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REVIEW PAPER

Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants

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Abstract

Nitrogen (N) availability is a major factor determining plant growth and productivity. Plants acquire inorganic N from the soil, mainly in the form of nitrate and ammonium. To date, researchers have focused on these N sources, and demonstrated that plants exhibit elaborate responses at both physiological and morphological levels. Mixtures of nitrate and ammonium are beneficial in terms of plant growth, as compared to nitrate or ammonium alone, and therefore synergistic responses to both N sources are predicted at different steps ranging from acquisition to assimilation. In this review, we summarize interactions between nitrate and ammonium with respect to uptake, allocation, assimilation, and signaling. Given that cultivated land often contains both nitrate and ammonium, a better understanding of the synergism between these N sources should help to identify targets with the potential to improve crop productivity.

Key words: Ammonium assimilation, ammonium uptake, metabolic flux, nitrate sensing, primary nitrate response, root-to-shoot transport.

Introduction

Nitrogen (N) availability is a strong determinant of plant growth and crop productivity. Plants use several forms of N in natural soils, and inorganic forms include nitrate, nitrite, and ammonium. Nitrate is the major form of N in most aerated soils, whereas ammonium can be a dominant form in some acidic and/or anaerobic environments (Miller and Cramer, 2004). Nitrite availability varies in soils worldwide, depending on the balance between nitrification and denitrification, although its concentration in soil is generally lower than that of nitrate and ammonium (summarized in Shen *et al.*, 2003; Kotur *et al.*, 2013). Plants also absorb organic N, and sources include urea, amino acids, and peptides (Kojima *et al.*, 2007; Tegeder and Rentsch, 2010; Forde, 2014*a*). In boreal habitats, the concentrations of amino acids available to plants can be comparable to that of inorganic N (Näsholm *et al.*, 2009). Plant growth is often limited by N availability in natural environments; therefore, plants have developed transport and signaling mechanisms specific to their respective N sources (Kiba and Krapp, 2016).

Of the different available N sources, researchers have focused on nitrate and ammonium, because these are often present in natural and cropland soils at much higher levels than other sources (Miller and Cramer, 2004). In addition to being a nutrient, nitrate is also a local and systemic

Abbreviations: AMT, ammonium transporter; GOGAT, glutamine 2-oxoglutarate aminotransferase; GS, glutamine synthetase; HATS, high-affinity transport system; LATS, low-affinity transport system; LR, lateral root; N, nitrogen; NPF, nitrate transporter 1/peptide transporter family; NR, nitrate reductase; NRT, nitrate transporter; TCA, tricarboxylic acid; PNR, primary nitrate response.

signal that regulates genome-wide gene expression (Wang *et al.*, 2000, 2003, 2004; Scheible *et al.*, 2004; Ho *et al.*, 2009; O'Brien *et al.*, 2016), leaf expansion (Walch-Liu *et al.*, 2000; Rahayu *et al.*, 2005), root morphology (Zhang *et al.*, 1999; Remans *et al.*, 2006), seed dormancy (Alboresi *et al.*, 2005; Matakiadis *et al.*, 2009), and floral induction (Castro Marín *et al.*, 2011). Several responses to nitrate are mediated via calcium and phytohormone signaling pathways, including auxin, cytokinin, and abscisic acid (ABA) (Sakakibara *et al.*, 1997; Signora *et al.*, 2001; Takei *et al.*, 2004; Krouk *et al.*, 2010*a*; Kiba *et al.*, 2011; Léran *et al.*, 2015; Riveras *et al.*, 2015; Krouk, 2016). Nitrate is reduced to ammonium by nitrate reductase (NR), and nitrite reductase (NiR) requires eight moles of electrons per mole of nitrate. Thus, ammonium utilization greatly decreases the energy consumption required to synthesize organic N compounds (Williams *et al.*, 1987). Recent evidence has demonstrated that in the leaves of C_3 plants, nitrate reduction is inhibited by elevated CO_2, whereas ammonium assimilation is little affected (Bloom *et al.*, 2010). Ammonium is thus a preferable N source for the future when global levels of CO_2 are predicted to increase; however, in excessive quantities, ammonium has detrimental effects on plant growth (i.e. ammonium toxicity). Ammonium acts as a signal that alters gene expression and root morphology (Lima *et al.*, 2010; Patterson *et al.*, 2010). Co-provision of nitrate and ammonium stimulates plant growth beyond that observed with either N source provided alone (Britto and Kronzucker, 2002). Studies have revealed that nitrate responses can be affected by the co-provision of ammonium, and ammonium responses are altered by nitrate. The interactions between nitrate and ammonium should optimize N utilization in the field, where nitrate and ammonium often coexist and are found at various concentrations within short distances (Lark *et al.*, 2004; Miller and Cramer, 2004). This review aims to summarize their interactions in physiological processes, focusing on N uptake, translocation, assimilation, and signaling.

For molecular and physiological responses to either nitrate or ammonium, readers are also referred to previously published reviews by Nacry *et al.* (2013), Krapp *et al.* (2014), Medici and Krouk (2014), Krouk (2016), and O'Brien *et al.* (2016). The interaction between nitrate and glutamate on root growth has been well documented in previous reviews by Forde and Walch-Liu (2009), and Forde (2014a, b). The physiological and molecular links between potassium and N sources (nitrate and ammonium) have also been discussed in detail by Coskun *et al.* (2016). In addition, recent advances regarding the mechanisms of toxicity of excessive ammonium in comparison with nitrate have been published by Li *et al.* (2014*a*), and Esteban *et al.* (2016).

