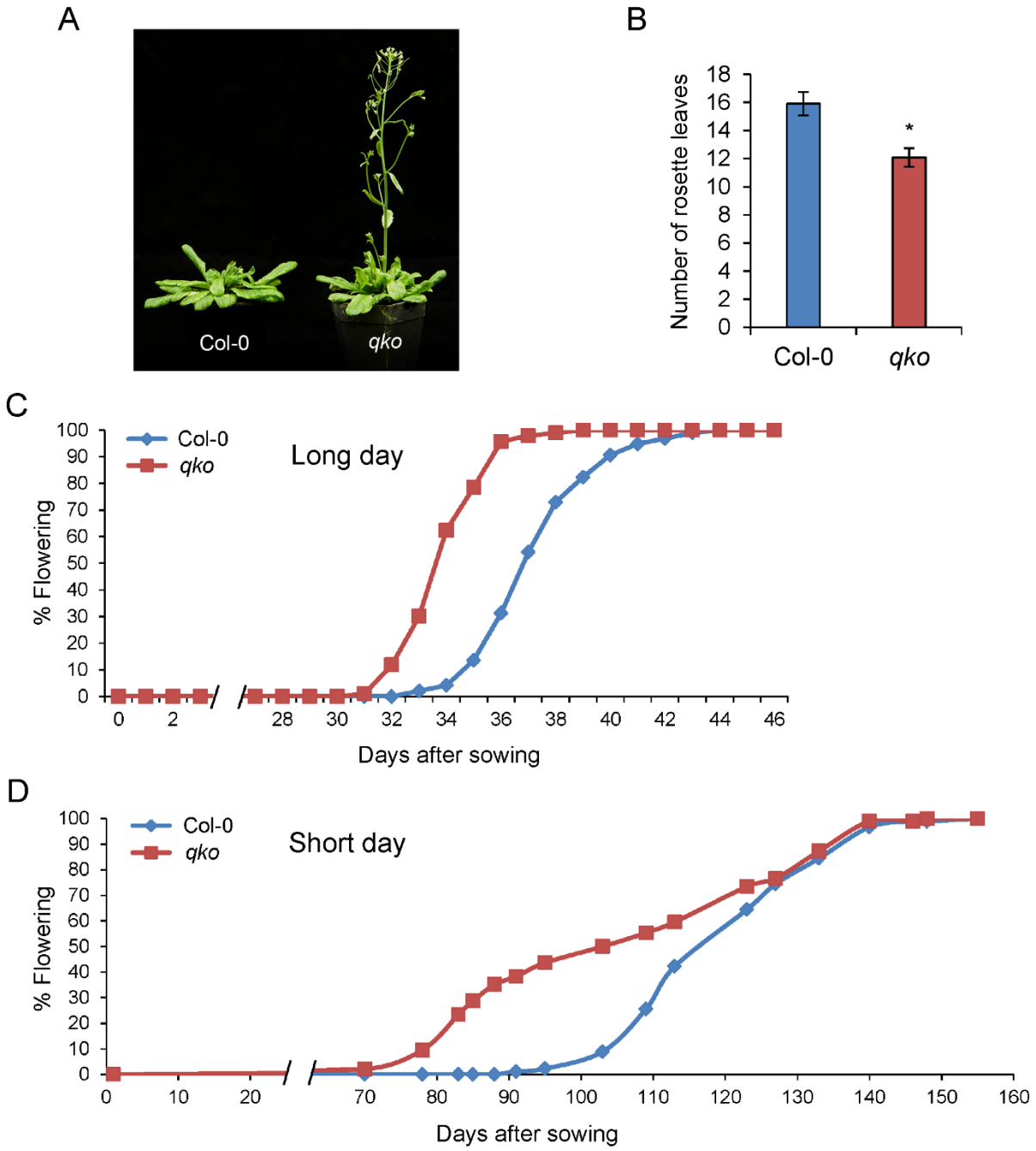


Figure 21. Influence of lacking *AMT* gene expression on the transition to flowering. Plants were grown on solid substrate in a 16 h / 8 h (A, B, C) or an 8 h / 16 h (D) light/dark rhythm, i.e. under long or short days, respectively. (A) Representative photograph of 33 day-old wild-type (Col-0) and *qko* plants. (B) Number of rosette leaves at the transition to flowering, i.e. when 30% of the plants had at least one visible petal (corresponding to 33 days for *qko* and 36 days for wild-type plants). (C, D) Percentage of plants with a visible petal (n = 92 – 100 plants). Shown are means ± SD (n = 11 – 12). Asterisk indicates a significant difference according to student's t-test ($P < 0.05$).

Wild-type plants had three days more to complete the vegetative growth phase (Figure 21C) and could therefore accumulate a significantly larger shoot biomass during this period (Figure 22A). The shoot fresh weight of Col-0 and *qko* plants at flowering was 672 mg and 283 mg, respectively (Figure 22A). In other words, *qko* had formed just 42% of the wild-type shoot biomass. Besides a lower biomass, *qko* plants exhibited a smaller rosette diameter (Figure 22B) and leaf area than Col-0 (Figure 22C). The diameter of the rosettes of *qko* plants was only 76% of the size of wild-type rosettes. The average area of the leaves of a wild-type plant was 37.8 cm² and of a *qko* plant 17.3 cm², corresponding to reduction by 54%.

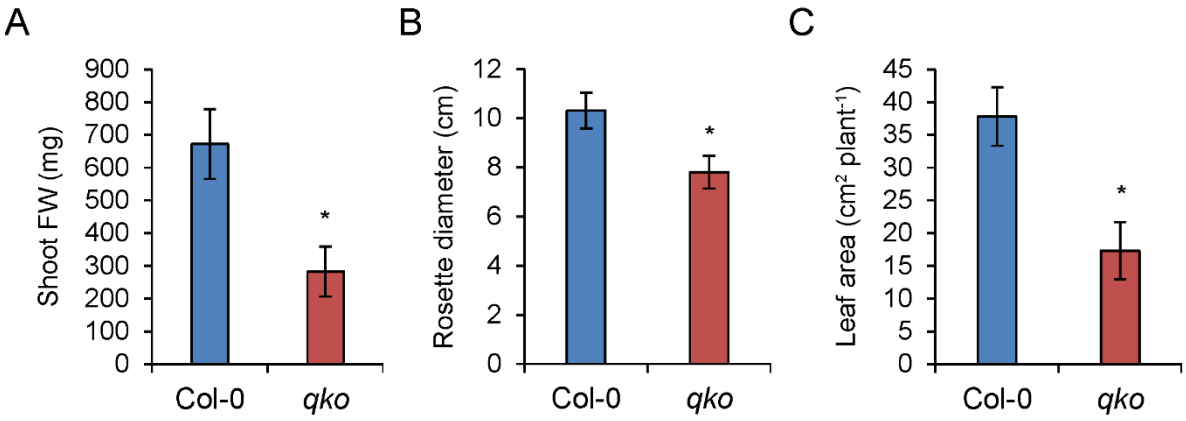


Figure 22. Influence of lacking *AMT* gene expression on the transition to flowering. Plants were grown on solid substrate in a 16 h / 8 h light/dark rhythm, i.e. under long days. Shoot fresh weight (FW) (A), rosette diameter (B) and leaf area (C) at the transition to flowering, i.e. when 30% of the plants had at least one visible petal (corresponding to 33 days for *qko* and 36 days for wild-type plants). Shown are means ± SD (n = 11 – 12). Asterisk indicates a significant difference according to student's t-test ($P < 0.05$).

4.4.2 Relative growth rates of wild-type, *qko* and triple *amt* insertion lines

In the vegetative phase, under short-day conditions, *qko* shoots grew larger than the shoots of wild-type plants (Figure 2 - Figure 5). Conversely, when cultivated under long-day conditions and compared at the transition to flowering, wild-type plants had built larger shoots (Figure 22). To shed more light on the growth behavior of wild-type and *qko* plants, they were cultivated on substrate under long-day conditions for non-destructive assessment of the leaf area on a daily basis and for calculation of relative growth rates (RGR) (Figure 23). The plants were grown in a climate-controlled growth chamber with a LemnaTec phenotyping system (Junker et al., 2015). In addition to wild-type and *qko* plants, the triple *amt* insertion lines *qko11*, *qko12*, *qko13* and

qko21 were included in the experiment. During the experiment the transition to flowering was additionally measured by determining the day at which the first petal was visible (Figure 24). 8 days after sowing the imaging-based phenotyping started. At this time point *qko* plants had a significantly larger leaf area than wild-type plants (Figure 23A and B, Table 3), resembling the phenotype under short-day conditions (Figure 2 - Figure 5). The leaf area of *qko11* and *qko12* was similar to that of *qko* and the leaf area of *qko21* similar to that of Col-0 (Figure 23A and B, Table 3). The leaf area of *qko13* was significantly larger than that of the other genotypes (Figure 23A and B, Table 3). During the course of the experiment, up to 22 days after sowing, *qko* exhibited a significantly larger leaf area than wild-type plants. 25 days after sowing the leaf area of wild-type plants nearly reached that of *qko*. The area of wild-type leaves had overtaken that of *qko* leaves after 27 days and was significantly larger 30 days after sowing (Figure 23A and B, Table 3).

