REVIEW PAPER

Plant nitrogen uptake and assimilation: regulation of cellular pH homeostasis

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Abstract

The enzymatic controlled metabolic processes in cells occur at their optimized pH ranges, therefore cellular pH homeostasis is fundamental for life. In plants, the nitrogen (N) source for uptake and assimilation, mainly in the forms of nitrate (NO$_3^-$) and ammonium (NH$_4^+$) quantitatively dominates the anion and cation equilibrium and the pH balance in cells. Here we review ionic and pH homeostasis in plant cells and regulation by N source from the rhizosphere to extra- and intracellular pH regulation for short- and long-distance N distribution and during N assimilation. In the process of N transport across membranes for uptake and compartmentation, both proton pumps and proton-coupled N transporters are essential, and their proton-binding sites may sense changes of apoplastic or intracellular pH. In addition, during N assimilation, carbon skeletons are required to synthesize amino acids, thus the combination of NO$_3^-$ or NH$_4^+$ transport and assimilation results in different net charge and numbers of protons in plant cells. Efficient maintenance of N-controlled cellular pH homeostasis may improve N uptake and use efficiency, as well as enhance the resistance to abiotic stresses.

Keywords: Ammonium, assimilation, ATPase, charge balance, cellular pH, homeostasis, nitrate, pump, transport, uptake.

Introduction

Nitrogen (N) is required for plants to complete their life cycles and is the most important nutrient acquired in greatest quantities by roots (Xu et al., 2012; Oosterhuis et al., 2014). NO$_3^-$ and NH$_4^+$ are the most prominent forms of inorganic N taken up by land plant species, and their root uptake rapidly causes primary effects on ionic and pH balance in plant cells. Cellular homeostasis of ions and pH is fundamental to basic cellular processes and is needed to maintain normal plant growth and development as well as responses to stresses (Basil and Blumwald, 2014; Reguera et al., 2015). In addition, pH varies within different intracellular compartments and the proton gradient is important for the viability of cells (Shen et al., 2013).

Within plant cells, several compartments with different pH exist in parallel. The cytosol has pH values at 7.2–7.4 to ensure proper biochemical reactions (Schumacher, 2014), while the vacuole and apoplast maintain more acidic pH levels at 5.0–5.5 (Felle, 2001; Martinière et al., 2013a; Shen et al., 2013; Schumacher, 2014). Cytoplasmic pH (pHc) homeostasis is the result of a variety of processes. First, cytoplasmic chemical buffering components, such as bicarbonate, phosphate, and protein...
buffers, play important roles in stabilizing pHc (Kurfđjian and Guern, 1989). Secondly, the physical pH-stat, which is proton transport across membranes, contributes to pHc homeostasis (Felle 2001; Britto and Kronzucker, 2005). The maintenance of optimal pH in plant cells has to be tightly regulated and is established by different primary active H⁺ pumping complexes, such as the plasma membrane (PM) or P-type H⁺-ATPase (PM-ATPase), vacuolar H⁺-ATPase (V-ATPase), and the vacuolar H⁺-pyrophosphatase (V-PPase) (Schumacher, 2006; Gaxiola et al., 2007; Marshansky and Futai, 2008). The P-type ATPases can be present in both the PM and vacuole (Li et al., 2016). The physical pH-stat is also determined by transport of other ions to maintain the electrochemical balance, and H⁺-coupled ion transporters contribute to intracellular pH homeostasis (Gerendás and Schurr, 1999; Reguera et al., 2000; Palmgren, 2001). In addition, the Cl⁻ concentration in leaves can be reduced by the NO₃⁻ supply (Glass and Siddiqi, 1985; Guo et al., 2017). Two maize nitrate transporters, ZmNPF6.4 and ZmNPF6.6, are permeable to both NO₃⁻ and Cl⁻ (Wen et al., 2017), indicating that the two anions could be facilitated by the similar transport systems in plants. There are also chloride-specific MATE transporters in the vacuolar membrane (Zhang et al., 2017). Diurnal changes in vacuolar malate have been observed to compensate for NO₃⁻ and K⁺ fluctuations (Niedziela et al., 1993).

**Instant response of cellular membrane potential and pH**

The cell membrane potential (ΔΨ, negative inside the cell compared with outside the cell) can be affected by fluxes of charged ions across the PM. An immediate physiological response of root cells to NH₄⁺ and NO₃⁻ exposure is a transient change of ΔΨ, which is caused by NH₄⁺ and NO₃⁻ influx carrying H⁺ into the cell and compensated by activation of the PM H⁺-ATPase to repolarize and maintain ΔΨ. The initial membrane depolarization was not commensurate with the increased influx of NH₄⁺/NO₃⁻ (pKₐ 9.25) at pH 6.25 in the medium in roots of barley, suggesting that the increased transport of electroneutral NH₄⁺ dominates uptake (Coskun et al., 2013). NO₃⁻ is co-transported with H⁺ through a symporter into cells, and the stoichiometry of NO₃⁻ and H⁺ is ~2 (Glass et al., 1992; Miller and Smith, 1996). Root NO₃⁻ acquisition commonly leads to ΔΨ depolarization of the cells suggesting an H⁺ stoichiometry >1 (Meharg and Blatt, 1995; Mistrik and Ullrich, 1996; Britto and Kronzucker, 2005).