N uptake from the soil

In the field, N availability often limits plant productivity, and hence uptake for N acquisition has attracted considerable research interest. Most plants benefit from a mixture of nitrate and ammonium in order to increase their N contents (Miller and Cramer, 2004; Hachiya *et al.*, 2012). Thus, it is crucial that we understand how nitrate and ammonium interact with each other and how this affects N uptake at physiological, morphological, and molecular levels. The net N influx via roots consists of two components, total N influx and total N efflux (Glass *et al.*, 2002). Thus, when the net N influx is increased, the total N influx increases and/or the total N efflux decreases. Specific transporters of nitrate (NRT) and ammonium (AMT) contribute to the total N influx (Nacry *et al.*, 2013), except under high ammonium conditions (Esteban *et al.*, 2016). The total efflux components include simple N leakage and those mediated by specific transporters and channels that facilitate N efflux (Segonzac *et al.*, 2007; Zheng *et al.*, 2015).

Inhibition of net nitrate uptake by ammonium

Kronzucker and co-workers determined the reciprocal effects of nitrate and ammonium on the components of N flux in rice and barley roots using a highly sensitive ^{13}N-labeled radiotracer. They found that net nitrate uptake was significantly inhibited by the co-provision of ammonium, as compared to that observed with nitrate alone (Kronzucker *et al.*, 1999*a*, *b*). The total nitrate influx followed the same tendency as the net nitrate influx, whereas the efflux rates were either decreased or not changed by ammonium. Thus, decreases in the net nitrate influx were determined by the total influx component. In barley, inhibition of total nitrate uptake by 1 mM ammonium was significant under low nitrate conditions (below 1 mM), suggesting the involvement of the high-affinity transport system (HATS) (Kronzucker *et al.*, 1999*a*). In *Arabidopsis thaliana*, evidence from genetic studies indicates that a major HATS component, *AtNRT2.1*, is the target for ammonium-dependent inhibition of nitrate uptake (Cerezo *et al.*, 2001). Levels of the *AtNRT2.1* transcript and AtNRT2.1 protein are decreased within hours in response to the coexistence of nitrate and ammonium, as compared to that observed with nitrate alone (Muños *et al.*, 2004; Wirth *et al.*, 2007). This repressive effect of ammonium might be mediated by the sensing of ammonium and/or amino acids (Zhuo *et al.*, 1999; Nazoa *et al.*, 2003). Some of the underlying molecular mechanisms have been identified. The ammonium-dependent repression of *AtNRT2.1* is more notable under conditions of 1 mM nitrate and 1 mM ammonium than under 0.1 mM nitrate and 1 mM ammonium (Krouk *et al.*, 2006). This observation seems reasonable given that AtNRT2.1 is a major HATS component that is more functional below 1 mM nitrate. The results of a split-root experiment indicated that the nitrate concentration (i.e. 0.1 mM or 1 mM) acts locally on the ammonium-dependent repression of *AtNRT2.1* (Krouk *et al.*, 2006). This local effect of nitrate is mediated by AtNRT1.1/NPF6.3/CHL1, a dual-affinity nitrate transporter/sensor (a transceptor: membrane proteins that belong to nutrient transporter families and act as sensors/receptors; see Gojon *et al.*, 2011). In *AtNPF6.3*-deficient mutants, higher expression of *AtNRT2.1* is maintained even under conditions of 1 mM nitrate and 1 mM ammonium (Muños *et al.*, 2004; Krouk *et al.*, 2006), indicating that AtNPF6.3 has a suppressive effect on *AtNRT2.1* expression. Nitrate

transport and signaling via AtNPF6.3 are differentially regulated depending on the phosphorylation status of the threonine 101 residue (i.e. phosphorylated form in the high-affinity state, non-phosphorylated form in the low-affinity state; Liu and Tsay, 2003; Ho *et al.*, 2009). The existence of phosphomimic AtNPF6.3 in the *AtNPF6.3*-knockout background can repress *AtNRT2.1* expression, while non-phosphomimic AtNPF6.3 alone cannot (Bouguyon *et al.*, 2015). Widiez *et al.* (2011) demonstrated that repression of *AtNRT2.1* expression due to high N (10 mM nitrate and 10 mM ammonium) requires *HIGH NITROGEN INSENSITIVE 9*/*INTERACT WITH SPT6* (*HNI9*/*ATIWS1*) in roots. This gene product leads to trimethylation of histone H3 lysine 27 at the *NRT2.1* locus in response to high N, which suggests an involvement of chromatin modifications in nitrate uptake. Taken together, the evidence suggests that ammonium-dependent repression of nitrate uptake occurs through the convergence of nitrate- and ammonium-dependent mechanisms.