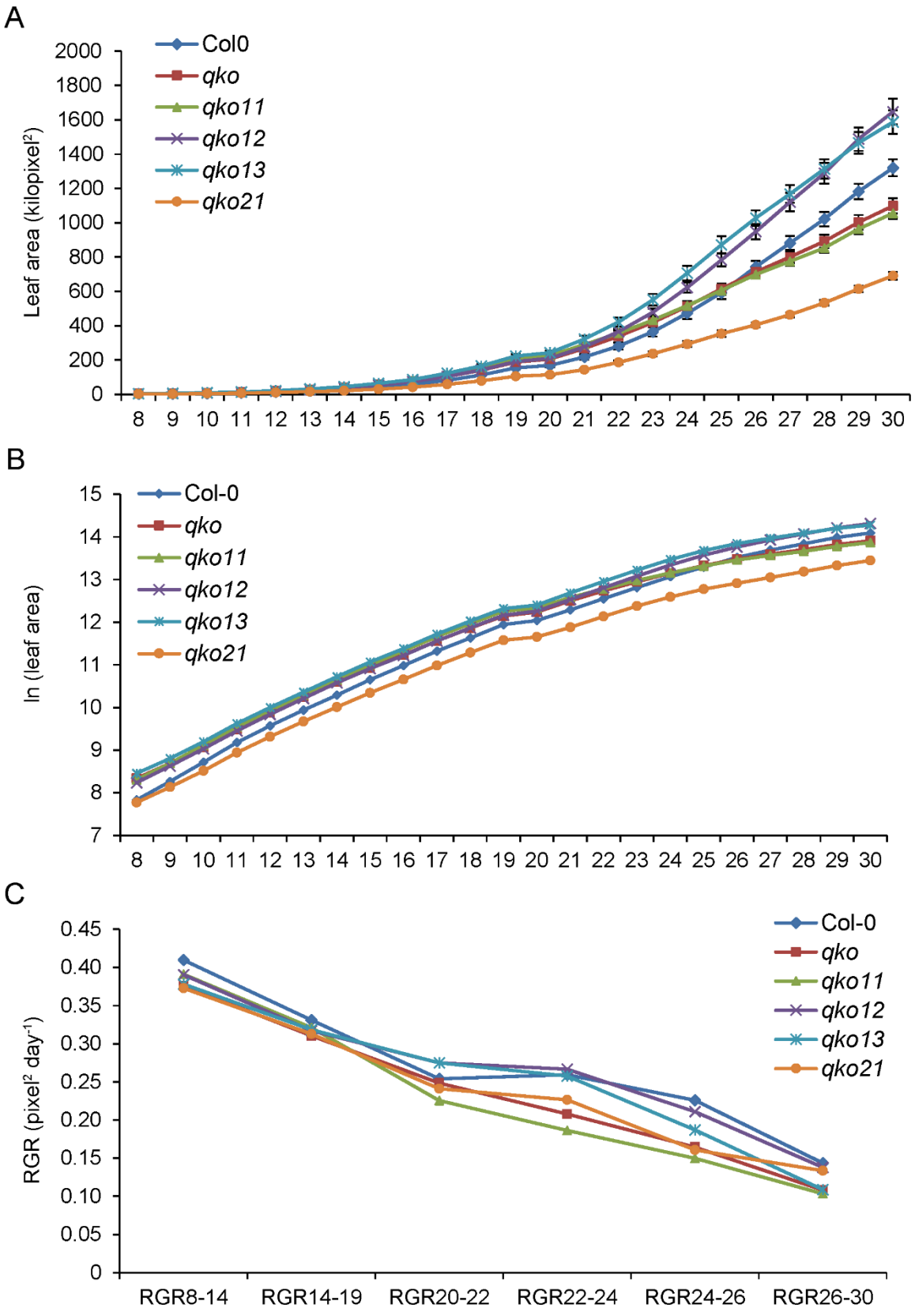


Figure 23. Time course of leaf area formation in wild-type and *amt* mutant lines. (A) Leaf area, (B) the natural logarithm of the leaf area and (C) the relative growth rate (RGR) of wild-type (Col-0) *qko*, *qko11*, *qko12*, *qko13* and *qko21* plants that had been cultured on solid substrate under long-day conditions. Leaf area was assessed

on a daily basis with a LemnaTec phenotyping system. Values represent means ± SE (n = 23 - 45). ln, natural logarithm; RGR, relative growth rate. RGR8-14 refers to the relative growth rate based on leaf area in pixel² day^{-1} between 8 and 14 days after sowing; RGRs between other time points are indicated in the same way.

The leaf area of *qko21* plants was smaller than that of the other genotypes at all analyzed time points, but not significantly at the first one (Figure 23A and B, Table 3). The leaves of *qko11* plants showed a similar area to that of *qko* and did not differ significantly at any time point (Figure 23A and B, Table 3). At every time point, but the last two days, *qko13* leaves exhibited the largest area (Figure 23A and B, Table 3). The area of *qko12* leaves were at the beginning of the phenotyping similar to that of *qko* and reached *qko13* levels at the end of the experiment (Figure 23A and B, Table 3).

Table 3. Leaf area of wild-type (Col-0) *qko*, *qko11*, *qko12*, *qko13* and *qko21* plants. Plants were grown on solid substrate under long day conditions and leaf area was assessed on a daily basis with a LemnaTec phenotyping system. Values represent means ± SE (n = 23 - 45). Different letters indicate significant differences according to Fisher's LSD test (P < 0.05).

	Leaf area (kilopixel² $plant^{-1}$)					
Genotype	8 DAS	17 DAS	22 DAS	25 DAS	27 DAS	30 DAS
Col-0	2.5 ± 0.2 c	82.4 ± 5.7 c	282 ± 21 c	593 ± 38 b	882 ± 40 b	1320 ± 49 b
qko	4.2 ± 0.2 b	104.7 ± 4.5 b	339 ± 16 b	615 ± 32 b	800 ± 33 b	1098 ± 45 c
qko11	4.0 ± 0.2 b	116.1 ± 4.4 ab	356 ± 16 b	607 ± 29 b	776 ± 27 b	1056 ± 33 c
qko12	3.8 ± 0.2 b	104.9 ± 6.1 b	365 ± 18 b	783 ± 37 a	1120 ± 54 a	1648 ± 75 a
qko13	4.7 ± 0.2 a	123.0 ± 6.7 a	422 ± 26 a	872 ± 50 a	1167 ± 51a	1587 ± 68 a
qko21	2.4 ± 0.1 c	58.7 ± 3.8 d	187 ± 12 d	354 ± 19 c	464 ± 14 c	691 ± 22 d

RGRs were computed according to Poorter and Lewis (1986) and calculated for different periods to obtain clear curves (Figure 23C). In the course of the experiment, all lines showed a decrease in RGRs, a natural event during plant development and life cycle completion. The RGRs of the six lines did not differ substantially from each other in the first three periods measured between 8 and 14 DAS, 14 and 19 DAS and between 20 and 22 DAS. However, at the later growth stages, between 22 and 24 DAS, 24 and 26 DAS and between 26 and 30 DAS, the RGR of *qko* plants were only 80.2%, 72.8% and 75.2% of the RGR of wild-type plants, respectively (Figure 23C), reflecting a steeper decline of RGRs in *qko* plants than in the wild-type. *qko11* and *qko21* showed a similar pattern of decline in RGRs as *qko*. The RGRs of *qko12* were almost identical to those of Col-0 at the later periods. The RGR of *qko13* was similar to that of Col-0 and *qko12* between 22 and 24 DAS, dropped to an intermediate level

between 24 and 26 DAS and reached *qko* levels between 26 and 30 DAS (Figure 23C).

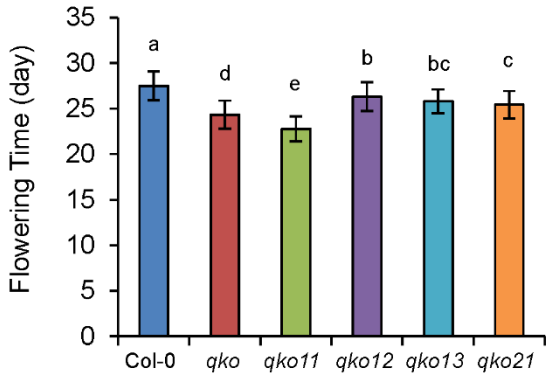


Figure 24. Flowering time of wild-type and *amt* mutant lines. Flowering time of wild-type (Col-0) *qko*, *qko11*, *qko12*, *qko13* and *qko21* plants that had been cultured on solid substrate under long-day conditions. Bars represent means ± SD (n = 24 - 35 replicates). Different letters indicate significant differences according to Fisher's LSD test ($P < 0.05$).

Assessment of the day of flowering in this experimental setup (Figure 24) revealed a similar pattern as previously observed (Figure 21). Wild-type plants began to flower 27.5 days after sowing, whereas *qko* plants began to flower significantly earlier, 24.3 days after sowing (Figure 24). *qko12*, *qko13* and *qko21* showed an intermediate flowering phenotype. Interestingly, *qko11* started to flower already 22.8 days after sowing, being the earliest line (Figure 24).

Interestingly, the flowering time of the different lines appeared to correlate with the RGRs around the transition to flowering (Figure 23C and Figure 24). *qko11* started to flower first, 22.8 DAS (Figure 24) and showed the lowest RGR between 22 and 24 DAS of 0.19 pixel² day^{-1} (Figure 23C). *qko* and *qko21* started to flower next, 24.3 and 25.4 DAS, respectively, and exhibited the second lowest RGRs between 24 and 26 DAS. Wild-type plants were the last which started flowering at 27.5 DAS and also had the highest RGRs at the later periods. *qko12* showed the most similar flowering and growth phenotype to the wild-type, followed by *qko13*.

4.4.3 Influence of N form on growth and development of wild-type and *qko* plants

After observing that *qko* plants flowered earlier than wild-type plants, when cultivated on substrate containing mixed N forms (Figure 21 and Figure 24), growth and development of wild-type (Figure 25) and *qko* plants (Figure 26) grown on agar plates containing either NH_4^+ or NO_3^- as the sole N source was monitored in a time course.

The first time point, at which plants were phenotyped was 10 days after sowing. Already there, the root (Figure 25A) and shoot (Figure 25B) fresh weights of wild-type plants were higher, when the plants were fed with NO_3^- instead of NH_4^+ as the sole N source. Also at the other analyzed time points, the biomass of NO_3^--fed wild-type plants was higher. From the first time point analyzed at 10 days after sowing to the last time point at 22 days after sowing, NH_4^+-fed wild-type plants increased their root and shoot fresh weight from 0.31 mg to 2.51 mg and from 0.73 mg to 4.76 mg, respectively. This corresponded to an 8-fold and 6-fold increase in root and shoot biomass, respectively. In contrast, NO_3^--fed wild-type plants increased their root and shoot biomass by 48-fold and 13-fold, respectively (Figure 25A and B), indicating that NO_3^- nutrition promotes plant growth in Arabidopsis much more than NH_4^+. 22 days after sowing, bolting and the formation of flower buds were observed in NO_3^--fed wild-type plants (Figure 25C). Interestingly, NH_4^+-fed wild-type plants did not show any sign of flowering 22 days after sowing (Figure 25C). NH_4^+ provision to wild-type plants inhibited plant growth and seemed to inhibit flowering (Figure 25).