It is controversial whether such transport mechanisms would lead to longer term cytosol alkalinization by NH₄⁺ uptake or acidification by NO₃⁻ uptake, but at least in the initial period after the addition of NH₄⁺ or NO₃⁻ some pH changes are generally accepted. For NO₃⁻ uptake, only small changes in cytoplasmic pH occurred in roots of maize seedlings growing in nutrient solutions at different pH and supplemented with NO₃⁻.
normal NO$_3^-$ (5 mM) (Gerendás et al., 1990). It is proposed that these results are attributed to the presence of tight regulatory mechanisms for intracellular pH. An important component of NH$_4^+$/NH$_3$ or NO$_3^-$ uptake in plants is the assimilatory consumption of these ions. An initial NO$_3^-$-induced cytosolic acidification was measured in Limnobium stoloniferum root hairs (Raven, 1985, 1986; Ullrich and Novacky, 1990). NO$_3^-$ assimilation, which is a proton-consuming process, might cause an increase of cytoplasmic pH and thus partially compensate for H$^+$ influx coupled with NO$_3^-$ uptake. In maize roots, the inhibition of NO$_3^-$ assimilation using tungstate, an inhibitor of NO$_3^-$ reductase activity, resulted in acidification of the cytosol (Espen et al., 2004). Another regulatory mechanism to prevent NO$_3^-$ uptake generating acidification of the cytoplasm is an increase in PM-ATPase activity. Decreased cytoplasmic pH is a signal triggering the PM-ATPase to pump H$^+$ out of the cytosol (Espen et al., 2004) and hyperpolarize the PM ΔΨ (Glass et al., 1992; McClure et al., 1990a, b). In contrast to NO$_3^-$, the effect of NH$_4^+$ uptake on intracellular pH is dependent on external medium pH (Gerendás et al., 1990; Kosegarten et al., 1997; Gerendás and Ratcliffe, 2000). Maize root tip intracellular pH showed no change at external pH 6, but decreased at pH 4 and increased at pH 8 with 5 mM NH$_4^+$ supply (Gerendás et al., 1990). At high external pH, the NH$_4^+/NH_3$ equilibrium shifts in favour of the NH$_3$ molecule that readily permeates the PM through aquaporins (Kleiner, 1981; Macfarlane and Smith, 1982; Coskun et al., 2013). At external pH 9, both the cytosol and vacuole were alkalized in 1 h with NH$_4^+$ supply from 5 mM to 20 mM (Gerendás and Ratcliffe, 2000). Both NH$_4^+$ transport and assimilation were assumed to contribute to the alkalization of cytosolic pH (Kosegarten et al., 1997). In the external pH range from 5 to 7, the cytoplasmic buffer capacity may be able to balance the NH$_4^+$-elicited pH changes (Kosegarten et al., 1997).

Some caution is needed when evaluating the influence of other accompanying cations (e.g. K$^+$, Mg$^{2+}$, or Ca$^{2+}$) and anions (e.g. Cl$^-$) on the alteration of cellular pH grown with NO$_3^-$ and NH$_4^+$ supply. For example, increased H$^+$/K$^+$ antiport at the PM under high K$^+$ supply may compensate for the NO$_3^-$ uptake-induced cytosolic acidification via 2H$^+$/NO$_3^-$ symport (Kurkdjian and Guern, 1989; Ullrich and Novacky, 1990; Guern et al., 1991; Briskin and Hanson, 1992; Sacchi and Cocucci, 1992; Nocito et al., 2002).

**Activity of ATPase and PPase in response to alternative supplies of N**

The activity of membrane ATPases, PPases, and H$^+$-coupled transporters establishes and can regulate cytoplasmic pH homeostasis. The PM H$^+$-ATPase plays an important physiological role in maintaining the plasma membrane electrical potential difference and generating a transmembrane H$^+$ chemical gradient (ΔΨ; acidic on the outside) during the uptake of nutrients (Palmgren, 2001; Falhof et al., 2016). For example, it was found that adding PM H$^+$-ATPase inhibitors dramatically decreased root NO$_3^-$ uptake (McClure et al., 1990b), and eliminated the NH$_4^+$ uptake–generated depolarization of ΔΨ (Wang et al., 1994). In early adjustment to N uptake, the PM H$^+$-ATPase plays an important role in maintaining cytosolic pH homeostasis. When compared with CaSO$_4$ solution, (NH$_4$)$_2$SO$_4$ induced the PM H$^+$-ATPase activity in roots of barley seedlings (Yamashita et al., 1995). Similarly, Ca(NO$_3$)$_2$ treatment also induced a significantly higher transcription of PM-ATPase genes after a 3 h exposure and a significantly higher protein concentration and activity after a 6 h exposure (Santi et al., 2003). Interestingly, PM H$^+$-ATPase activity including both hydrolytic and H$^+$-pumping activity and its related gene expression showed no difference in rice plants grown in 2.5 mM NH$_4^+$ or NO$_3^-$ solution when the solution was buffered at the same pH (Zhu et al., 2009).