Acceleration of net ammonium uptake by nitrate

Experiments utilizing the ^{13}N radiotracer technique have demonstrated that, in rice, net ammonium uptake becomes higher with the co-provision of nitrate, compared with ammonium alone (Kronzucker *et al.*, 1999*b*). Using the microelectrode technique, a similar facilitating effect of nitrate on net ammonium uptake was confirmed in *Brassica napus* and *Populus popularis* roots (Babourina *et al.*, 2007; Luo *et al.*, 2013), whereas no positive effect was observed in wheat roots (Zhong *et al.*, 2014). In rice, increased total ammonium uptake and decreased total ammonium efflux concomitantly contribute to the enhanced net ammonium uptake in the presence of nitrate (Kronzucker *et al.*, 1999*b*). However, it remains unknown how nitrate facilitates the net ammonium uptake. In plants, specific ammonium transporters, AMTs, have the features of HATS that function efficiently in the micromolar range (Pantoja, 2012) (Fig. 1). Nitrate does not induce the expression of *AtAMT*s in the presence of ammonium (Wang *et al.*, 2004). When ammonium is the sole N source, a major ammonium transporter, AtAMT1;1, is inactivated via phosphorylation, which limits the ammonium influx (Engelsberger and Schulze, 2012). It is unknown whether the co-provision of nitrate and ammonium could restore the activity of AtAMT1;1 via dephosphorylation. It should be noted that ammonium can also move across the plasma membrane through non-specific systems, including potassium channels/transporters, aquaporins, and non-selective cation channels (Britto and Kronzucker, 2006; Lima *et al.*, 2010; ten Hoopen *et al.*, 2010; Coskun *et al.*, 2013) (Fig. 1). The potassium channel/transporter AtAKT1 and AtHAK5 are activated via phosphorylation by the CBL-INTERACTING PROTEIN KINASE 23 (CIPK23) in *A. thaliana* (Ragel *et al.*, 2015; Coskun *et al.*, 2016). *AtCIPK23* is highly induced by nitrate application under the control of AtNPF6.3 (Ho *et al.*, 2009). Potassium channels/transporters might facilitate ammonium uptake in the presence of nitrate. At higher concentrations of ammonium, gaseous ammonia transport via the aquaporins represents an indispensable component of influx at the plasma membrane in barley roots (Ariz *et al.*, 2011; Coskun *et al.*, 2013). Nitrate increases root hydraulic conductivity, possibly via the activation and/or elevation of aquaporins in *A. thaliana* (Li *et al.*, 2016). This implies that aquaporins are involved in the nitrate-dependent enhancement of total ammonium uptake, although aquaporins also facilitate the total ammonium efflux (Coskun *et al.*, 2013). The enhancement of net ammonium uptake by the coexistence of nitrate can overcome the repressive effects of ammonium on net nitrate uptake, which results in improved N acquisition under the application of both nitrate and ammonium, as compared to that with nitrate or ammonium alone (Kronzucker *et al.*, 1999*b*). This phenomenon is worthy of attention as a potential target to improve N acquisition.

Complementary effects of nitrate and ammonium on lateral root growth

The diffusion coefficients of nitrate and ammonium in water are similar. However, nitrate and ammonium ions differ in their behavior in soil water, because the soil has complex properties, including its negative ion charge and viscosity (Miller and Cramer, 2004). The diffusion coefficient of nitrate is estimated to be 10–100-fold higher than that of ammonium in

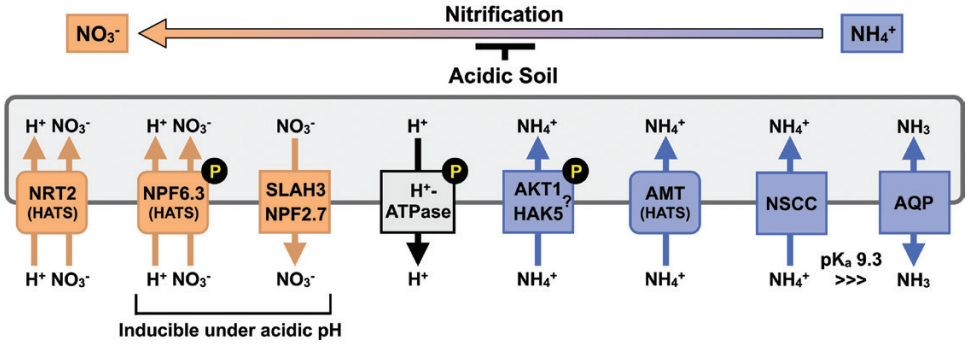

Fig. 1. Components of the influx and efflux of nitrate and ammonium in roots. Under acidic pH, microbial nitrification is suppressed, resulting in an accumulation of ammonium and a lowering of nitrate in the soil. NPF6.3, SLAH3, and NPF2.7 are induced or activated by acidic pH. NPF6.3 is phosphorylated under low nitrate conditions, indicating activity of the high-affinity transport system (HATS). Plasma membrane H^+-ATPase is activated via phosphorylation under ammonium conditions (Zheng *et al.*, 2015; Menz *et al.*, 2016). Ammonium is imported by specific transporters (AMT), and non-specific components including potassium channels (AKT1, HAK5), aquaporins (AQP), and non-selective cation channels (NSCC).

certain soil waters. Nitrate can reach the root surface by rapid mass flow, whereas cationic ammonium is easily adsorbed by soil particles (Giehl and von Wirén, 2014). Plants are required to adapt their root morphology in response to nitrate or ammonium in order to optimize N absorption from the soil. Lateral roots (LRs) account for a large part of the total root length, and many NRTs and AMTs are expressed in the epidermis and cortex of LRs, permitting efficient N uptake (Yuan *et al.*, 2007; Nacry *et al.*, 2013; Kiba and Krapp, 2016). Hence, the morphological responses of LRs to nitrate and ammonium have been documented with particular interest in *A. thaliana* grown using the split-root system with two separate patches containing different N sources (Remans *et al.*, 2006; Lima *et al.*, 2010; Bisseling and Scheres, 2014; Li *et al.*, 2014*b*; O'Brien *et al.*, 2016). Overall, application of ammonium stimulates LR branching, whereas nitrate stimulates LR elongation. Interestingly, application of nitrate and ammonium together enhances branching and elongation of LRs concomitantly, suggesting that the application of nitrate and ammonium has local, complementary effects on LR development. Lima *et al.* (2010) suggested that this complementary response may reflect an adaptation of LRs to the distinct mobilities of nitrate and ammonium, as described above. For example, finely branched LRs are efficient at absorbing ammonium fixed on the soil surface, whereas longer LRs can explore highly mobile nitrate sources. These independent responses to local nitrate and ammonium are dependent on a nitrate transporter, AtNPF6.3, and an ammonium transporter, AtAMT1;3, respectively. Since AtNPF6.3 and AtAMT1;3 can change root morphology independent of N uptake, these transporters are considered sensors (transceptors; Gojon *et al.*, 2011) of external N availability. The transceptors allow plant roots to forage N-rich patches with an adequate morphology, optimizing N acquisition from heterogenous N conditions.