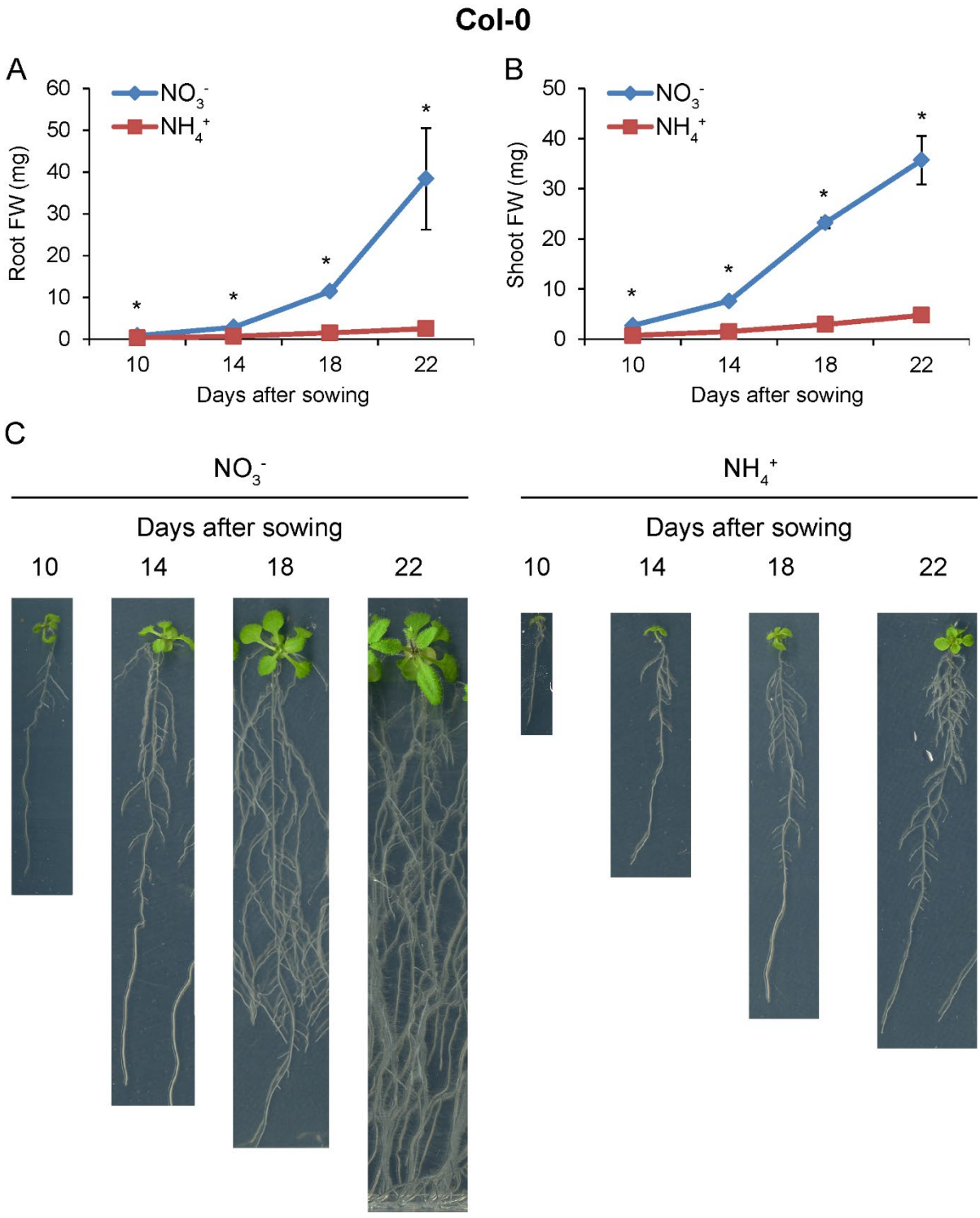


Figure 25. Influence of N form on growth and development of wild-type plants. Shoot (A) and root (B) biomass of wild-type (Col-0) plants that had been cultured under long-day conditions on 0.5 MS agar medium containing either 2 mM NH_4^+ or 2 mM NO_3^- as the sole N source for 10, 14, 18 or 22 days. Values represent means ± SD (n = 4 replicates, each consisting of 8 - 40 plants). Asterisk indicates a significant difference according to Student's t-test ($P < 0.05$) between NO_3^- and NH_4^+-fed plants at the indicated time point of harvest. FW refers to fresh weight. (C) Representative images for the developmental stages of plants shown in (A) and (B).

Similar to wild-type plants (Figure 25A and B), also *qko* plants showed a much higher biomass accumulation when NO_3^- instead of NH_4^+ was used as the sole N source (Figure 26A and B). NH_4^+-fed *qko* plants increased their root and shoot fresh weight 8-fold and 6-fold, respectively, from 10 to 22 days after sowing. In contrast, NO_3^--fed *qko* plants showed a 38-fold and 12-fold biomass increase in roots and shoots, respectively (Figure 26A and B), confirming that also in *qko* plants NH_4^+ nutrition inhibited plant growth.

In contrast to wild-type plants (Figure 25C), bolting was observed 22 days after sowing in *qko* plants independent of whether they were fed with NO_3^- or NH_4^+ as the sole N source (Figure 26C). These qualitative observations indicated that NH_4^+ nutrition inhibited the transition to flowering in wild-type plants, but not in *qko* plants. Compared to NO_3^- nutrition, NH_4^+ nutrition inhibited plant growth in wild-type plants and *qko* plants (Figure 25 and Figure 26).

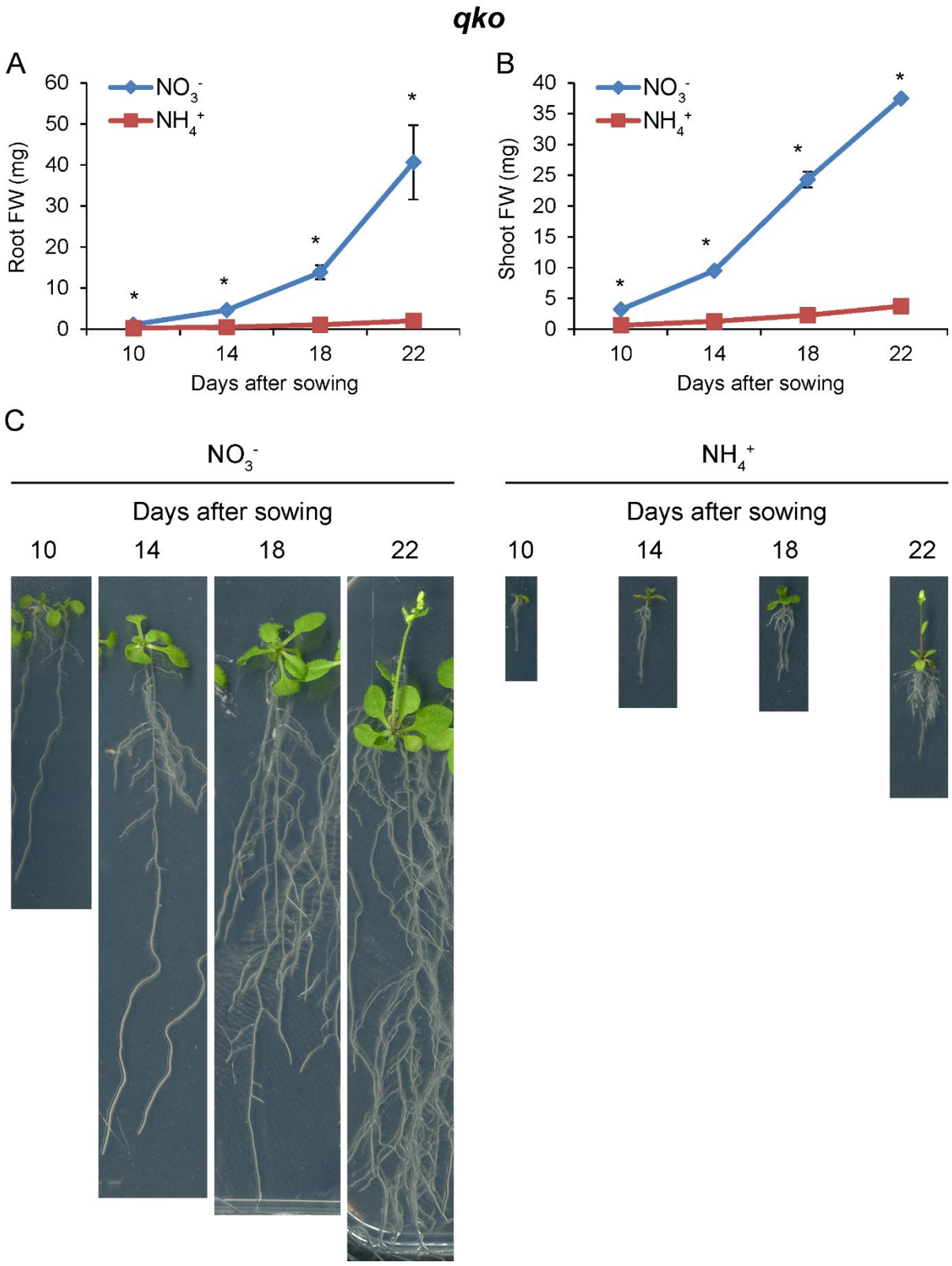


Figure 26. Influence of N form on growth and development of *qko* plants. Shoot (A) and root (B) biomass of *qko* plants that had been cultured under long-day conditions on 0.5 MS agar medium containing either 2 mM NH_4^+ or 2 mM NO_3^- as the sole N source for 10, 14, 18 or 22 days. Values represent means ± SD (n = 4 replicates, each consisting of 8 - 40 plants). Asterisk indicates a significant difference according to Student's t-test ($P < 0.05$) between NO_3^- and NH_4^+-fed plants at the time

point of harvest. FW refers to fresh weight. (C) Representative images indicating the developmental stage of plants shown in (A) and (B).

4.4.4 Influence of N form on the expression of flowering time genes in wild-type and *qko* plants

After observing that NH_4^+ nutrition inhibited growth in wild-type and *qko* plants, but only seemed to inhibit flowering in the wild-type (Figure 25 and Figure 26), expression of genes regulating flowering time were analyzed. Therefore, wild-type and *qko* plants were cultivated in agar medium containing either NH_4^+ or NO_3^- as the sole N source. Shoot samples were collected 10, 14, 18 and 22 days after sowing for gene expression analysis. Expression of the flower organ identity gene *APETALA1* (*AP1*) was measured as a molecular marker for flowering (Figure 27A). During the growth period *AP1* levels strongly increased in NO_3^--fed wild-type and *qko* plants as well as in NH_4^+-fed *qko* plants. In contrast, in NH_4^+-fed wild-type plants *AP1* levels remained low (Figure 27A), which was in agreement with the delayed flowering phenotype (Figure 25 and Figure 26). Also the expression of *FLOWERING LOCUS T* (*FT*) was analyzed, which integrates over several central floral pathways (integrator gene) and acts as a long-distance flowering signal (florigen) (Figure 27B). *FT* mRNA levels steadily increased during development in NO_3^--fed wild-type and *qko* plants. In NH_4^+-fed *qko* plants *FT* expression increased up to 18 days after sowing when it reached maximum levels. In NH_4^+-fed wild-type plants *FT* levels hardly increased during growth (Figure 27B). The expression of *TWIN SISTER OF FT* (*TSF*), the closest homolog of *FT* in Arabidopsis, increased only slightly during development and did not differ greatly between the treatments (Figure 27C). The expression of *CONSTANS* (*CO*), a gene encoding a key component of the photoperiod pathway, increased in NO_3^--fed wild-type and *qko* plants as well as in NH_4^+-fed *qko* plants during growth in a similar fashion (Figure 27D). Interestingly, the increase of *CO* mRNA levels during development was inhibited in wild-type plants by the supply of NH_4^+ (Figure 27D).