NO$_3^-$ transport into the vacuole from the cytosol is mediated by an H$^+$/NO$_3^-$ antiport mechanism, which is driven by P- and V-type ATPases and V-PPase activity (Granstedt and Hufnaker, 1982; Blumwald and Poole, 1985; Schumaker and Sie, 1987; Glass et al., 1992; Miller and Smith, 1992; Krebs et al., 2010). High concentrations of NO$_3^-$ could inhibit V-ATPase activity in isolated vacuoles (Blumwald and Poole, 1985). Inhibiting the activity of V-ATPase or V-PPase or knock-out of their encoding genes significantly decreased NO$_3^-$ storage and influx into vacuoles of Brassica napus plants (Han et al., 2016).

**Factors dominating N supply effects on rhizosphere pH**

Soil alkalinity above pH 8.0 or acidity below pH 5.5 limits plant growth and development (Schubert et al., 1990; Koyama et al., 2001; Cha–Um et al., 2009; Patil et al., 2012). Uptake of NH$_4^+$ or NO$_3^-$ (i.e. transport and assimilation) results in rapid acidification or alkalization of the apoplasm (Geißus, 2017) and rhizosphere (Taylor and Bloom 1998; Kosegarten et al., 1997; Gerendás and Ratcliffe, 2000; Ruan et al., 2000; Hinsinger et al., 2003). It has been shown that decreasing external pH to acidic levels can up-regulate the expression of 20–40% of the NH$_4^+$-responsive genes in Arabidopsis thaliana, suggesting that apoplastic acidification is a component of NH$_4^+$-induced stress (Patterson et al., 2010).

The N supply factors causing changes in rhizosphere or apoplastic pH include N concentrations and forms, balance of N with other major nutrients, and plant species. (i) High NH$_4^+$ supply induced rhizosphere acidification and high NO$_3^-$ induced alkalization (Marschner and Römheld, 1983; Römheld, 1986; Hinsinger et al., 2003) controlled by the processes of N transport (see ‘Extra- and intracellular pH regulation at short- and long-distance N distribution’) and assimilation (see ‘Cellular pH homeostasis during N assimilation’). (ii) For charge balance, NO$_3^-$ may increase, while NH$_4^+$ decreases, cation uptake by root cells. The imbalanced uptake of cations and anions triggers release of H$^+$ or OH$^-$ (or HCO$_3^-$) into the apoplasm, resulting in opposing pH changes in the rhizosphere (Haynes, 1990; Marschner, 1995; Hinsinger et al., 2003). (iii) The extent of the N supply-induced pH change in the rhizosphere or apoplasm is also dependent on plant species. For example, the rhizosphere of lentils and chickpea could be acidified even at relatively high NO$_3^-$ supply (Römheld, 1986). The effects of N supply on rhizosphere pH can be simply shown using pH indicators in agar (see Fig. 1 for rice).
Fig. 1. Rhizosphere pH regulated by uptake of NH₄⁺ and NO₃⁻ in rice roots. (A) The rhizosphere pH of rice roots shown with a colour pH indicator. (B) Agar profile showing rhizosphere pH after removing the roots. Rice seedlings (Oryza sativa L. ssp. japonica, Nipponbare) were grown in full nutrient solution containing 1.25 mM NH₄NO₃ for 4 weeks and then transferred to 2.5 mM NH₄⁺ or 2.5 mM NO₃⁻ for 72 h. After 72 h N treatment, the plant root was washed by dipping in 0.2 mM CaSO₄ for 1 min before placement on the agar. An intact plant was placed on agar (0.9 g l⁻¹, containing the pH indicator (0.03 g l⁻¹ bromocresol purple). The initial pH was 5.2–5.3 from 11.00 h to 11.30 h, roots were kept in darkness covered with a moist paper tissue and under a 0.5×12×12 cm³ Plexiglas plate, and the picture was taken after 2–4 h in contact with the pH indicator agar. (C) pH of the hydroponic growth medium during 2.5 mM NH₄⁺ or 2.5 mM NO₃⁻ solution after 24, 48, and 72 h. The initial pH was 5.2–5.3.
Extra- and intracellular pH regulation at short- and long-distance N distribution

A variety of root and shoot NH$_4^+$ and NO$_3^-$ transporters may be involved in cellular pH homeostasis through the processes of H$^+$ production or consumption within cellular compartments (Fig. 2). Cellular pH homeostasis is also dependent on the activity of the proton pumps, the PM-ATPase, V-ATPase, and V-PPase (Fig. 2). NH$_4^+$ transport is controlled by NH$_4^+$ transporters (AMTs) and non-saturable low-affinity uptake systems (i.e. aquaporins TIPs or cation channels) in plants. NO$_3^-$ transport is mediated by the NO$_3^-$ Transporter (NRT1 and NRT2) family, and the NRT1 family is renamed the NO$_3^-$ Transporter1/Peptide Transporter Family (NPF) (Léran et al., 2013). The Chloride Channel (CLC) family also function as anion/proton exchangers or anion channels (De Angeli et al., 2006), mediating NO$_3^-$ transport at the vacuole or in endomembrane vesicles (Zifarelli and Pusch, 2010).