Responses of root hairs to nitrate and ammonium

Root hairs increase the surface area of roots and enhance their ability to uptake N. However, few studies have analyzed the root-hair responses to different N sources. Both the density and average length of root hairs are increased by a decrease in N availability in some grass species and vegetables (Foehse and Jungk, 1983; Robinson and Rorison, 1987). In *A. thaliana*, root-hair development in response to different magnitudes of nitrate, ammonium, or their combination varies depending on the accession (Vatter *et al.*, 2015). Interestingly, cell-specific transcriptome analysis in *A. thaliana* roots has revealed that *AtNPF6.3* and *AtNRT2.1* are significantly enriched in trichoblasts as compared to that in the cells from other parts of the roots (Lan *et al.*, 2013). This suggests that root hairs are important for nitrate uptake and/or sensing.

Alteration in the external pH via nitrate and ammonium uptake

Nitrate and protons are co-transported into the cytosol via NRTs, whereas ammonium uptake is accompanied by proton extrusion via the plasma membrane H^+-ATPase to maintain the charge balance (Meharg and Blatt, 1995; Britto and Kronzucker, 2005) (Fig. 1). Thus, the extracellular environments are alkalinized and acidified following the application of nitrate and ammonium, respectively (Escobar *et al.*, 2006). The microbial conversion of ammonium to nitrate (i.e. nitrification) is suppressed by acidic pH, thus decreasing nitrate availability in the soil. Notably, acidic conditions induce *AtNPF6.3* expression in roots irrespective of the N source (Tsay *et al.*, 1993; Lager *et al.*, 2010). Under nitrate conditions, *AtNPF6.3*-deficient plants exhibit impaired growth and nitrate uptake at pH 4.5 and 5.0, but not at pH 6.5, and the mutants fail to elevate the pH of acidic media (Fang *et al.*, 2016). This observation suggests that acidic induction of *AtNPF6.3* may ensure better uptake/sensing of the nitrate that accumulates at lower levels under acidic pH, acting to elevate the external pH and thus avoiding proton toxicity. When high ammonium is used as the sole N source at low pH, the *AtNPF6.3*-deficient mutants show enhanced growth (Hachiya *et al.*, 2011). AtNPF6.3 could suppress growth under conditions that are unfavorable for *A. thaliana* plants that prefer nitrate, allowing the plants to wait for more optimal conditions. Knockout mutants of *AtSLAH3* (*SLOW ANION CHANNEL ASSOCIATED 1 Homologue 3*) show hypersensitivity under high ammonium and low nitrate conditions at low pH (Zheng *et al.*, 2015). Expression of *AtSLAH3* is increased under conditions of acidic pH, which facilitates nitrate efflux without being accompanied by proton export. Furthermore, the passive nitrate excretion transporter, AtNAXT1/NPF2.7, is activated by acidification of the cytosol and/or apoplast in roots (Segonzac *et al.*, 2007). Thus, NPF6.3, SLAH3, and NPF2.7 may perform nitrate cycling across the plasma membrane in roots to increase extracellular pH, ensuring the acclimation of plants to the acidic soil (Fig. 1). A recent study has demonstrated that OsNRT2.3b possesses a pH-sensing motif on the cytosolic side, and as a result the nitrate uptake ability via this transporter is quickly modulated depending on the cytosolic pH (Fan *et al.*, 2016). Interestingly, overexpression of this gene enhances nitrate uptake in the presence of nitrate alone, but represses it under a mixture of nitrate and ammonium. Ammonium uptake/assimilation would modulate the activity of OsNRT2.3b owing to a change in the cytosolic pH. Taken together, these findings show that molecular interactions exist between intra/extracellular pH and uptake/assimilation of N.

N transport from root to shoot

Root-to-shoot transport of nitrate

There are two alternative fates of nitrate following its absorption from the soil. One involves immediate nitrate reduction in the roots, and the other involves root-to-shoot transport via the xylem followed by nitrate reduction in the leaves. In herbal species, most of the nitrate is reduced predominantly in the shoots via the reducing equivalents derived from photosynthesis (Scheurwater *et al.*, 2002; Hachiya *et al.*, 2016). Low-affinity nitrate transporters AtNRT1.5/NPF7.3,

AtNPF2.3, and OsNPF2.4 have been identified in *A. thaliana* and *O. sativa* for nitrate loading in the xylem (Lin *et al.*, 2008; Taochy *et al.*, 2015; Xia *et al.*, 2015). There proteins are expressed in the pericycle cells of roots, and loss-of-function mutants have been shown to decrease the root-to-shoot nitrate transport. Nitrate transported to the shoots via the transpiration stream is imported to the petiole for nitrate storage by AtNRT1.4/NPF6.2 or to the mesophyll cells for nitrate reduction by several transporters that are expressed in leaves (Chiu *et al.*, 2004; Wang *et al.*, 2012).