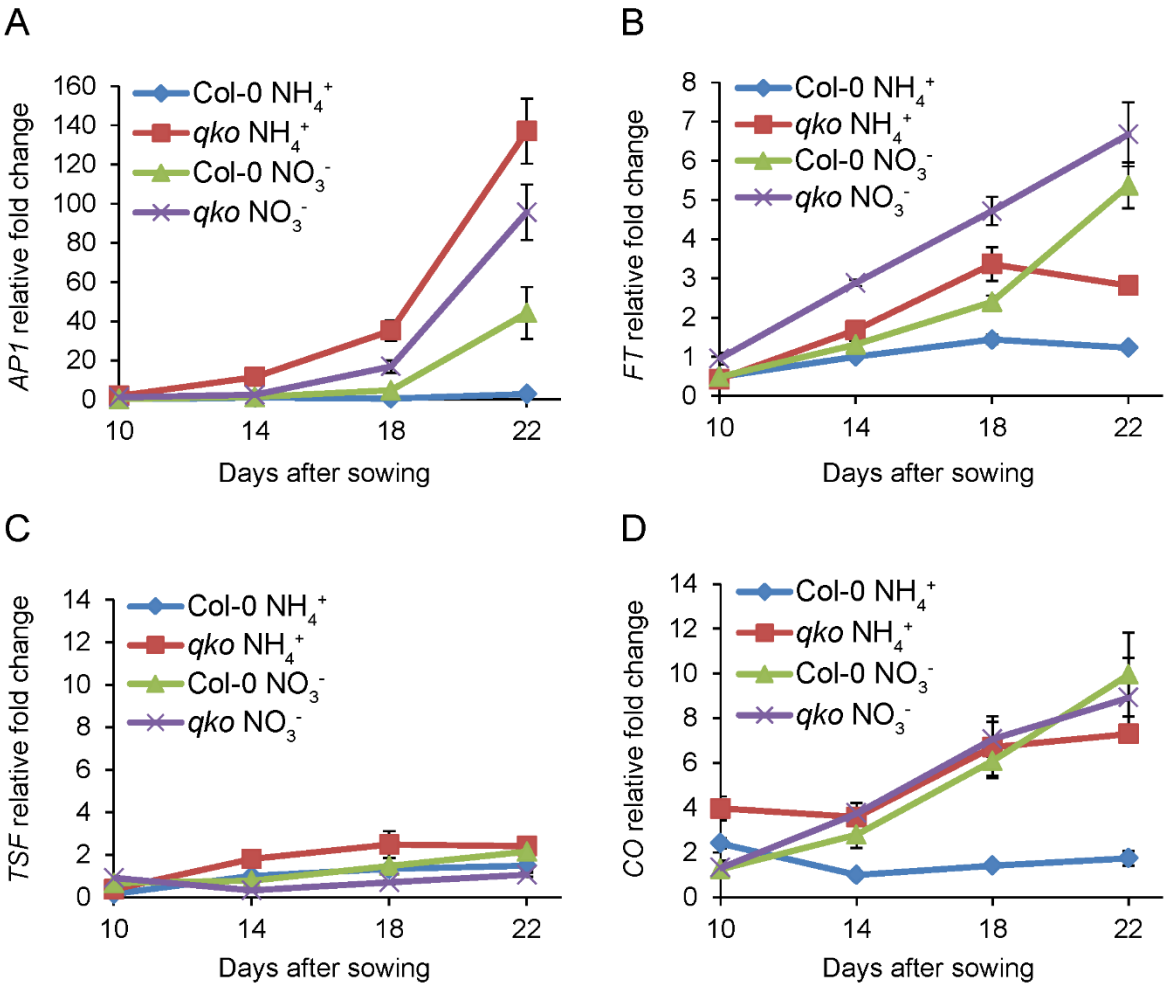


Figure 27. Expression analysis of flowering time genes in wild-type and *qko* plants. Transcript levels of the flowering time genes *APETALA1* (*AP1*) (A), *FLOWERING LOCUS T* (*FT*) (B), *TWIN SISTER OF FT* (*TSF*) (C) and *CONSTANS* (*CO*) (D) were determined in shoots of wild-type (Col-0) and *qko* plants that had been cultured under long-day conditions on 0.5 MS agar medium containing either 2 mM NH_4^+ or 2 mM NO_3^- as the sole N source for 10, 14, 18 or 22 days. Values represent means ± SE (n = 4).

4.4.5 N-form dependent expression of *NRT2.1* during development in wild-type and *qko* roots

In adult, vegetative growing *qko* plants, high-affinity NO_3^- influx was higher than in wild-type plants (Figure 7B and Figure 12A) and correlated with root *NRT2.1* expression (Figure 8B and Figure 12B). Later, it was shown that *qko* plants began to flower earlier than wild-type plants (Figure 21 and Figure 24). It is known that root *NRT2.1* transcript levels change with plant development (Nazoa et al., 2003). Analyses of microarray data using Genevestigator v3 (Hruz et al., 2008) confirmed that root *NRT2.1* levels increase during the vegetative phase and drop after the onset

of flowering (Figure 28A). In addition to a peak at bolting, a second peak of *NRT2.1* transcript levels was observed at the seed filling stage (Figure 28A). Considering these results, the hypothesis was set up that *NRT2.1* might peak at an earlier time point in *qko* than in the wild-type and thereby cause an increased NO_3^- uptake. To test this hypothesis, *NRT2.1* transcript levels were determined in roots of wild-type and *qko* plants, which were cultivated under long-day conditions in agar medium containing either NH_4^+ or NO_3^- as the sole N source (Figure 28B).

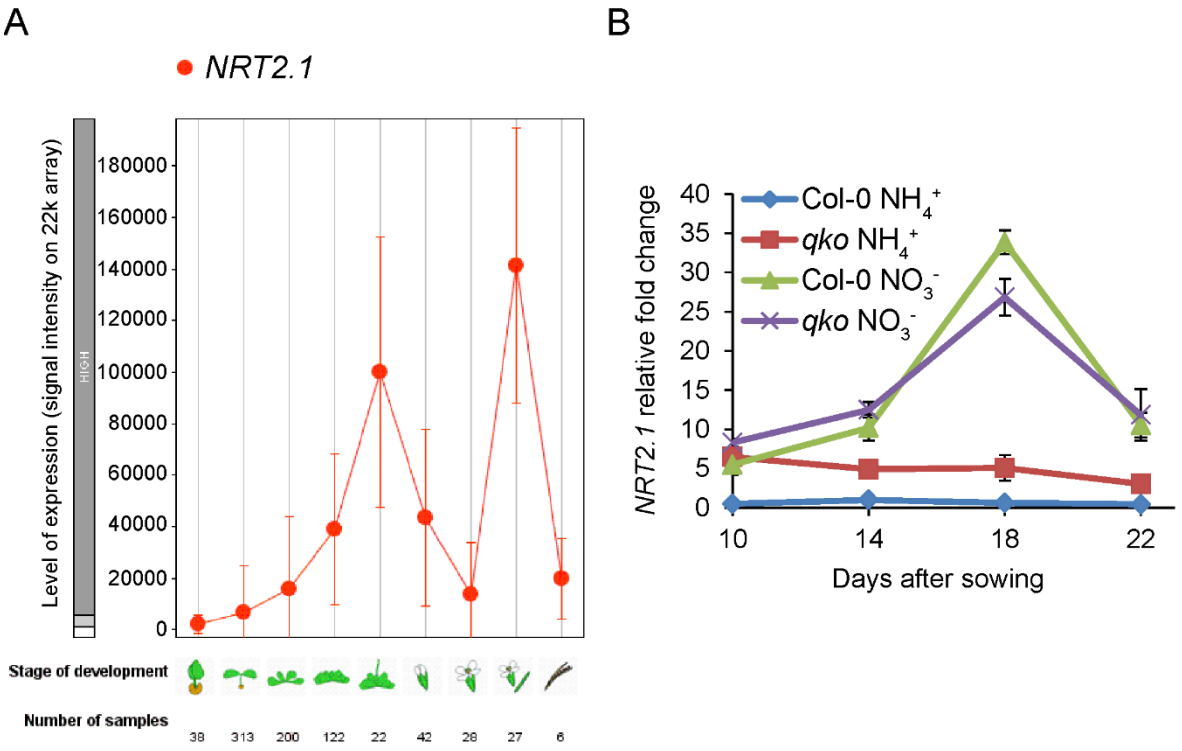


Figure 28. Gene expression analysis of *NRT2.1* in Arabidopsis roots during plant development. (A) Microarray and expression analysis of *NRT2.1* as determined in Genevestigator v3 **(Hruz et al., 2008)** (B) Transcript levels of *NRT2.1* in roots of wild-type (Col-0) and *qko* plants that had been cultured under long-day conditions on 0.5 MS agar medium containing either 2 mM NH_4^+ or 2 mM NO_3^- as the sole N source for 10, 14, 18 or 22 days. Values represent means ± SE (n = 4).

In roots of NO_3^--fed wild-type plants *NRT2.1* expression increased during development and reached a maximum before bolting (Figure 28B). After the onset of bolting *NRT2.1* levels dropped, resembling the microarray data obtained from Genevestigator (Figure 28A). In the roots of NO_3^--fed *qko* plants *NRT2.1* levels showed a similar expression pattern, however at 10, 14 and 22 days after sowing the levels were higher but at 18 days after sowing lower than in wild-type roots (Figure 28B). In NH_4^+-fed wild-type and *qko* plants mRNA levels of *NRT2.1* remained low and

did not change much during the development, with *qko* exhibiting higher *NRT2.1* levels at all analyzed time points (Figure 28B). With the time resolution used in this experiment, it could not be shown that *NRT2.1* expression levels increased earlier in *qko* roots.

5 Discussion

5.1 Influence of AMTs on plant growth and physiology in the vegetative phase

5.1.1 Under N-sufficient conditions *qko* plants grow larger shoots in the vegetative phase

A previous study has shown that *qko* plants accumulated a lower shoot biomass than wild-type plants, when grown on low N medium (500 µM N as NH_4^+ or NH_4NO_3) or on medium with NH_4^+ as the main N source in concentrations up to 2 mM (Yuan et al., 2007a). One of the aims of the present study was to verify the contradictory not yet documented observation that the shoots of *qko* plants are larger than wild-type shoots, when grown under standard conditions with mixed N forms and plenty of NO_3^- (personal communication with several colleagues). The present results confirmed that vegetative growing *qk*o plants had considerably larger shoots (Figure 2 - Figure 4, Figure 23B and Table 3), when N was supplied at millimolar concentrations in the form of NO_3^- or NH_4NO_3 (equimolar NH_4^+ and NO_3^- concentrations). Superior growth expressed in the development of rosettes with a larger diameter (Figure 2 and Figure 3) and a larger leaf area (Figure 4B, Figure 23B and Table 3). Considering that *qko* plants just have about 10% of the capacity for high-affinity NH_4^+ uptake of Col-0 (Yuan et al., 2007a), it is not surprising that the provision of low to moderate concentrations of NH_4^+ as sole N source leads to growth penalties. The supply of NH_4^+ concentrations higher than 2 mM resulted in similar shoot growth phenotypes in wild-type and *qk*o plants (Yuan et al., 2007a). At millimolar NH_4^+ concentrations supplied to Arabidopsis or rice, NH_4^+ uptake is passive and shows linear concentration-dependent kinetics (Wang et al., 1993; Rawat et al., 1999). Under these conditions, the *qko* mutant, which is defective in high-affinity NH_4^+ uptake, still took up sufficient NH_4^+ via the low-affinity transport system to sustain growth (Yuan et al., 2007a). However, the observation that *qko* plants exhibited larger shoots than wild-type plants, when sufficient N was supplied in the form of NO_3^- or as NH_4NO_3 (Figure 2 - Figure 4, Figure 23B and Table 3) merited further investigations. Root and shoot fresh weight of NO_3^--grown wild-type and *qko* plants did not differ significantly from each other (Figure 3C and D and Figure 4A).