**Fig. 2.** Protons are involved in NH$_4^+$ and NO$_3^-$ fluxes. Different transporters or channels for the fluxes of NH$_4^+$ (red arrow), NO$_3^-$ (blue arrow), and H$^+$ (black arrow). Potassium channels (AKT1), non-selective cation channels (NSCC), and aquaporins (AQP, TIP) are NH$_4^+$/NH$_3$ channels (Hachiya and Sakakibara, 2017; Liu and von Wirén, 2017). AMT1 is an NH$_4^+$ transporter functioning as an NH$_4^+$ or NH$_3$ channel, NH$_4^+$ uniporter, or H$^+$/NH$_4^+$ antiporter (Giehl et al., 2017; Duan et al., 2018; reviewed by Tegeder and Masclaux-Daubresse, 2018). NPF and NRT2 are plasma membrane (PM) or tonoplast NO$_3^-$ transporters functioning as an H$^+$/NO$_3^-$ symporter or an NO$_3^-$ excretion transporter (reviewed by Fan et al., 2017; Wang et al., 2018). CLCa and CLCb are tonoplast-localized chloride transporters functioning as H$^+$/NO$_3^-$ antiporters (reviewed by Zifarelli and Pusch, 2010). Intracellular pH maintenance is also established by different primary active H$^+$ pumping complexes, such as the PM H$^+$-ATPase (PM-ATPase), the vacuolar H$^+$-ATPase (V-ATPase), and V-PPase (reviewed by Gaxiola et al., 2007). Cytosol, vacuole.
For inorganic N transporters in plants, readers are also referred to previously published reviews (Lérant et al., 2013; Fan et al., 2017; Tegeder and Masclaux-Daubresse, 2018; Wang et al., 2018).

Here we focus on the plant NH₄⁺ and NO₃⁻ transporters which are involved in maintaining pH balance both in vitro and in vivo.

**H⁺/NO₃⁻ symporters are involved in regulation of cellular pH and ion homeostasis**

Both NO₃⁻ and NH₄⁺ can be imported into root cells by H⁺-coupled symporters across the PM through energetically uphill processes. Most members of the nitrate transporter families NPF/NRT1 and NRT2 showed characteristics of pH-dependent NO₃⁻ transport when expressed in Xenopus laevis oocytes. After injection of the NPF/NRT1 and NRT2 genes, the oocytes showed NO₃⁻-elicited inward current and the pH dependency (i.e. NO₃⁻-induced current is larger at pH 5.5 than at pH 7.4) that is associated with a H⁺-symport mechanism (Søgaard et al., 2009; Ortiz-Ramirez et al., 2011; Fan et al., 2017; Wang et al., 2018). Many results indicate that the NPFs function as H⁺/NO₃⁻ co-transporters, which mediate the influx with the H⁺/NO₃⁻ ratio being greater than one (Zhou et al., 1998; Lin et al., 2008). AtNPF6.3/NRT1.1/CHL1 is one of the exceptions, which is identified as both a pH-dependent importer (Tsya et al., 1993; Liu et al., 1999; Wang et al., 2018) and a pH-independent exporter (Lérant et al., 2013). AtNPF6.3/NRT1.1/CHL1 knockout (point mutation of P492L, chl1-9) led to impaired H⁺ tolerance and the disappearance of alkalinization in NO₃⁻-sufficient growth medium (Fang et al., 2016), indicating that NRT1.1-mediated NO₃⁻ uptake contributes to plant H⁺ tolerance by alkalization of the rhizosphere. However, knockout of other nitrate transporters such as AtNPF4.6/AT11/NRT1.2, AtNRT2.1, AtNRT2.2, and AtNRT2.4 did not alter the plant H⁺ tolerance (Fang et al., 2016). Since NRT1.1 may contribute to root NO₃⁻ uptake by 70–80% (Huang et al., 1996; Wang et al., 1998; Orsel et al., 2004; Krouk et al., 2010; Kiba et al., 2012), it is possible that the activity of NRT1.1 masked the effect of other H⁺-coupled NO₃⁻ transport in the tolerance to rhizosphere acidity. Furthermore, the mechanism of H⁺ movement via water molecules in the peptide-binding site for some members of the NRT1/NPF/POT family of secondary active transporters was suggested to provide a mechanism enabling the proteins to transport many diverse substrates (Parker et al., 2017). Effectively, this mechanism separates substrate recognition from H⁺ translocation in this family of transporters.

Two members of the plant AMT family, common bean AMT1;1 and wheat AMT1;1, are characterized as H⁺-coupled importers. Expression of common bean PvAMT1;1 in oocytes led to NH₄⁺-elicited inward currents and cytosolic acidification, indicating that it functions as an H⁺/NH₄⁺ symporter in a 1:1 ratio (Ortiz-Ramirez et al., 2011). The activity of PvAMT1;1 was enhanced by low extracellular pH (pH 5.5), and this was demonstrated by changes in the reversal potential and by increased cytoplasm acidification measured with pH-selective microelectrodes (Ortiz-Ramirez et al., 2011). However, there was no direct evidence to show whether PvAMT1;1 was related to H⁺ exchange in both the cytosol and rhizosphere in vivo.