Root-to-shoot transport of ammonium

A high proportion of moderate-concentration ammonium is assimilated in the roots. Cytosolic isoforms of glutamine synthetase (GS), AtGLN1;2, and OsGS1;2 are expressed largely in roots, are induced by ammonium, and contribute to primary ammonium assimilation in the roots (Ishiyama *et al.*, 2004; Funayama *et al.*, 2013; Guan *et al.*, 2016). AtGLN1;2 is localized in the endodermis and vasculature of roots (Guan *et al.*, 2015), whereas OsGS1;2 is detected in the dermatogen, epidermis, and exodermis (Ishiyama *et al.*, 2004). Thus, with relatively lower concentrations of ammonium (e.g. submillimolar) as the N source, amino acids converted from ammonium in the roots are loaded into the xylem via specific transporters (Tegeder, 2014). When the root GS capacity is exceeded by a large amount of ammonium, it can be directly loaded into the xylem (Husted *et al.*, 2000; Schjoerring *et al.*, 2002). The molecular mechanisms responsible for xylem loading of ammonium remain unknown. In barley seedlings, the addition of 10 mM ammonium significantly inhibits the xylem loading of potassium, whereas equimolar nitrate has little effect (Kronzucker *et al.*, 2003), suggesting that there is competition between ammonium and potassium. In *A. thaliana*, the stelar K^+ outward rectifier (SKOR) or non-selective outwardly rectifying current (NORC) xylem-loaders of potassium might non-specifically facilitate the loading of ammonium (Gaymard *et al.*, 1998; Pottosin and Dobrovinskaya, 2014). Ammonium delivered to the shoots will flow directly out to the apoplast, because the ammonium concentrations in the xylem sap are similar to those in the apoplast in barley (Schjoerring *et al.*, 2002). Ammonium is preferentially distributed to younger leaves compared with older leaves, implying that ammonium distribution might be under the control of transpiration (Kiyomiya *et al.*, 2001; Schjoerring *et al.*, 2002). In *A. thaliana* leaves, ammonium will be imported to the cytosol by AtAMT1;1 and AtAMT2;1, which are expressed in the leaves, or by other pathways via diffusion or unknown transporters/channels (Ludewig *et al.*, 2007).

Nitrate enhances root-to-shoot transport of ammonium and/or its assimilate

Kiyomiya *et al.* (2001) visualized the real-time movement of an isotopic signal from root-fed $^{13}NH_4^+$ in rice, and showed that the co-provision of nitrate enhances the shoot-ward distribution of the signal. A possible explanation is that the enhancement of net ammonium uptake by nitrate in roots can increase the amount of N loaded into the xylem. Kronzucker *et al.* (1999*b*) analyzed the partitioning of the $^{13}NH_4^+$ signal after its import to rice roots. Interestingly, they found that in the presence of nitrate, a larger proportion of the isotopic signal was allocated to the xylem compared with that observed in the presence of ammonium only. It should be noted that these analyses do not clarify the molecular identity of the N compound in xylem. In *A. thaliana*, nitrate acts as a signal to induce the expression of genes related to ammonium assimilation such as *AtGLN1;2* or *AtGLN2*, although it does not increase the expression of an amino acid exporter, *SIAR1*, which is involved in xylem loading of amino acids, including glutamine (Wang *et al.*, 2004; Sakakibara *et al.*, 2006; Ladwig *et al.*, 2012). This suggests that nitrate may stimulate the xylem loading of ammonium and/or its assimilate through the enhanced biosynthesis of amino acids. However, in the rice examined by Kronzucker *et al.* (1999*b*) and Kiyomiya *et al.* (2001), glutamine or a related compound (not nitrate) would be potent candidates to induce the expression of genes involved in ammonium assimilation (Tabuchi *et al.*, 2007; Kamada-Nobusada *et al.*, 2013). Plants of *A. thaliana* subjected to high levels of ammonium and nitrate as N sources accumulate ammonium in shoots at much higher levels than those subjected to high levels of nitrate, suggesting that ammonium can move from the roots to shoots (Barth *et al.*, 2010; ten Hoopen *et al.*, 2010; Hachiya *et al.*, 2012; Li *et al.*, 2012). Interestingly, in barley grown with nitrate and ammonium, the addition of potassium reduces ammonium accumulation in the shoots more effectively than in the roots (ten Hoopen *et al.*, 2010). In *A. thaliana*, gene expression of *AtSKOR*, whose product exports potassium from the pericycle to the xylem in roots (as mentioned previously), is induced by nitrate application in the presence of ammonium (Wang *et al.*, 2004). Under ammonium and nitrate conditions, the steady-state level of *AtSKOR* expression is maintained under the control of *AtNPF6.3* (Muños *et al.*, 2004). AtSKOR might be involved in the root-to-shoot transport of ammonium under both nitrate and ammonium conditions. Given that remobilized N from vegetative tissues accounts for 50–90% of N in the grain of rice, wheat, and maize (Masclaux *et al.*, 2001), how nitrate co-existence stimulates shoot-ward fluxes of ammonium and/or its assimilate is an important issue in agriculture.

Nitrate and ammonium independently affect leaf apoplastic pH and alter phytohormone uptake

The pH in leaf apoplasts changes rapidly in response to several environmental stimuli, including external CO_2 concentrations and light intensity (Mühling *et al.*, 1995; Hedrich *et al.*, 2001). The N source is also known to be a determinant of apoplastic pH. In ryegrass, switching the root N source from 3 mM nitrate to ammonium causes a rapid increase in the ammonium concentration in the leaf apoplast and a concomitant decrease in apoplastic pH (Schjoerring *et al.*, 2002). Perfusion experiments utilizing petioles of detached sunflower leaves revealed that nitrate application increases apoplastic pH, whereas ammonium decreases it, as observed in