The specific leaf area of NO_3^--fed *qko* plants was not significantly different compared to the wild-type (Figure 4C). However, when *qko* was grown on NH_4NO_3, its specific leaf area was larger compared to the wild-type (Figure 4C). Considering that NH_4^+ nutrition decreases specific leaf area in several plant species (Magalhaes and Wilcox, 1984; Raab and Terry, 1994; Bowler and Press, 1996; Guo et al., 2002), reduced NH_4^+ uptake into *qko* roots may explain this phenotype. Histological analyses of cotyledons of NO_3^--fed wild-type and *qko* plants revealed no significant difference in leaf thickness between the two genotypes (Figure 5C and D), supporting that under this condition the specific leaf area of wild-type and *qko* plants did not differ from each other (Figure 4C). In addition to the larger total and average leaf area of *qko* plants (Figure 4B and E), the area of their cotyledons was also larger (Figure 5B). Microscopic analyses identified a larger number of epidermal cells in cotyledon cross sections of *qko* plants as compared to the wild-type (Figure 5E). Also in tobacco (Walch-Liu et al., 2000) and *Festuca arundinacea* (MacAdam et al., 1989) altered leaf expansion mediated by N treatments was attributed mainly to an increase in cell number. In this study, the lacking expression of four AMTs in the *qko* mutant resulted in an increase of the cotyledon size by 16% and increased the number of epidermal cells by 29%, suggesting that also here the increased cell number caused the greater expansion in *qko* cotyledons. Whether the greater leaf expansion in *qko* plants was also caused by an enhanced number of cells, cannot be concluded. Interestingly, Esashi and Leopold (1969) used cotyledon expansion measurements as a bioassay for cytokinins. Maybe the greater cotyledon and leaf expansion in *qko* was also caused by higher cytokinin levels or responsiveness. During the vegetative growth phase no considerable difference in relative growth rate (RGR) between wild-type and *qko* plants (Figure 23C) was observed. But it must be considered that RGR measurements started only 8 days after sowing. The observation, that the cotyledons of *qko* plants had a larger area and that the shoot size of *qko* plants was already early larger suggests that *qko* seedlings established faster than wild-type seedlings. Differences in seedling establishment may indicate differences in seed traits rather than in nutrition-dependent traits.

Another interesting observation was that *qko* plants produced less leaves than wild-type plants (Figure 4D). The average area of an individual *qko* leaf was larger compared to the wild-type (Figure 4E). These results showed that the rosettes of vegetative growing *qko* plants were larger, because *qko* plants were producing less

leaves with a larger area (Figure 3 and Figure 4). In tobacco (Walch-Liu et al., 2000) and Arabidopsis (Helali et al., 2010) NH_4^+ nutrition also diminished leaf number compared to NO_3^- nutrition. These findings led to the hypothesis that *qko* plants might show accelerated development, since the number of leaves is indicative of flowering time. The influence of AMTs on the transition to flowering is discussed in chapter 5.2.

The supply of NH_4^+ as sole N source, instead of NO_3^- or a mixed supply, diminishes plant growth and/or leaf expansion in many plant species (Chaillou et al., 1986; Magalhaes and Huber, 1991; Raab and Terry, 1994; Walch-Liu et al., 2000; Guo et al., 2002), including *Arabidopsis thaliana* (Helali et al., 2010). In tobacco and tomato NO_3^--mediated leaf expansion was associated with altered cytokinin homeostasis (Walch-Liu et al., 2000; Rahayu et al., 2005). The increased leaf expansion of *qko* (Figure 2, Figure 3 and Figure 4) resembled the phenotype of plants grown under NO_3^- nutrition. For that reason, it was assumed that *qko* plants might compensate for the reduced capacity for NH_4^+ uptake by increasing the capacity for NO_3^- uptake and thereby enhance cytokinin-mediated leaf expansion. The influence of AMTs on NO_3^- uptake and on metabolism is discussed in section 5.1.2 and 5.1.3 respectively.

The growth phenotype of *qko* plants compared to wild-type plants that were cultivated with NO_3^- or NH_4NO_3 as N source can be summarized as follows: *qko* seedling establishment resulted in plants with larger cotyledons and subsequently in larger juvenile and adult vegetative plants. The larger shoot size of vegetative growing *qko* plants was reflected in larger rosettes with less leaves that had a larger area. Both, cotyledons and leaves of NO_3^--fed *qko* plants were larger, but not thicker, and they did not display a larger specific leaf area. Cotyledons of *qko* plants produced a larger number of epidermal cells, indicating a higher cell division rate at least in cotyledons of NO_3^--fed *qko* plants.

Exploration of the growth phenotype, namely of the shoot morphological differences between wild-type and *qko* plants revealed novel physiological and developmental roles for AMT-dependent NH_4^+ nutrition that are further discussed below.

5.1.2 The role of AMTs in modulating NO_3^- uptake

Previous studies have shown that the shoot growth of NO_3^--fed plants is stronger than that of NH_4^+-fed plants (Walch-Liu et al., 2000; Helali et al., 2010). Since *qko* plants showed a reduced capacity for NH_4^+ uptake (Yuan et al., 2007a), a putatively increased capacity for NO_3^- uptake might compensate for this and be linked to the increased shoot growth of *qko* plants. To test this hypothesis, NO_3^- influx studies in wild-type and *qko* plants were carried out. The capacity for NO_3^- uptake at 2 mM $^{15}NO_3^-$ supply was unaffected by the absence of AMTs in *qko* plants grown under N-deficient conditions or under the continuous supply of 2 mM NO_3^- (Figure 7A). In plants of both genotypes that had been precultured in the absence of N for three days, the capacity for NO_3^- uptake at 2 mM external $^{15}NO_3^-$ was lower than that of plants grown under continuous supply of NO_3^- (Figure 7A). Under N deficiency there is no necessity to increase the capacity for low-affinity NO_3^- uptake. Under this condition the ability to absorb NO_3^- at lower external concentrations is more important (Forde, 2000). At a supply level of 0.2 mM $^{15}NO_3^-$, which is indicative for the high-affinity NO_3^- transport system (HATS), *qko* plants responded differently to the absence of AMTs. High-affinity NO_3^- influx into the roots of the *qko* mutant was significantly higher as compared to Col-0, when plants were pre-grown under N-sufficient conditions (2 mM KNO_3 or 1 mM NH_4NO_3) (Figure 7B, Figure 10, Figure 12A and Figure 13), suggesting that components regulating high-affinity NO_3^- uptake were affected by the absence of AMTs in *qko* plants. In Arabidopsis NRT2.1 is the main component of the HATS making the greatest contribution to high-affinity NO_3^- uptake in roots (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007). Accordingly, *NRT2*.1 transcript levels (Figure 8B and Figure 12B) and subsequently NRT2.1 protein levels (Figure 9) were strongly derepressed in roots of N-sufficient *qko* plants. This suggested that the enhanced capacity for high-affinity NO_3^- uptake in N-sufficient *qko* plants was caused primarily by weaker systemic repression of *NRT2.1* transcription or *NRT2.1* transcript degradation by the N nutritional status of the plants.

The regulation of *NRT2.1* is complex and not yet fully understood. It is well established that *NRT2.1* is induced by short-term NO_3^- supply (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999; Ho et al., 2009) and feedback repressed by reduced N (NH_4^+ and amino acids) or prolonged provision of high NO_3^- concentrations (Lejay

et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003; Muños et al., 2004; Girin et al., 2007). Interestingly, *NRT2.1* repression by reduced N is relieved in mixed N media, when NO_3^- concentrations decrease to low levels (< 0.5 mM), causing an induction of *NRT2.1* expression and high-affinity NO_3^- uptake (Muños et al., 2004; Krouk et al., 2006). In *nrt1.1* mutants repression of *NRT2.1* by high NO_3^- provision in the presence of a reduced N source was absent, showing that this repression is mediated by NRT1.1 (Muños et al., 2004; Krouk et al., 2006). The mechanism by which NRT1.1 modulates *NRT2.1* expression in response to high N provision is not fully understood.

Interestingly, *NRT1.1* levels were substantially repressed in *qko* under N-sufficient conditions as compared to the wild-type, but not under N-deficient conditions (Figure 8A). Under N-deficient conditions *NRT1.1* mRNA levels were similar and strongly reduced in both, wild-type and *qko* plants (Figure 8A). Under this condition, the differences between wild-type and *qko* plants in *NRT2.1* levels and high-affinity NO_3^- influx vanished (Figure 7B and Figure 8B). Differences in *NRT2.1* expression and high-affinity NO_3^- influx between both genotypes became prominent only under N-sufficient conditions, when *NRT1.1* levels were high in the wild-type and repressed in *qko*. This suggests that AMTs play a role in NRT1.1-dependent repression of *NRT2.1* and high-affinity NO_3^- uptake. This is consistent with the fact that NRT1.1 expression is necessary for NO_3^--mediated repression of *NRT2.1* under high N provision (e.g. 1 mM NH_4NO_3) (Muños et al., 2004; Krouk et al., 2006; Wang et al., 2009). The present study shows that NRT1.1-dependent repression of *NRT2.1* by high N provision is attenuated in *qko*, indicating that AMTs or AMT-mediated NH_4^+ transport might be involved in this repression. In roots of plants grown under NO_3^--supply, the NH_4^+ transported may originate amongst others from NO_3^- reduction, protein catabolism and/or NH_4^+ retrieval.

The question then arose, which of the four AMTs absent in *qko* could be responsible for repressing *NRT2.1* and high-affinity NO_3^- uptake? Although the present results did not allow pinpointing a single AMT being responsible for the effect seen in *qko*, AMT1;1 showed the largest contribution to repression of *NRT2.1* transcript levels and high-affinity NO_3^- uptake (Figure 12). In both tested conditions, namely continuous supply of 2 mM KNO_3 or treatment with 1 mM NH_4NO_3 for 7 d, *qko11,* expressing functional AMT1;1 in the *qko* background, showed the greatest reduction in high-affinity NO_3^- uptake compared to *qko* and reached in the NH_4NO_3 treatment even

wild-type levels (Figure 12A). Accordingly, in roots of NH_4NO_3-treated *qko11* plants *NRT2.1* transcript abundance was reduced to wild-type levels (Figure 12B). However, *NRT2.1* levels were not reduced in the roots of *qko11* plants grown under KNO_3 (Figure 12B). Although *AMT1;3* and *AMT2;1* expression in the *qko* background substantially reduced *NRT2.1* transcript levels, they were not able to repress high-affinity NO_3^- uptake to wild-type levels. AMT1;2 showed a weaker contribution to repression of *NRT2.1* and high-affinity NO_3^- uptake (Figure 12). AMT1;1 and AMT1;3 were shown to be involved in NH_4^+ sensing or N signaling (Loqué et al., 2007; Lanquar et al., 2009; Lima et al., 2010; Wang et al., 2013; Yuan et al., 2013). To avoid NH_4^+ toxicity AMT1;1 is phosphorylated at the cytosolic C terminus after NH_4^+ exposure, resulting in an allosteric inactivation of AMT1 trimers. Recently, the same kinase that regulates the NO_3^- transceptor NRT1.1, namely CIPK23, was identified as the enzyme responsible for phosphorylating AMT1;1 and AMT1;2 (Straub et al., 2017). Although feedback repression of *NRT2;1* by reduced N is thought to be under systemic regulation (Gansel et al., 2001), NRT1.1-dependent derepression of *NRT2.1* by low NO_3^- concentrations in the presence of NH_4^+ is under local regulation (Krouk et al., 2006). Together with the fact that high-affinity NH_4^+ influx and *AMT1;1* expression are also regulated locally (Gansel et al., 2001), suggests that AMT1;1 may play an important role in modulating local and NRT1.1-dependent regulation of *NRT2.1* and high-affinity NO_3^- uptake.