Currently, it is not clear if xylem pH is regulated by H⁺/NO₃⁻ co-transport. A PM NO₃⁻ transporter, AtNPF7.3/NRT1.5, which is abundantly expressed in the pericycle or xylem parenchyma cells, mediates both pH-dependent NO₃⁻ influx and efflux in oocytes, and release of NO₃⁻ from the Arabidopsis root pericycle (Lin et al., 2008). These authors proposed that there is a potential link between xylem pH and root-to-shoot NO₃⁻ transport. However, AtNPF7.3/NRT1.5 is also identified as a H⁺-coupled H⁺/K⁺ antiporter in Xenopus oocytes, and functions in facilitating K⁺ loading into the xylem (Li et al., 2017). Thus, it is unclear whether the long-distance transport of NO₃⁻ and/or K⁺ contributed by NPF/NRT1s such as AtNPF7.3/NRT1.5 can alter pH in the xylem.

The NRT2s are another important family of NO₃⁻ transporters, mediating uptake from the soil and transport to leaf cells and developing seeds (Xu et al., 2012; Fan et al., 2017). One of the first members of this family to be functionally characterized in oocytes was suggested not only to be an H⁺-coupled NO₃⁻ symporter, but also to operate in an NO₃⁻ transport mode uncoupled to H⁺ movement (Zhou et al., 2000). This alternative mechanism may be beneficial when external NO₃⁻ is very abundant, avoiding the pH problems that might be associated with H⁺ influx and cytosolic acidification. Some of the NRT2 transporters require a partner protein (NAR2) for function (Orsel et al., 2006; Feng et al., 2011; Yan et al., 2011). In both Arabidopsis and rice, it has been shown that NAR2 is required for the targeting of the NRT2 protein from internal membrane vesicles to the PM (Wirth et al., 2007; Liu et al., 2014). The accumulation of the NRT2 transporter protein may provide a mechanism for altering the pH of these endomembrane vesicles.

In the rice genome, the OsNRT2.3 gene encodes two members of a H⁺-coupled nitrate transporter family, OsNRT2.3a and OsNRT2.3b (Feng et al., 2011; Yan et al., 2011). OsNRT2.3a is located in root stellar cells and plays an important role in distribution of NO₃⁻ from root to shoot (Tang et al., 2012), while OsNRT2.3b is expressed in phloem and contributed to phloem pH and ion homeostasis (Fan et al., 2016). OsNRT2.3b expression in oocytes elicited a depolarized membrane potential and cytosolic acidification in response to NO₃⁻ supply (Fan et al., 2016). Notably, OsNRT2.3b functions only at a slightly alkaline cytosolic pH, and a pH-sensitive motif of OsNRT2.3b facing the cytosolic side determines its activity to acquire NO₃⁻ from the external medium (Fan et al., 2016). In rice, OsNRT2.3b overexpression decreased the phloem sap pH from 8 to 7.1 under NO₃⁻ supply, and from 7.4 to 6.8 under NH₄⁺ supply, resulting in significantly increased grain yield and nitrogen use efficiency (NUE) at different N levels in field conditions (Fan et al., 2016). The sensing of cytosolic pH by OsNRT2.3b provides an explanation for plant adaptation to changes in the form of N supply. This finding highlights the important link between N transport, pH regulation, and NUE.

**NO₃⁻ excretion transporters may be involved in cellular pH regulation**

In contrast to NO₃⁻ influx, NO₃⁻ efflux from root cells is energetically a downhill process which is also dependent on the activity of the PM H⁺-ATPase pump. It was shown that in isolated root PMs, NO₃⁻ efflux is tightly coupled to H⁺
excretion by the H⁺-ATPase, and that both activities of NO₃⁻ efflux and H⁺ excretion share similar acidic optimum pH at the cytosolic face of the PM (Vara and Serrano, 1982; De Michiels and Spanswick, 1986; Grouzis et al., 1997; Pouliquen et al., 2000). It has been shown that the Nitrate Excretion Transporter AtNPF2.7/NAXT1 mediates passive NO₃⁻ efflux across the isolated PM of plant root cells in acidic medium in vitro (Segonzac et al., 2007), suggesting that the NO₃⁻ excretion transporter can mediate both NO₃⁻ and H⁺ efflux in combination with PM proton pumps, thus re-balancing the acidification of cytosol to some extent.

**Intracellular H⁺/NO₃⁻ antiporters involved in pH regulation of cellular organelles**

NO₃⁻ can be stored in, and remobilized from, vacuoles. NO₃⁻ transport into vacuoles is mediated by an H⁺/NO₃⁻ antiporter, and the H⁺/NO₃⁻ symport systems also serve in NO₃⁻ efflux from the vacuole to the cytosol, which are energized by V-ATPases pumping H⁺ to vacuoles (De Angeli et al., 2006). Arabidopsis AtCLCa is expressed in leaf mesophyll cells; disruption of AtCLCa led to an ~50% decrease of vacuolar NO₃⁻ organelles. As members of all the NO₃⁻-antiporter, the H⁺/NO₃⁻ exchanger, which transports NO₃⁻ from the cytosol to the vacuolar lumen (De Angeli et al., 2006). AtCLCa expression in oocytes indeed induced intracellular alkalization at both pH 5.5 and pH 7.5 when oocytes were pulsed to positive voltages (Bergsdorf et al., 2009). In vitro, although transport processes such as the H⁺/NO₃⁻ exchanger AtCLCa play a role in alkalization of the vacuole or acidifying the cytosol, the active accumulation of H⁺ in the vacuole is also accomplished by P- and V-type ATPases, which function as ‘proton pumps’. There are two ATP-binding sites, at His620 and Asp750 in the C-terminus CBS domain of AtCLCa. Adding micromolar concentrations of ATP could inhibit AtCLCa activity in isolated A. thaliana vacuoles, resulting in a decrease of NO₃⁻ influx by up to 60% (De Angeli et al., 2009). It is possible that the V-ATPases can work together with the CLC antiporter in the tonoplast to balance cytoplasmic pH during the process of vacuolar NO₃⁻ accumulation.