the rhizosphere (Hoffmann *et al.*, 1992). Within 12 h of treatment, there is a significant difference of 0.5 in the apoplastic pH between nitrate and ammonium conditions. The simultaneous application of nitrate and ammonium results in an intermediate pH value, suggesting that these N species act on apoplastic pH independently. Notably, in detached leaves of *Commelina communis*, the sensitivity of the stomatal response to ABA is increased with nitrate application, and decreased in response to ammonium (Jia and Davies, 2007). Since ABA is a weak acid (pK_a 4.75), it can be protonated in the physiological range of the apoplastic pH. Protonated ABA with no ionic charge diffuses into the cells through the plasma membrane. Thus, the apoplastic pH is a determinant of the cellular compartment of ABA. It has been demonstrated that AtNPF4.6/NRT1.2/AIT1 can import both nitrate and ABA to the cytosol, which regulates stomatal behavior and seed dormancy (Kanno *et al.*, 2012). Although nitrate does not competitively suppress the import of ABA via AtNPF4.6 (Kanno *et al.*, 2013), nitrate uptake might affect ABA import indirectly via a local increase in apoplastic pH. Additionally, indole-3-acetic acid (IAA) is a phytohormone with a pK_a of 4.75, similar to that of ABA. Several reports have shown that apoplastic pH can alter IAA import to the cytosol, which regulates organ development and guard-cell movement in *A. thaliana* (Li *et al.*, 2005; Cho *et al.*, 2012). A nitrate transceptor, AtNPF6.3, is believed to be an IAA transporter (Krouk *et al.*, 2010*a*; Mounier *et al.*, 2014). Nitrate inhibits IAA import via AtNPF6.3 in a concentration-dependent manner. Independently of this direct link between nitrate and auxin uptake, AtNPF6.3-mediated uptake of nitrate could indirectly decrease IAA import into the cells through the elevation of extracellular pH. Local changes in pH associated with N transport represent a missing link in our understanding of the different responses to nitrate and ammonium.

Metabolic alterations in the response to different N sources

Stimulation of carbon and nitrogen flow by ammonium co-provision

In herbal species grown under moderate N concentrations, nitrate and ammonium are assimilated mainly in shoots and roots, respectively (see above). Under conditions of abundant N supply, which is often the case in cultivated fields, nitrate and ammonium are likely to be processed in the same organs (Guan *et al.*, 2016). N assimilation requires the reducing equivalents and carbon skeletons that are derived from carbon metabolism. Masakapalli *et al.* (2013) compared the steady-state metabolic carbon fluxes in heterotrophic cell suspensions of *A. thaliana* cultivated in media containing nitrate either with or without ammonium using carbon isotope-labeled glucose and gluconate with substrates. Supplementation with ammonium significantly redirects carbon fluxes (Fig. 2). The first important change is a decreased flux through the oxidative pentose phosphate pathway (oxPPP) in the presence of ammonium [see (a) in Fig. 2]. oxPPP produces NADPH to reduce ferredoxin as

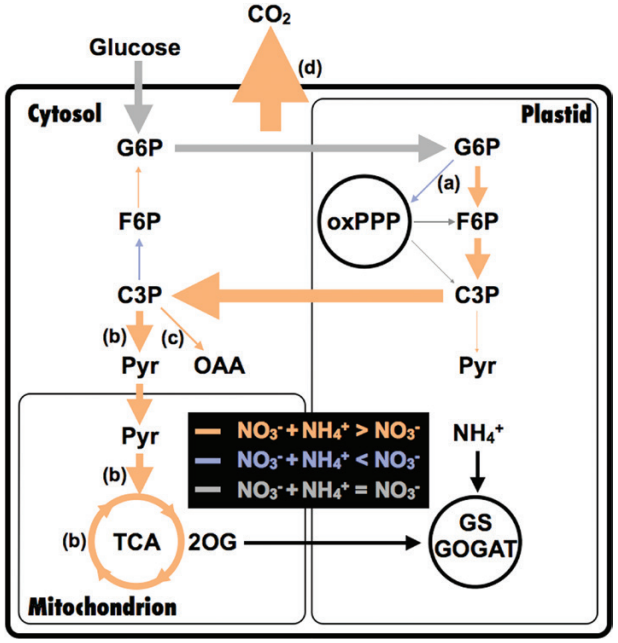

Fig. 2. Net metabolic carbon fluxes of primary carbon metabolism in cell suspensions of heterotrophic *Arabidopsis thaliana* in Murashige and Skoog (MS) medium containing nitrate with or without ammonium. Orange arrows indicate increased fluxes (more than 10% difference) when both nitrate and ammonium are present compared to when only nitrate is present (i.e. $NO_3^- + NH_4 > NO_3^-$). Blue arrows indicate decreased fluxes (more than 10% difference) when both nitrate and ammonium are present compared to when only nitrate is present (i.e. $NO_3^- + NH_4 < NO_3^-$). Gray arrows indicate no substantial effect on fluxes (less than 10% difference) between the two conditions (i.e. $NO_3^- + NH_4 = NO_3^-$). The thickness of the arrows is proportional to the size of the flux. Carbon fluxes that are markedly altered depending on the nitrogen source are indicated as follows: (a) through the oxidative steps of the oxidative pentose phosphate pathway (oxPPP); (b) production of organic acids by pyruvate kinase and enzymes of the tricarboxylic acid cycle; (c) oxaloacetate production by phospho*enol*pyruvate carboxylase; and (d) carbon dioxide emission. Abbreviations: C3P, three-carbon phosphate ester pool; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GOGAT, glutamine: 2-oxoglutarate aminotransferase; GS, glutamine synthetase; OAA, oxaloacetate; 2OG, 2-oxoglutarate; Pyr, pyruvate; TCA, tricarboxylic acid cycle. The figure has been redrawn with modifications from Masakapalli *et al.* (2013), with permission.