Although AMT1;1 showed the greatest contribution in repressing *NRT2.1* and high-affinity NO_3^- uptake, its reconstitution in the *qko* background could not account for the entire effect (Figure 12). Interestingly, increasing intracellular NH_4^+ concentrations in the roots of NO_3^--fed *qko* plants by applying the glutamine synthetase inhibitor MSX, could reduce high-affinity NO_3^- influx into *qko* roots almost to wild-type levels (Figure 13). This suggests that lower high-affinity NO_3^- uptake in the wild-type is likely caused by higher concentrations of intracellular NH_4^+ than in *qko* plants, even when plants were continuously grown on KNO_3. This may be indicative for a role of the AMTs in NH_4^+ retrieval, because also NO_3^--grown plants show continuous losses of NH_4^+ or NH_3 (Britto et al., 2001a; Lanquar et al., 2009) and also NO_3^- induces AMT-type transporters like *LeAMT1;2* (Lauter et al., 1996). Indeed, roots of *qko* plants showed reduced NH_4^+ concentrations (Figure 17) and weaker induction of NH_4^+-responsive genes (Figure 18) when NH_4^+ was present in the medium. NH_4^+ originating from NO_3^-

reduction and leaking out of cells might be retrieved to a lower extent into cells of *qko* roots and is thus less available for repression of NO_3^- transport systems.

The large shoot phenotype of *qko* could not be associated to an increased accumulation of NO_3^- in the shoot of NO_3^--fed plants (Figure 6E), despite the higher capacity for high-affinity NO_3^- uptake. Also, the amount of total N was not substantially affected in roots and shoots of the NH_4^+ uptake-defective *qko* line (Figure 6), suggesting that the shoot phenotype and the impaired regulation of NO_3^- uptake in *qko* were not caused by systemic regulation via the nutritional status of the plant. However, since *qko* is defective in high-affinity NH_4^+ uptake (Yuan et al., 2007a), the increased capacity for high-affinity NO_3^- uptake could be a compensatory mechanism. The uptake of NH_4^+ and NO_3^- are tightly regulated at multiple levels and interactions between NH_4^+ and NO_3^- in their uptake have been predicted (Hachiya and Sakakibara, 2017) and confirmed by posttranslational regulation of AMT1;1 and NRT1.1 by CIPK23 (Straub et al., 2017). NH_4^+ and NO_3^- are the predominant N forms taken up by plants and their uptake comprises up to 80 % of the total mineral elements taken up by plants (Marschner, 2012). As these two N forms differ in charge, the form of N has a substantial effect on the cation/anion uptake balance, cellular pH regulation and rhizosphere pH (Marschner, 2012). NH_4^+ and NO_3^- nutrition differ in their effect on rhizosphere pH: NO_3^- uptake results in an alkalinization of the rhizosphere, because NO_3^- is cotransported with protons into the cell. In contrast, NH_4^+ uptake results in an acidification of the rhizosphere, because protons are pumped out of the cell by a H^+-ATPase to maintain the intracellular charge balance and the electrochemical potential gradient across the plasma membrane (Bloom et al., 2003; Escobar et al., 2006; Patterson et al., 2010). The rhizosphere pH has a great impact on plant growth. It influences the solubility and the acquisition of nutrients and toxic elements. Furthermore, many plant species are sensitive to acid soils and exhibit growth suppression on low pH (Marschner, 2012). When NH_4^+ is the exclusive N source, it can cause toxicity symptoms, resulting in growth suppression, reduced leaf expansion and severe yield depression (Walch-Liu et al., 2000; Britto and Kronzucker, 2002). NH_4^+ toxicity has been linked to rhizosphere acidification associated with NH_4^+ uptake (Britto and Kronzucker, 2002). Transcriptome analyses revealed that many NH_4^+-induced genes are also upregulated by low pH, tightening the link between NH_4^+ nutrition and H^+ toxicity (Lager et al., 2010; Patterson et al., 2010). Interestingly, *AMT1;1* expression was shown to be repressed by exposure to

acidic pH (Lager et al., 2010). Recently, NRT1.1-mediated NO_3^- influx has been shown to contribute to tolerance to proton toxicity by increasing the rhizosphere pH (Fang et al., 2016). *NRT1.1* expression is induced by acidic pH and the contribution of NRT1.1 to NO_3^- influx is greater under this condition than under alkaline pH (Tsay et al., 1993; Wang et al., 1998; Fang et al., 2016). Furthermore, the contribution of NRT1.1 to NO_3^- uptake depends on the N form used for preculture. *nrt1.1* mutants show a larger reduction in NO_3^- uptake when grown in the presence of NH_4^+ than of KNO_3, indicating that the contribution of NRT1.1 is higher when plants are grown in the presence of NH_4^+ (Huang et al., 1996; Touraine and Glass, 1997; Crawford and Forde, 2002). Moreover, *NRT1.1* is induced by NH_4^+ supply (Patterson et al., 2010; Hachiya et al., 2012). All these studies tightly link NH_4^+ and NO_3^- nutrition to changes in the intracellular charge balance and to the regulation and activity of NH_4^+ and NO_3^- transporters, especially AMT1;1 and NRT1;1. Therefore, it can be hypothesized that rhizosphere acidification mediated by AMT-dependent NH_4^+ uptake is also involved in regulating NRT1.1 expression and activity.

Since *qko* plants are defective in high-affinity NH_4^+ uptake, it is probable that the rhizosphere of *qko* plants is locally acidified to a lower extent by NH_4^+ originating from supply or leakage after NO_3^- reduction. This could have led to weaker *NRT1.1* expression and subsequently to derepression of *NRT2.1* under high N provision and concomitant induction of high-affinity NO_3^- uptake. Recent data showing that *qko* plants acidify the rhizosphere to a lower extent than wild-type plants (Meier, 2018), indicate that the expression of AMTs influence rhizosphere pH, supporting this hypothesis. A pH-dependence of the expression of genes involved in N acquisition would also explain why the growth differences (Figure 3 and Figure 4) and differences in *NRT1.1* and *NRT2.1* expression levels (Figure 11) in plants grown on pH-buffered agar medium were not so prominent as in hydroponically-grown plants. Buffering media might attenuate phenotypes caused by differences in NH_4^+ and/or NO_3^- uptake capacities. It is tempting to postulate that changes in intra- and/or extracellular pH or membrane potential induced by the uptake of inorganic N are key components in N sensing and/or signaling. CIPK23 might also be regulated by pH, depending on the N form available and thereby phosphorylate AMT1;1 and NRT1.1 accordingly.

Taken together these experiments showed that the absence of AMTs in the *qko* mutant resulted in derepression of high-affinity NO_3^- uptake, caused by an upregulation of NRT2.1 at the transcript and subsequently at the protein level, when plants were grown under repressive N-sufficient conditions. Under this condition, *NRT1.1* levels were additionally repressed in the roots of *qko* plants. This suggests that AMT-mediated NH_4^+ transport may be involved in NRT1.1-dependent repression of *NRT2.1* by high N provision. AMT1;1 showed the greatest contribution to modulation of the NO_3^- HATS. It is hypothesized that rhizosphere acidification mediated by AMT-dependent NH_4^+ uptake is involved in regulating NRT1.1 expression, activity and downstream processes like *NRT2.1* expression. Since plants predominantly supplied with NO_3^- as a N source exhibit better shoot growth (Walch-Liu et al., 2000; Britto and Kronzucker, 2002; Helali et al., 2010), the larger shoots of *qko* can be associated to an increased capacity for high-affinity NO_3^- uptake. Krouk et al. (2006) demonstrated that NRT2.1-mediated high-affinity NO_3^- uptake is crucial for preventing Arabidopsis plants from the detrimental effects of NH_4^+ supply on shoot growth, when NO_3^- availability is low.

5.1.3 The influence of AMT expression on metabolism

The supply of NH_4^+ as sole N source, instead of NO_3^- or a mixed supply, diminishes plant growth and/or leaf expansion in many plant species (Chaillou et al., 1986; Magalhaes and Huber, 1991; Raab and Terry, 1994; Walch-Liu et al., 2000; Guo et al., 2002), including *Arabidopsis thaliana* (Helali et al., 2010). In tobacco and tomato NO_3^--mediated leaf expansion was associated with an altered cytokinin homeostasis (Walch-Liu et al., 2000; Rahayu et al., 2005). NO_3^- nutrition induces the accumulation (Takei et al., 2001; Miyawaki et al., 2004; Takei et al., 2004a) and root-to-shoot translocation (Walch-Liu et al., 2000; Takei et al., 2001; Rahayu et al., 2005) of cytokinin. The genes *IPT3* and *IPT5* encoding adenosine phosphate-isopentenyltransferases (IPTs), which are involved in cytokinin biosynthesis, are induced by NO_3^- (Miyawaki et al., 2004; Takei et al., 2004a). The increased leaf expansion of *qko* (Figure 2 - Figure 4) resembled such a NO_3^--dependent phenotype with higher cytokinin levels. For that reason, it was assumed that *qko* plants might compensate for the reduced capacity for NH_4^+ influx by increasing the capacity for NO_3^- uptake and thereby enhancing cytokinin-mediated leaf expansion. Unfortunately, it was not possible to detect the cytokinins tZ and cZ. Although *qko*

plants displayed an increased capacity for high-affinity NO_3^- uptake (chapter 5.1.2), the analysis of further cytokinins, cytokinin precursors and conjugates did not reveal an involvement of cytokinins in increased leaf expansion in *qko* plants (Figure 14). The quantification of auxins and ABA in wild-type and *qko* plants did also not allow the identification of AMT-dependent patterns. Unfortunately, the measurement of phytohormones showed partially a high variation and did not allow the detection of differences associated with N nutrition. The action of most phytohormones is not exclusively determined by their tissue concentration. Relative concentration gradients and the different sensitivity of various cell types are usually more relevant (Davies, 2010). Therefore, the use of transgenic plants, expressing reporters responsive to the investigated phytohormones and measuring the expression of phytohormone-responsive genes might be a more successful approach.

An interesting observation of the phytohormone measurements was that the shoots of N-sufficient *qko* plants had higher salicylic acid concentrations as compared to the wild-type (Figure 16). The main role of salicylic acid is to mediate responses to pathogen attack (Davies, 2010). The involvement of salicylic acid in mediating responses to N nutrition has not been reported yet. Interestingly, Lager et al. (2010) found by transcriptome analysis that *At1g74710* (*ICS1/SID2*), the key gene involved in salicylic acid biosynthesis is up-regulated by 5-fold after shifting plants to acidic pH. Furthermore, the pH-regulated gene set clustered with transcriptional responses to a variety of pathogens and pathogen elicitors. Moreover, the transcriptional response to salicylic acid also showed a positive correlation to pH responses (Lager et al., 2010). The fact that NH_4^+ nutrition induces rhizosphere acidification and genes induced by NH_4^+-supply partially overlap with genes induced by acidic pH (Lager et al., 2010; Patterson et al., 2010), could link altered salicylic acid levels to NH_4^+ nutrition. Further investigations are needed to explore a potential connection between NH_4^+ nutrition and salicylic acid.