Currently, it is not known if there are nitrate transporters involved in NO₃⁻ flux and pH homeostasis in other cellular organelles. As members of all the NO₃⁻-transporter families (NRT1, NRT2, and CLCs) can be located in endomembrane systems, they may have important roles in the generation of compartmental pH gradients within the cell.

**pH regulatory sites in N transporters**

The activity of many NO₃⁻ transporters is affected by pH; however, the regulatory mechanism is not clear. Interestingly, many plant N transporters including H⁺/NO₃⁻ symporters, H⁺/NO₃⁻ antiporters, and H⁺/NH₄⁺ symporters contain putative pH-sensing sites (Table 1), indicating that these transporters may sense either external (i.e. apoplastic) or internal pH. Both the ExxER motif and histidine residues are essential for H⁺ binding in plant NPs (Jorgensen et al., 2015; Longo et al., 2018). Removal of charged residues in the ExxER motif of AtNPF6.3/NRT1.1 abolished both H⁺ binding and NO₃⁻ transport activity (Sun et al., 2014). The stoichiometry of H⁺/NO₃⁻ transport through AtNPF6.3/NRT1.1 is at least 2H⁺:1NO₃⁻, and it was proposed that the ExxER motif in TM1 binds one H⁺, leaving His356 on TM7 to bind another H⁺ and NO₃⁻ (Parker and Newstead, 2014).

It is well known that histidine residues are important H⁺-binding amino acids involved in the regulation or activity of pH-dependent transporters in Escherichia coli, yeast, mammals, and plants, because they can ionize within the physiological pH range (Wiebe et al., 2001; Ortiz-Ramirez et al., 2011). PvAMT1;1 is an NH₄⁺ transporter of common bean, for which the mutation of its conserved His211 to glutamic acid (H211E) results in altering the transport mechanism to be pH independent, with its affinity for NH₄⁺ decreasing while increasing the transport capacity (Ortiz-Ramirez et al., 2011). Exposure of PvAMT1;1 H211E-expressing oocytes to NH₄⁺ did not affect the cytoplasmic pH but caused depolarization of the membrane potential at both pH 5.5 and pH 7.0 (Ortiz-Ramirez et al., 2011). For a rice NO₃⁻ transporter, OsNRT2.3b, His167 (H167) was located on the cytoplasmic side and has been confirmed to play a critical role in sensing cytosolic pH (Fan et al., 2016). The H167R mutation does not fully eliminate the basic activity of OsNRT2.3b as a H⁺-coupled NO₃⁻ transporter but results in the loss of cytosolic pH sensing (Fan et al., 2016).

Certain gating glutamate residues of some channel proteins may be involved in sensing cellular pH. Mutation of AtCLCa ‘gating glutamate Glu203 or the ‘H⁺ glutamate site’ Glu270 to alanine prevented its activity in generating NO₃⁻ flux-elicited currents or depolarization-induced H⁺ transport in oocytes (Bergsdorf et al., 2009; Miller and Nguitra, 2009), suggesting that the two Glu sites are H⁺-binding sites in AtCLCa.

| Table 1.  pH-sensing sites in plant ammonium and nitrate transporters |
| Transporter | Transport mode | pH sensing site | Localization | References |
|---------------------------------|----------------|----------------|-------------|------------|
| AtNPF6.3/NRT1.1/CHL1 | 2 H⁺/1 NO₃⁻ symport | ExxER (E141, E144), His365 (H365) | PM | Sun et al. (2014); Parker and Newstead (2014) |
| PvAMT1;1 | 1 H⁺/1 NH₄⁺ symport | His211 (H211) | PM | Ortiz-Ramirez et al. (2011) |
| OsNRT2.3b | 2 H⁺/1 NO₃⁻ symport | His167 (H167) | PM | Fan et al. (2016) |
| AtCLCa | 1 H⁺/2 NO₃⁻ antiport | Glu203 (E203), Glu270 (E270) | Tonoplast | Bergsdorf et al. (2009); Miller and Nguitra (2009) |

At, Arabidopsis; Pv, common bean; Os, rice. PM, plasma membrane; E, glutamate; H, histidine.
Table 2. Proton changes in the processes of N transport and assimilation

| N utilization processes | Equation of H⁺ change in cytoplasm |
|-------------------------|-----------------------------------|
| NH₄⁺ transport          | NH₄⁺ (out) + NH₃ + H⁺ (out) |
| NH₃ protonation          | NH₃ + H⁺ = NH₄⁺ |
| NH₄⁺ assimilation        | NH₄⁺ + C₅H₈NO₄ + 1.5O₂ → CO₂ + 3H₂O + 2H⁺ |
| NO₃⁻ transport          | NO₃⁻ (out) + H⁺ (out) + NO₃⁻ + 1H⁺ |
| NO₃⁻ reduction           | NO₃⁻ + 2/3C₅H₈O₄ + 2O₂ + 2H⁺ → NH₄⁺ + 4CO₂ + 3H₂O |
| NH₄⁺ assimilation        | NH₄⁺ + C₅H₈NO₄ + 1.5O₂ → CO₂ + 3H₂O + 2H⁺ |