the electron donor for NiR in heterotrophic plastids. Nitrate reduction from nitrate to ammonium via nitrite is the second largest sink for reducing equivalents following carbon fixation (Noctor and Foyer, 1998). Nitrite reduction requires six moles of electrons per mole of nitrite, which accounts for 75% of the electrons consumed during nitrate reduction. The coexistence of ammonium with nitrate suppresses nitrate uptake, thereby decreasing the relative demand on NADPH for nitrite reduction. The second notable finding is the increased glycolytic production of pyruvate through pyruvate kinase (PK) and fluxes via the tricarboxylic acid (TCA) cycle in response to ammonium application [see (b) in Fig. 2]. 2-oxoglutarate in the TCA cycle furnishes the GS/GOGAT cycle with the C skeleton required for amino acid biosynthesis. Thus, the ammonium-dependent enhancement of C input to the TCA cycle is associated with increased N assimilation, which is supported by the higher levels of amino acids and proteins observed when both ammonium and nitrate are present

compared with nitrate alone in *A. thaliana* shoots (Hachiya *et al.*, 2012; Masakapalli *et al.* 2013; Sato and Yanagisawa, 2014). Addition of ammonium stimulates ammonium assimilation by bypassing nitrate reduction as the rate-limiting step of N assimilation, which, in turn, requires high levels of the C skeleton. In the *A. thaliana* cell suspension, the anaplerotic C fluxes via phospho*enol*pyruvate carboxylase (PEPC) [see (c) in Fig. 2] are much lower than the C fluxes to pyruvate biosynthesis [see (b) in Fig. 2] although, in *A. thaliana* and rice, genetic defects of *AtPEPC* cause impaired ammonium assimilation (Matsumoto *et al.*, 2010; Shi *et al.*, 2015). Yanagisawa *et al.* (2004) demonstrated that concomitant induction of both *AtPK* and *AtPEPC* by the introduction of the *DNA-binding with One Finger 1* (*ZmDof1*) transcription factor into *A. thaliana* enhances organic acid production and amino acid biosynthesis with nitrate and ammonium used as N sources. Elevated carbon dioxide further enhances the ammonium-dependent stimulation of N assimilation in *A. thaliana* (Sato and Yanagisawa, 2014). Carbon availability is crucial in order to maximize organic N biosynthesis when both nitrate and ammonium are available as N sources.

Stimulation of respiratory carbon loss by ammonium co-provision

Masakapalli *et al.* (2013) found that ammonium co-provision stimulates the efflux of carbon dioxide [see (d) in Fig. 2], which lowers the accumulation of biomass per unit glucose input. This respiratory enhancement is a typical response to abundant ammonium supply (Britto and Kronzucker, 2002). Hachiya *et al.* (2010) found that the ammonium levels in *A. thaliana* shoots are positively correlated with the rates of respiratory oxygen uptake under varying combinations of nitrate and ammonium. The ammonium-dependent respiratory increase occurs via the mitochondrial cytochrome pathway, which is coupled to ATP production. This phenomenon may be explained by the futile cycling of ammonium across the plasma membrane, which requires a large amount of ATP for the plasma membrane H^+-ATPase (Britto and Kronzucker, 2002). Coskun *et al.* (2013) observed that, in barley roots, the respiratory increase is abolished by elevated pH in the external solution in spite of stimulated ammonium influx to the cells. Under high pH, the ratio of NH_3 to NH_4^+ rises in the media, enhancing diffusional ammonia influx. This implies that the ammonium-dependent respiratory increase might be related to the energetic demand of intracellular pH regulation. Since the suppression of respiratory carbon loss leads directly to biomass accumulation, further studies would be valuable to improve crop productivity in the presence of both nitrate and ammonium.

Primary nitrate response with or without ammonium

Early responses of gene expression following the application of nitrate to plants are referred to as the primary nitrate response (PNR) (see Medici and Krouk, 2014). Approximately 600 genes are regulated even in the *A. thaliana* NR-null mutant (Wang *et al.*, 2004), indicating that the detection of nitrate initiates the PNR. Currently, several transcription factors, kinases, and transporters are known to govern the PNR (Medici and Krouk, 2014; O'Brien *et al.*, 2016). However, it is difficult to obtain an integrated perspective of the key players, because the so-called nitrate-responsive genes alter depending on the experimental conditions. There are some transcriptome data available on the PNR, which can be classified according to the preculture conditions, i.e. with or without ammonium (Tables 1, 2). Wang *et al.* (2009) found that in *AtNPF6.3*-deficient mutants, nitrate application fails to induce the expression of some marker genes for the PNR in the presence of ammonium. Interestingly, following N deprivation, nitrate application can induce the expression of these genes in the same mutants. This clearly demonstrates that distinct mechanisms for the PNR operate depending on the preculture conditions. Recently, genetic screening with mutant lines expressing the nitrate-responsive promoter fused to a reporter gene has identified *NITRATE REGULATORY GENE2* (*NRG2*) (Xu *et al.*, 2016). The *nrg2* mutants exhibited an impaired PNR in both the presence and absence of ammonium. Under nitrate and ammonium conditions, AtNRG2 acts upstream of AtNPF6.3 in the PNR, and AtNPF6.3 in turn regulates other PNR components, including *TCACG SEQUENCE-SPECIFIC BINDING PROTEIN1* (*TGA1*), *CALCINEURIN B-LIKE INTERACTING SER/THE-PROTEIN KINASE8* (*CIPK8*), and *23* (*CIPK23*), *AUXIN SIGNALING F-BOX3* (*AFB3*), and phospholipase C (PLC) activity (Ho *et al.*, 2009; Hu *et al.*, 2009; Riveras *et al.*, 2015). The NIN-LIKE PROTEIN7 (NLP7) transcription factor drives the PNR irrespective of the presence of ammonium.