An interesting observation was that the concentration of amino acids was lower (Table 2 and Figure 19A) and the concentration of sucrose higher (Figure 20E) in roots of *qko* plants as compared to the wild-type. It is well established that *NRT2.1* expression and high-affinity NO_3^- uptake are feedback repressed by reduced N metabolites including amino acids (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003). Several genes encoding root ion transporters, including the NO_3^- transporters

NRT1.1 and *NRT2.1* and the NH_4^+ transporters *AMT1;1, AMT1;2* and *AMT1;3* as well as high-affinity NO_3^- and NH_4^+ influx into plant roots are diurnally regulated (Gazzarrini et al., 1999; Lejay et al., 1999; Lejay et al., 2003) and stimulated by sugar supply. *NRT2.1* expression and high-affinity NO_3^- uptake are induced by light and by the supply of the sugars sucrose, glucose and fructose. Since induction by light and induction by sucrose are strongly correlated, it is believed that photoassimilates and not light per se are the positive regulators of root NO_3^- uptake (Lejay et al., 1999; Lejay et al., 2003). Although the total N concentration was only slightly reduced in the roots of N-sufficient *qko* plants (Figure 6C), the reduced levels of amino acids (Table 2 and Figure 19A) together with the increased sucrose concentration (Figure 20E) and the non-reduced dry matter content in *qko* roots (Figure 6A), suggests that the C:N ratio might be elevated in roots of *qko* plants. This increase in the C:N ratio on the one hand, might have been a direct consequence of the suppressed NH_4^+ uptake in *qko* roots and thus a lower consumption of C skeletons for subsequent NH_4^+ assimilation. On the other hand, NO_3^- reduction and N assimilation in the root requires sucrose import from the shoot to generate reducing equivalents via the OPP (Hawkesford et al., 2012). The increased capacity for high-affinity NO_3^- uptake in *qko*, probably compensating for defective in NH_4^+ influx, might require a higher flow of C to the roots for NO_3^- assimilation than the wild-type. Taken together, depression of *NRT2.1* expression and increased high-affinity NO_3^- uptake was accompanied by a decreased amino acid and an increased sucrose concentration in *qko* roots.

Recently, Chen et al. (2016) found that the protein HY5 is a shoot-to-root mobile transcription factor mediating light-responsive coupling of C assimilation and shoot growth with root NO_3^- uptake. HY5 is required for sugar-induced up-regulation of *NRT2.1* expression and high-affinity NO_3^- uptake (Chen et al., 2016). It will be interesting to see in future how different N regimes and how the expression of NO_3^- and NH_4^+ transporters can influence HY5-dependent N and C balances.

5.2 Influence of AMTs on plant growth in the reproductive phase and on flowering time

5.2.1 *qko* plants start flowering earlier than the wild-type and accumulate a lower biomass at the transition to flowering

Observing the growth phenotype in the vegetative phase (section 5.1.1) and reproduction of wild-type and *qko* seeds, led to the hypothesis that *qko* plants might be altered in development, especially in flowering time. Indeed, characterization of the flowering behavior revealed that *qko* plants induced flowering earlier than wild-type plants (Figure 21, Figure 24 - Figure 26) and produced less leaves at the transition to flowering (Figure 21B). The number of leaves is often correlated with the time of floral initiation and is widely used as a measurement to compare the time to flowering between genotypes and/or treatments (Koornneef et al., 1991). *qko* plants developed larger shoots in the vegetative phase than wild-type plants (section 5.1.1). During the vegetative growth phase no considerable difference in relative growth rate (RGR) between wild-type and *qko* plants (Figure 23) was observed. This trait was reverted in the reproductive phase, as *qko* plants started to flower earlier than wild-type plants, and accordingly the RGR of *qko* plants decreased earlier (Figure 23). During plant development and life cycle completion plants decrease RGR. The earlier decrease of RGR and the earlier flowering of *qko* indicated that *qko* had a shorter life cycle than wild-type plants. As a consequence, *qko* plants had less time for biomass accumulation and developed smaller shoots with less and smaller leaves at the transition to flowering (Figure 21 - Figure 23 and Table 3).

In many studies, the supply of low N concentrations promoted the induction of flowering in comparison to the supply of high N concentrations (Dickens and Van Staden, 1988; Loeppky and Coulman, 2001; Kant et al., 2011; Marín et al., 2011; Liu et al., 2013; Yuan et al., 2016). Recently, Lin and Tsay (2017) suggested that flowering time describes a U-shaped curve in dependence of the NO_3^- or N concentration. Plants supplied with low or optimal NO_3^- concentrations exhibiting the earliest flowering times were at the base of the curve, whereas plants supplied with high concentrations of NO_3^- or grown under NO_3^--limiting and starving conditions were delayed in flowering and located at the right or left side of the U-shaped curve respectively (Lin and Tsay, 2017).

In the present study, the *qko* mutant, which showed an increased *NRT2.1* expression and a higher capacity for high-affinity NO_3^- uptake, initiated flowering earlier than the wild-type. Neither the concentration of total N nor of NO_3^- were increased in the tissues of *qko* plants (chapter 5.1.2). It is known that *NRT2.1* is upregulated by low to moderate external NO_3^- concentrations and repressed by high NO_3^-/N provision (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003; Krouk et al., 2006; Ho et al., 2009). These observations connect *NRT2.1* expression with an early flowering phenotype. This link is further supported by the fact that root *NRT2.1* transcript levels change with plant development (Nazoa et al., 2003). *In silico* (Figure 28A) and transcript (Figure 28B) analysis showed that *NRT2.1* levels increase during the vegetative phase and drop after the onset of flowering, when NO_3^- is the predominant N source. Interestingly, *NRT2.1* expression levels were higher in the root of *qko* plants than in the wild-type before bolting (Figure 28B), strengthening the correlation between *NRT2.1* expression and floral induction.

Since *NRT1.1* expression was reduced in the *qko* mutant (chapter 5.1.2) and NRT1.1 participates in the regulation of *NRT2.1* (Muños et al., 2004; Krouk et al., 2006; Ho et al., 2009), it is probable that NRT1.1 is involved in regulating flowering time. Interestingly, *nrt1.1* mutants displayed a late-flowering phenotype in an earlier study (Guo et al., 2001), indicating that NRT1.1 might be involved in regulating the time of flowering in dependence of the N availability.

Taken together, the NH_4^+ uptake-deficient mutant *qko* showed an early flowering phenotype, when NO_3^- was the predominant N source. This phenotype may be linked to a reduced *NRT1.1* and an increased *NRT2.1* expression prior to floral induction. New insight on the role of AMT-dependent NH_4^+ nutrition on flowering time is discussed in the next section. More studies are needed in the future to elucidate the contribution of NRT1.1 and NRT2.1 in regulating flowering time under varying N availabilities.

5.2.2 NH_4^+ nutrition delays flowering and inhibits *CONSTANS* in an AMT-dependent manner

The NH_4^+ uptake-defective mutant *qko* exhibited a higher capacity for high-affinity NO_3^- uptake and initiated flowering earlier than the wild-type, when cultivated on mixed N forms or on NO_3^- as the sole N source. This observation raised the question

if the supply of different forms of inorganic N may exert different effects on the time of flowering. In a time-course experiment conducted under long day conditions and on NO_3^- as the sole N source, wild-type plants were bolting and flower buds were visible 22 days after sowing (DAS) (Figure 25C). Interestingly, NH_4^+-fed wild-type plants did not show any signs of flowering 22 DAS (Figure 25C), indicating that NH_4^+ provision inhibited the induction of flowering. Furthermore, root and shoot biomass production were strongly reduced, when NH_4^+ was supplied as the sole N source compared to NO_3^- supply (Figure 25). Growth retardation caused by exclusive NH_4^+ nutrition is a commonly observed phenomenon (Walch-Liu et al., 2000; Britto and Kronzucker, 2002; Rahayu et al., 2005; Helali et al., 2010; Hachiya et al., 2012). However, the inhibitory effect of NH_4^+ on the induction of flowering in Arabidopsis plants has apparently not been documented yet. In tobacco and Arabidopsis reduction of shoot growth was associated with a lower leaf number and a reduced leaf expansion rate, when grown on NH_4^+ instead of NO_3^- (Walch-Liu et al., 2000; Helali et al., 2010). Helali et al. (2010) proposed that NH_4^+ supply may repress leaf initiation. Although leaf number is a widely used parameter for describing flowering behavior, as mentioned before, no connection between the N form and the induction of flowering has been made yet. Despite the circumstance that the leaf number was not counted and quantified in the time-course experiment comparing the effect of NH_4^+ and NO_3^- on growth and development, the visual phenotype suggests that also here the supply of NH_4^+ reduced leaf number (Figure 25). The inhibitory effect of NH_4^+ on floral induction is further supported by the analysis of the expression of flowering time genes. The expression of *CONSTANS* (*CO*), the florigen gene *FLOWERING LOCUS T* (*FT*) and the flower organ identity gene *APETALA1* (*AP1*) strongly increased during the course of the experiment in wild-type plants grown on NO_3^- (Figure 27). In contrast, in NH_4^+-fed plants the expression of these genes barely changed, confirming the inhibitory effect of NH_4^+ supply on floral induction at a molecular level.

Similar to wild-type plants, also *qko* plants showed a considerably higher biomass accumulation when grown on NO_3^- as the sole N source instead of on NH_4^+ (Figure 26). In contrast to wild-type plants, bolting and visible flower buds were observed 22 DAS in *qko* plants, irrespective of whether they were fed with NO_3^- or NH_4^+ as the sole N source (Figure 26C). The observation that NH_4^+ nutrition inhibited the transition to flowering in wild-type, but not in *qko* plants indicates that AMTs absent in *qko* might be involved in mediating NH_4^+-dependent inhibition of flowering. Observing

flowering time in wild-type, *qko* and triple insertion lines encoding either functional *AMT1;1* (*qko11*), *AMT1;2* (*qko12*), *AMT1;3* (*qko13*), or *AMT2;1* (*qko21*) in the *qk*o background did not allow to pin point a single AMT responsible for delaying flowering time (Figure 24). *qko12*, *qko13* and *qko21* exhibited an intermediate flowering time phenotype, indicating that AMT1;2, AMT1;3 and AMT2;1 contribute partially to delayed flowering time in the presence of NH_4^+. Initiation of flowering occurred earlier in the *qko11* than in the *qko* line, indicating that AMT1;1 does not contribute to delayed flowering time (Figure 24).

The observation that the expression of the floral integrator gene *FT* and of *AP1* were not considerably affected by NH_4^+ supply in the *qko* mutant (Figure 27), supports the hypothesis that AMTs might be involved in mediating NH_4^+-dependent inhibition of flowering. Interestingly, the expression of *CONSTANS* (*CO*) increased during the life cycle in NH_4^+-fed *qko* plants in a similar fashion as NO_3^--fed *qko* and wild-type plants (Figure 27D). However, *CO* transcript levels remained relatively stable at a low level over the course of the experiment in NH_4^+-fed wild-type plants. CO is a transcriptional regulator and key component of the photoperiod pathway that integrates inputs from the circadian clock and light receptors, thereby promoting flowering in long days (Suárez-López et al., 2001; Valverde et al., 2004; Fornara et al., 2010). CO promotes floral initiation by inducing expression of the integrator genes *FT* and *TWIN SISTER OF FT* (*TSF*) genes (Turck et al., 2008). The suppression *CO* in the wild-type und derepression in *qko* when NH_4^+ is the sole N source, indicates that NH_4^+ nutrition delays flowering time by affecting the photoperiod pathway in an AMT-dependent manner.