H⁺, H⁺ production and H⁺, H⁺ consumption in the cytoplasm. In the process of NH₄⁺ transport, it is assumed that 1NH₄⁺ counterbalances 1 extra H⁺, released to outside the cell (out). In the process of NH₄⁺ assimilation, if the glucose is ample, 2H⁺ will be produced in the cytoplasm. For 1NO₃⁻/2H⁺ co-transport into the cytoplasm, it is assumed that 1H⁺ is pumped out of the cell by the PM H⁺-ATPase. For NO₃⁻ reduction, 2H⁺ will be produced when plenty of carbon is available. Combining the NO₃⁻ transport, reduction, and assimilation, if 1NO₃⁻ is totally incorporated into 1 glutamate (Glu), it yields 1H⁺ in the cell, and 1H⁺ extra (Britto and Kronzucker, 2005). If 1NH₄⁺ is transported and assimilated to 1Glu, it generates 1H⁺ in the cell, and 1H⁺ extra (Britto and Kronzucker, 2005).

Cellular pH homeostasis during N assimilation

The ‘proton economy’ in N transport and assimilation

The majority of root acquired NH₄⁺ is rapidly assimilated in roots, whereas NO₃⁻ is mainly assimilated in shoots depending on different plant species and the external N level, requiring both ATP and carbon (C) skeletons (Fig. 3; Table 2; Raven and Smith, 1976; Andrews, 1986; Bloom et al., 1992; Rachmilevitch et al., 2004; Nunes-Nesi et al., 2010; Britto and Kronzucker, 2005).

Reduction of NO₃⁻ to NH₄⁺ is catalysed by nitrate reductase (NR) and nitrite reductases (NiRs) in the cytosol and plastids or chloroplasts, respectively, with the consumption of 2H⁺ (molecule) per 1NO₃⁻ in the cytosol (Fig. 3; Table 2; Lea and Miflin, 1974; Xu et al., 2012). In general, NH₄⁺ assimilation into amino acids occurs quickly under NH₄⁺ supply and is conducted in root plastids or shoot chloroplasts by the GS/GOGAT cycle, producing 2H⁺ per 1NH₄⁺ (Fig. 3; Table 2; Masclaux-Daubresse et al., 2010). However, there is some controversy as to whether the GS/GOGAT pathway of NH₄⁺ assimilation is net H⁺ consuming or producing in plants. Three conditions need to be considered for predicting the consumption or production of H⁺ in NH₄⁺ assimilation. (i) If ATP and NAD(P)H for the reaction are regenerated only by other processes, the GS/GOGAT pathway is H⁺ consuming (Kosegarten et al., 1997). (ii) If the C skeletons can be continually provided for the regeneration of ATP and NAD(P)H, it appears to be an H⁺-releasing process (Gerendás and Ratcliffe, 2000). (iii) If the C skeletons are limited, then the 2-oxoglutarate pool is replenished by re-utilization of malate (stored in the vacuole), and NH₄⁺ assimilation may rapidly consume H⁺ (Gerendás and Ratcliffe, 2000). In addition, different plant species showed diverse cytoplasmic pH changes in response to NH₄⁺; for example, rice, which has stronger GS activity than maize, showed a larger increase of cellular pH during NH₄⁺ assimilation (Magalhaes and Huber, 1989, 1991; Kosegarten et al., 1997).

The combination of NH₄⁺ or NO₃⁻ transport and assimilation results in different net changes of H⁺ numbers in plant cells (Table 2; Bloom et al., 1992; Rachmilevitch et al., 2004; Britto and Kronzucker, 2005). Incorporation of one NH₄⁺ to glutamate produces one H⁺ in the cell, while assimilation of one NO₃⁻ to glutamate produces one H⁺. However, when NO₃⁻ or NH₄⁺ is not immediately assimilated and presumed to accumulate, it is expected that the uptake of NO₃⁻ is a transient cytosol-acidifying process whereas that of NH₄⁺ is a transient cytosol-alkalinizing process.

Biochemical malate pH-stat due to NO₃⁻ assimilation

In the process of NO₃⁻ reduction to NH₄⁺, a substantial amount of the dicarboxylic malate can accumulate in the cytosol due to the anion deficit (van Beusichem et al., 1988; Lütge et al., 2000; Pasqualini et al., 2001). Cellular malate synthesis and degradation is important for regulation of the cytosolic pH (Smith and Raven, 1979; Hurth et al., 2005). For example, knockout of the tonoplast malate transporter AttDT reduced the capacity of the mutant plant to overcome cytosolic acidification in leaf protoplasts (Hurth et al., 2005). However, these mutants did not have a strong phenotype, but the effect of changing N supply form was not tested.