Table 1. *Transcriptome data on the primary nitrate response in* A. thaliana *in the presence of* NH_4^+

Reference	Genotype	Organ	Experimental method	NH_4^+ conditions	NO_3^- treatment
Wang *et al.*, 2000	Col	Seedling	Microarray	10 mM $(NH_4)_2$ succinate	0.25, 5, 10 mM KNO_3, 0, 20, 120 min
Wang *et al.*, 2003	Col	Shoot, Root	Microarray	2.5 mM $(NH_4)_2$ succinate	250 μM KNO_3, 20 min
Wang *et al.*, 2004	Col, *nia1-2/chl3-5* (NR-null)	Shoot, Root	Microarray	2.5 mM $(NH_4)_2$ succinate	5 mM KNO_3, 120 min
Wang *et al.*, 2007	Col	Root	Microarray	2.5 mM $(NH_4)_2$ succinate	5, 250 μM KNO_3, 20 min
Gifford *et al.*, 2008	Cell-specific reporter lines	Root cells	Microarray	0.5 mM $(NH_4)_2$ succinate	5 mM KNO_3, 3.5 h
Hu *et al.*, 2009	Col, *chl1-5*	Root	Microarray	12.5 mM $(NH_4)_2$ succinate	25 mM KNO_3, 30 min
Wang *et al.*, 2009	Col, *nrg1*	Root	Microarray	2.5 mM $(NH_4)_2$ succinate	1 mM KNO_3, 30 min
Xu *et al.*, 2016	Col, *nrg2-2, chl1-13, nlp7-4*	Root	RNA -Seq	2.5 mM $(NH_4)_2$ succinate	10 mM KNO_3, 120 min

Table 2. *Transcriptome data on the primary nitrate response in* A. thaliana *after N depletion*

References	Genotype	Organs	Experimental method	Depleting duration	NO_3^- treatment
Scheible *et al.*, 2004	Col	Seedling	Microarray	2 d	3 mM KNO_3, 0, 0.5, 3 h
Krouk *et al.*, 2010*b*	Col	Root	Microarray	24 h	1 mM KNO_3, 0, 3, 6, 9, 12, 15, 20 min
Patterson *et al.*, 2010	Col	Root	Microarray	26 h	1 mM KNO_3, 0, 0.5, 1.5, 8 h
Marchive *et al.*, 2013	Col, *nlp7-1*, *nlp7-3*	Seedling	Microarray	3 d	3 mM KNO_3, 0, 10, 20 min
Vidal *et al.*, 2013	Col, *afb3-1*	Root	Microarray	14 d* (gradual depletion)	5 mM KNO_3, 120 min
Alvarez *et al.*, 2014	Col, *tga1/tga4*	Root	Microarray	14 d (gradual depletion)	5 mM KNO_3, 120 min

* Information about N status is not available in Vidal *et al.* (2013), but the conditions of pre-culture were similar to that in Alvarez *et al.* (2014).

AtNLP7 physically interacts with AtNRG2 *in vivo*, although AtNRG2 and AtNLP7 regulate separate downstream genes. Given that *AtNRG2* and *AtNLP7* are never responsive to nitrate, these components should function upstream of the PNR (Wang *et al.*, 2004; Castaings *et al.*, 2009; Xu *et al.*, 2016).

It remains unclear how the presence of ammonium could act on the PNR. Ammonium itself may modulate the PNR following its sensing by possible ammonium sensors such as AtAMT1;3 (Lima *et al.*, 2010). Rhizosphere acidification accompanied by ammonium uptake might induce *AtNPF6.3* expression, enhancing its contribution (Tsay *et al.*, 1993; Lager *et al.*, 2010) (see Fig. 1). Some effects of ammonium on gene expression are mediated via downstream products of ammonium assimilation, such as glutamine (Patterson *et al.*, 2010; Kamada-Nobusada *et al.*, 2013). Given that all of the experiments detailed in Table 1 included ammonium at millimolar concentrations, it is highly possible that amino acids would have accumulated in those plants. Plants might systemically modulate the PNR depending on the internal N status.

Future perspectives

Recent evidence from laboratory and field studies strongly suggests that nitrate uptake and reduction will be suppressed in the future when atmospheric CO_2 levels are predicted to increase (Bloom *et al.*, 2010, 2014). Nitrate reduction requires a large amount of energy. Ammonium seems to be the preferable N source, but ammonium application as the sole N source often results in the suppression of plant growth. It is widely accepted that the co-provision of nitrate with ammonium eliminates this toxicity, and that plant growth is maximized at a certain ratio of ammonium to nitrate depending on the species (Britto and Kronzucker, 2002). It remains a challenge to determine how the optimal nitrate and ammonium ratio is determined in association with their uptake, translocation, assimilation, and signaling. Hachiya *et al.* (2012) found that, in *A. thaliana* shoots, changes in amino acid and organic acid concentrations are not a prerequisite for nitrate-dependent growth enhancement in the presence of concentrated ammonium. Notably, a marked decrease in NR activity has little influence on this growth enhancement (Konishi and Yanagisawa, 2013). This strongly suggests that nitrate signaling in the presence of ammonium – and thus components associated with the PNR – would play crucial roles in maximizing plant growth. Therefore, the PNR components are potential candidates for the improvement of plant growth, which is confirmed by the recent finding that constitutive overexpression of *NLP7* improves plant growth and N assimilation when both nitrate and ammonium are used as N sources (Yu *et al.*, 2016). Phosphoproteome analysis has shown that the addition of nitrate or ammonium to N-depleted *A. thaliana* seedlings results in a distinct phosphorylation pattern within tens of minutes (Engelsberger and Schulze, 2012). The strong association between the transcriptional PNR and protein phosphorylation will attract much interest among researchers.

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