How N signaling interacts with the flowering pathways to induce flowering is still poorly understood. Marín et al. (2011) observed that low NO_3^- still promoted flowering in late-flowering mutants impaired in the photoperiod, temperature, GA and autonomous flowering pathways as well as in mutants of floral integrators. The authors therefore proposed that NO_3^- modulates flowering time via a novel signaling pathway acting in parallel with the photoperiod, GA and autonomous pathways and entering downstream of the known floral integrators. Conversely, Liu et al. (2013) found that plants grown under low NO_3^- conditions induced the expression of the GA biosynthesis gene *GA1* and exhibited higher concentrations of GA_3. In addition, they observed an increased expression of *CO* and of the integrator gene *SOC1* under low

NO_3^- conditions. In another study, the expression of the flowering repressor *FLC* (a component of the vernalization and autonomous pathway) was repressed and of the positive regulators of flowering *FT*, *AP1* and *LEAFY* (*LFY*) induced under the supply of low NO_3^- (Kant et al., 2011). Together, these studies indicate that N nutrition may interact with components of various flowering pathways to modulate the induction of flowering as a function of N availability. Some recent studies supported the involvement of genes from the photoperiod pathway in N-dependent flowering (Gutiérrez et al., 2008; Yuan et al., 2016; Weber and Burow, 2018). Gutiérrez et al. (2008) showed that the master clock control gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) is regulated by Glu or a Glu-derived metabolite and this in turn affects the expression of key N-assimilatory genes, partially by direct binding to their promoter regions. Distinct N-metabolites were able to advance or delay the phase response of the circadian clock as measured by *CCA1* expression. Therefore, the authors proposed that regulation of *CCA1* by organic N signals may represent a novel input mechanism for N nutrition to affect the circadian clock function of plants (Gutiérrez et al., 2008). Another study identified ferredoxin-$NADP^+$-oxidoreductase and the blue light receptor cryptochrome1 CRY1 as key factors involved in N-regulated flowering time control (Yuan et al., 2016). Both genes are transcriptionally upregulated by low N provision and their loss-of-function mutants are insensitive to altered N supply levels. High N provision decreased, whereas low N supply increased the expression of several key components of the central oscillator (e.g., *CCA1*, *LHY* and *TOC1*) and the flowering output genes (e.g., *GI* and *CO*) (Yuan et al., 2016). Yuan et al. (2016) proposed that N signaling acts as a modulator of nuclear CRY1 protein abundance to regulate the amplitude of the circadian clock and thereby controlling flowering time. A meta-analysis conducted by Weber and Burow (2018) revealed that the photoperiod pathway is strongly represented as a pathway with genes responding to N treatments and genes involved in the control of flowering time.

Taken together, flowering and the expression *CO*, a key output gene of the photoperiod pathway, were repressed in wild-type but not in *qko* plants when NH_4^+ was used as N form, indicating that NH_4^+ nutrition delays flowering time by affecting the photoperiod pathway in an AMT-dependent manner.

5.3 Conclusion

The present study could confirm the previous undocumented observation that the NH_4^+ uptake-defective mutant *qko* develops larger shoots than the wild-type under certain growth conditions. Under non-limiting NO_3^- or NH_4NO_3 availability, *qko* produced larger shoots with less leaves that had a larger area. Exploration of the distinct shoot growth phenotype between wild-type and *qko* plants revealed novel physiological and developmental roles for AMT-dependent NH_4^+ nutrition in *Arabidopsis thaliana*. The absence of AMTs in the *qko* mutant increased the capacity for high-affinity NO_3^- uptake by a derepression of NRT2.1 at the transcript and protein level, when plants were grown under repressive N sufficient conditions. Under this condition *NRT1.1* levels were additionally repressed in the roots of *qko* plants. The NO_3^- transceptor NRT1.1 is known to mediate the induction of *NRT2.1* by short-term exposure to NO_3^- and the repression of *NRT2.1* by high N provision. Therefore, AMT-mediated NH_4^+ transport is proposed to be involved in NRT1.1-dependent repression of *NRT2.1* by high N provision. It is hypothesized that rhizosphere acidification mediated by AMT-dependent NH_4^+ uptake is involved in regulating NRT1.1 expression, activity and downstream processes like *NRT2.1* expression. Among the *AMTs* disrupted in the *qko* mutant AMT1;1 showed the greatest contribution to modulation of high-affinity NO_3^- uptake. Modulation of the NO_3^- uptake capacity by AMT-mediated NH_4^+ transport may represent a mechanism to ensure the cation-anion balance of the cell and to stabilize the electrochemical gradient across the plasma membrane of root cells involved in ion uptake from the soil solution. It is a commonly observed phenomenon that the shoot growth is promoted by NO_3^- or mixed N supplies and suppressed by exclusive NH_4^+ provision. In this study, the increased shoot size of vegetative growing *qko* plants was associated with an increased capacity for high-affinity NO_3^- uptake and elevated *NRT2.1* levels. Disruption of *AMTs* also affected primary metabolism. The roots of *qko* plants exhibited decreased amino acid concentrations and elevated sucrose levels. Consistent with previous findings, reduced N metabolites repress and sugars induce the expression of *NRT2.1* and the NO_3^- HATS. In addition to physiological processes, plant development was also affected by AMT-dependent NH_4^+ transport. *qko* plants exhibited a shorter life cycle with an earlier flowering time and decreased leaf number. *NRT2.1* is developmentally regulated and was higher expressed in *qko* roots

in the pre-flowering phases. Elevated *NRT2.1* expression seems to be correlated to floral induction by low NO_3^- supply levels. Interestingly, NH_4^+ but not NO_3^- supplied as the sole N source suppressed floral induction and the expression of the flowering time genes *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *APETALA1* (*AP1*) in wild-type plants. Conversely, *qko* plants initiated flowering independent of whether they were fed with NH_4^+ or NO_3^- as the sole N source. The expression of the flowering time genes was also not considerably affected by NH_4^+ supply in the *qko* mutant. Flowering and expression of flowering time genes including *CO*, a key output gene of the photoperiod pathway, were repressed in wild-type but not in *qko* plants when NH_4^+ was used as N form, indicating that NH_4^+ nutrition delays flowering time by affecting the photoperiod pathway in an AMT-dependent manner. The involvement of *CO* in N-dependent flowering control is consistent with recent findings proposing that the photoperiod pathway is a major target for N-dependent signals modulating flowering time in dependence of N availability.

In conclusion, investigation of the *AMT*-quadruple knock-out line *qko* allowed uncovering novel roles of AMT-type transporters that go beyond their function in NH_4^+ uptake. Especially the new findings about the modulation of the expression of *NRT*-type transporters and of flowering time by AMT-dependent NH_4^+ nutrition are of great significance. N use efficiency and flowering are important agricultural traits influencing crop productivity. Understanding the mechanisms by which plants regulate N acquisition is of pivotal importance for the improvement of N use efficiency. Exploitation of flowering responses to varied N availabilities could lead to the development of fertilizing and breeding strategies for flowering time control.

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7 Abbreviations

%	percent
°C	degree Celsius
µ	micro
µg	microgram
µl	microliter
µM	micromolar AA amino acid
ABA	abscisic acid
ACQ	aminoquinolyl-N-hydroxysuccimidyl carbamate
AMT	ammonium transporter
AP1	APETALA 1
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
cm	centimeter
CO	CONSTANS
Col-0	Columbia-0, ecotype of Arabidopsis thaliana
cZ	cis-zeatin
cZR	cis-zeatin-riboside
DAS	days after sowing
DNA	deoxyribonucleic acid
dpi	dots per inch
DW	dry weight
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FLD	fluorescence detector
FT	FLOWERING LOCUS T
FW	fresh weight
g	gram
GA	gibberellic acid
GC	gas chromatography
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
Gln	glutamine
Glu	glutamate
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour
HATS	high-affinity transport system
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
iP	isopentenyladenine
iP9G	isopentenyladenin-9-glucoside
iPR	isopentenyladenine-riboside
IPT	isopentenyl transferase
LATS	low-affinity transport system
ln	natural logarithm
m	milli
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MS	mass spectrometry
MSX	methionine sulfoximine
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
pH	‘power of hydrogen’, measure of the acidity or basicity of an aqueous solution
qko	ammonium transporter quadruple knock-out line
RGR	relative growth rate
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SE	standard error
sec	second
T-DNA	transfer-DNA
TSF	TWIN SISTER OF FT
t-test	Student’s t distribution test
tZ9G	trans-zeatin-9-glucoside
tZOG	trans-zeatin-O-glucoside
tZOGR	trans-zeatin-O-glucoside riboside
tZR	trans-zeatin-riboside
UPLC	ultra performance liquid chromatography
v/v	volume-to-volume ratio
x *g*	gravitation force

8 Curriculum Vitae

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Education

01/2011 - 03/2015	PhD study on 'Contribution of Ammonium Transporters to Growth, Nitrate Uptake, Metabolism and Development in *Arabidopsis thaliana*' 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
10/2004 - 10/2010	Studies in Agricultural Biology at the University of Hohenheim, Stuttgart, Germany (Diploma, Dipl.-Agr.Biol.) Diploma thesis in the Institute for Plant Nutrition at the University of Hohenheim on „Physiological characterization of the ammonium transporter AMT2;1 in *Arabidopsis thaliana*"
09/1995 - 06/2004	Secondary school (Wirtemberg-Gymnasium in Stuttgart, Abitur)

Work experience

since 08/2018	Agricultural Biologist at Aurora Deutschland GmbH, Berlin, Germany
05/2016 - 07/2018	Freelance Scientist and Consultant

05/2016 – 05/2017	Project Manager
04/2015 - 12/2016	Research Scientist at the Resources Genetics and Reproduction Group, Department of Genebank (IPK)
11/2010 - 12/2010	Research Assistant at the IPK
04/2010 - 10/2010	Student Assistant at the IPK

Publications

Laginha, A.M. (2018) High-Tech-Anbau von Cannabis - Gesteuerte Wachstumsbedingungen sichern gleichmäßige Blütenqualität. Deutsche Apotheker Zeitung. 158. Jahrgang, Nr. 21: 36-43

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Presentations in scientific conferences during the PhD study

Investigations on the Role of Ammonium Transporters in Modulating Nitrate Uptake. XVII. International Plant Nutrition Colloquium, Istanbul - Turkey, August 19 - 22, 2013

Investigations on the role of ammonium transporters in modulating nitrate uptake. 9th Plant Science Student Conference 2013, Leibniz Institute of Plant Biochemistry in Halle (Saale) - Germany, May 28 - 31, 2013

Investigations on the role of ammonium transporters in modulating nitrate uptake. 26. Tagung Molekularbiologie der Pflanzen, Dabringhausen - Germany, February 26 - March 01, 2013

Investigations on the mechanism underlying the preferential uptake of ammonium in Arabidopsis roots. 8th Plant Science Student Conference 2012, IPK Gatersleben - Germany, June 4 - 7, 2012

Physiological characterization of the ammonium transporter AMT2;1 in *Arabidopsis thaliana*. Botanikertagung 2011, Berlin - Germany, September 18 - 23, 2011

Supervision activity

Supervision of an undergraduate student from the Martin-Luther University Halle-Wittenberg for research in molecular plant nutrition on ‘Cloning and heterologous expression of plant genes in yeast’ at the IPK (15.07.2013 - 26.07.2013)

Co-supervision of a practical course in ‘Transport Processes Across Plant Membranes’ for undergraduates from the Martin-Luther University Halle-Wittenberg at the IPK (2013).

Alberto Manasse Laginha

Quedlinburg, September 07th, 2018

9 Affirmation

I hereby declare that the submitted work has been completed by me, the undersigned, and that I have not used any other than permitted reference sources or materials or engaged any plagiarism. All the references and the other sources used in the presented work have been appropriately acknowledged in the 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 diese 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.

Alberto Manasse Laginha

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