pH regulation during amino acid transport

In addition to inorganic N, amino acids in the soil solution can also be directly taken up by roots (Tegeder and Masclaux-Daubresse, 2018). Inside the plant, amino acids are the major form of N for transport and re-distribution, particularly in NH₄⁺-supplied plants (Tegeder and Hammes, 2018). Most amino acid transporters function with a H⁺ co-transport mechanism and this has been shown for a broad range of amino acids, including neutral, cationic, and anionic amino acids (Tegeder and Masclaux-Daubresse, 2018; Tegeder and Hammes, 2018). The plant amino acid transporters show characteristic pH dependence in oocytes (Boorer et al., 1996; Boorer and Fischer, 1997; Hinrner et al., 1998, 2006; Fischer et al., 2002). Although there is no evidence for their direct involvement in pH regulation, the root amino acid transporters can lead to a slight increase in rhizosphere pH (Näsholm et al., 2009). In sterile conditions, amino acids can be used as a positive control for experiments comparing NO₃⁻ and NH₄⁺ as N sources.

Future perspectives

Developing the techniques to instantly monitor in real-time the dynamic changes of cellular pH by either N transport or H⁺ pumps in plants

For a better understanding of the underlying mechanisms of cellular pH homeostasis during N uptake and assimilation, it is essential to develop more molecular tools enabling in vivo measurements of pH in different intracellular compartments. Changes in proton concentrations are associated with both the N transporter and H⁺-pumping activity of ATPase (De Angeli...
et al., 2009; Bassil et al., 2011; Shen et al., 2013), thus both factors should be taken into account for pH regulation in plant cells. In tobacco, Martinière et al. (2013a) used a pHluorin-based pH sensor to directly measure pH of the endomembrane system, and found that luminal pH homeostasis in the trans-Golgi Network (TGN) and pre-vacuolar compartment (PVC) involved both V-ATPase–dependent acidification and H⁺ efflux mediated by the activity of the Arabidopsis Na⁺(K⁺)/H⁺ exchanger NHX5. In Arabidopsis protoplasts, Shen et al. (2013) used a modified pHluorin targeted to different organellar compartments for visualization and quantification of pH in vivo. Other pH sensors are also available for measurement of intracellular pH in plants (Martinière et al., 2013b). Some H⁺-coupled NO₃⁻ transporters (e.g. AtCLCa) and NH₄⁺ transporters (e.g. PvAMT1;1) have also been identified as transporters leading to cytosolic pH changes in the oocyte system (Bergsdorf et al., 2009; Ortiz-Ramírez et al., 2011). However, there is still a lack of information about direct measurement of intercellular pH in nitrate transporter mutant plants. With the available tools for in vivo pH measurement using pH sensors (Shen et al., 2013; Reguera et al., 2015), it will now be possible to determine how they affect pH in the cytosol and endomembranes in the future.

Role of N-controlled cellular pH homeostasis in enhancing abiotic stress tolerance

In acidic media, H⁺ and NO₃⁻ excretion are tightly coupled. AtANPF2.7/NAXT1 mediated root NO₃⁻ excretion, and
PM-ATPase stimulated H⁺ excretion (Segonzac et al., 2007). H⁺ stress enhanced NO₃⁻ uptake mediated by NRT1.1 in Arabidopsis and caused significant rhizosphere alkalization (Fang et al., 2016), and thus decreased some heavy metal toxicity such as that of Cd and Pb (Mao et al., 2014; Zhu et al., 2019). It would be interesting to examine how much N-controlled cellular pH homeostasis and effects on rhizosphere pH can regulate plant tolerance to other abiotic stresses, such as heavy metals, drought, or flooding and salinity.

Using natural genetic variation or point mutation of key H⁺-binding residues in N transporters to enhance the cellular pH homeostasis within plants for improving N uptake and utilization

It is known that intracellular pH can be a signal for modulating downstream responses (Roos, 2000; Felle, 2001; Kader and Lindberg, 2010). In rice, overexpression of OsNRT2.3b, a cellular pH-sensing nitrate transporter, could buffer N transport-induced phloem alkalization, and thus improve NUE, phosphate and iron mobilization, C metabolism, and grain yield (Fan et al., 2016). This provides an exciting example for the possibility of utilizing pH-sensing transporters to improve plant NUE and growth. It is worth checking in other N transporters if there is a tight link between H⁺-binding residues and N transport activity at different medium pH. In future, utilizing the natural genetic variation among germplasm collections or making point mutations by gene-editing techniques of pH-sensing transporters may be a pathway for enhancing crop production at varied N supply levels and improving NUE.

Revealing the molecular regulatory mechanisms of synergism, antagonism, and interaction of NO₃⁻ and NH₄⁺ on potassium and other nutrients

In plants, the transport and assimilation of NO₃⁻ and NH₄⁺ can dominate cellular pH homeostasis, which in turn affects the availability and utilization of other nutrients. The synergism, antagonism, and interaction among N and other major nutrients, such as K⁺, Ca²⁺, Mg²⁺, and Cl⁻, are known to be physiologically relevant, while the regulatory mechanisms linking these nutrients to cellular pH homeostasis are unclear. Inactivation of some nitrate transporters, such as AtNPF7.3/AtNPF7.4, is tight linked to H⁺-binding residues and N transport activity. However, more thorough investigation of the interactions between N and other nutrients are needed.

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