



Reducing the Nitrate Content in Vegetables Through Joint Regulation of Short-Distance Distribution and Long-Distance Transport

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As an important nitrogen source, nitrate (NO_3^-) absorbed by plants is carried throughout the plant *via* short-distance distribution (cytoplasm to vacuole) and long-distance transportation (root to shoot), the two pathways that jointly regulate the content of NO_3^- in plants. NO_3^- accumulation within the vacuole depends on the activities of both tonoplast proton pumps and chloride channel (CLC) proteins, and less NO_3^- is stored in vacuoles when the activities of these proteins are reduced. The ratio of the distribution of NO_3^- in the cytoplasm and vacuole affects the long-distance transport of NO_3^- , which is regulated by the proteins NPF7.3 and NPF7.2 that play opposite but complementary roles. NPF7.3 is responsible for loading NO_3^- from the root cytoplasm into the xylem, whereas NPF7.2 regulates the unloading of NO_3^- from the xylem, thereby facilitating the long-distance transport of NO_3^- through the roots to the shoots. Vegetables, valued for their nutrient content, are consumed in large quantities; however, a high content of NO_3^- can detrimentally affect the quality of these plants. NO_3^- that is not assimilated and utilized in plant tissues is converted *via* enzyme-catalyzed reactions to nitrite (NO_2^-), which is toxic to plants and harmful to human health. In this review, we describe the mechanisms underlying NO_3^- distribution and transport in plants, a knowledge of which will contribute to breeding leafy vegetables with lower NO_3^- contents and thus be of considerable significance from the perspectives of environmental protection and food safety.

Keywords: nitrate, proton pumps, chloride channel protein, NPF7.3, NPF7.2, vegetable, health

INTRODUCTION

Nitrogen (N) is an essential element that affects the growth, yield, and quality of crops (Peng et al., 2010; Ju et al., 2015). It is required for the synthesis of macromolecular compounds such as proteins and nucleic acids, which play important roles in the metabolism and energy production of organisms (Li and Gong, 2011). Ammonium nitrogen (NH_4^+ -N) and nitrate nitrogen (NO_3^- -N) are the main forms of nitrogen absorbed and utilized by crops, with the latter being the main nitrogen source for dryland crops (Tang et al., 2013; Zhao et al., 2018). Exogenous concentrations of NO_3^- are among the most important factors affecting the accumulation of NO_3^- in plants. Plants actively take up NO_3^- from the environment through a proton/nitrate-coupled mechanism (Wang

et al., 2018). Several NPF/peptide transporter (PTR) family and NO_3^- transporter 2 (NRT2) members in *Arabidopsis* are involved in NO_3^- uptake, including components of the high-affinity transport system [HATS: NRT2.1, NRT2.2 (Filleur et al., 2001), NRT2.4 (Kiba et al., 2012), and NRT2.5 (Lezhneva et al., 2014)] and low-affinity transport system [LATS: NPF4.6 (Huang et al., 1999)]. Furthermore, NPF6.3 is known to function as a dual-affinity NO_3^- transporter (Liu et al., 1999), the affinity for NO_3^- of which is determined by external NO_3^- concentrations (Ho et al., 2009). Previous studies have shown that differences in NO_3^- accumulation in different lettuce varieties are largely attributable to differences in NO_3^- uptake (Burns et al., 2011). In a study examining two varieties of Chinese cabbage with high and low NO_3^- accumulation, respectively, a stronger NO_3^- absorption capacity and higher expression of NPF and NRT2 genes were detected in the high NO_3^- -accumulating variety (Zhao et al., 2011). The NO_3^- absorbed by plants can be directly assimilated into amino acids by the activities of NO_3^- reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) in the cell cytosol (Williams et al., 1987). An imbalance in the efficiency of NO_3^- absorption and reduction in vegetables has been demonstrated to be an important factor in determining NO_3^- accumulation, and the activity of NR has been shown to be significantly negatively correlated with the NO_3^- content in Chinese cabbage leaves (Du et al., 2008). Moreover, a proportion of NO_3^- is secreted into the rhizosphere, mediated by the NO_3^- transporter NPF2.7 (Wang et al., 2018).

The NO_3^- that remains unutilized by plants is distributed throughout the plant *via* short-distance distribution and long-distance transportation (Han et al., 2015; Zhang, 2017). Short-distance distribution of NO_3^- occurs at the subcellular level and involves the distribution of NO_3^- between the cytoplasm and vacuoles, a process regulated by members of the chloride channel (CLC) protein family. Initially, it was believed that CLC proteins were involved only in the transport of Cl^- , functioning as chloride channels and Cl^-/H^+ anti-transporters. However, in a study examining the *Arabidopsis clca* mutant, De Angeli et al. (2006) demonstrated that CLC family proteins could also transport NO_3^- across the tonoplast by acting as $2\text{NO}_3^-/\text{H}^+$ antiporters. Compared with wild-type plants, the *clca* mutant was found to accumulate 50% less NO_3^- in the vacuole, thereby indicating that *AtCLCa* plays a key role in regulating the vacuolar storage and short-distance distribution of NO_3^- in plants (De Angeli et al., 2006).

The long-distance transport of NO_3^- mainly relies on the coordination of the NPF proteins with low affinity. *AtNPF7.3* is a pH-dependent NO_3^- transporter protein located in the cytomembrane and expressed in the pericycle cells near the xylem. It has been found that in mutants lacking *AtNPF7.3*, the content of NO_3^- in the xylem sap and shoots is significantly reduced, thereby indicating that *AtNPF7.3* plays an important role in promoting the long-distance transport of NO_3^- from roots to aerial parts (Lin et al., 2008). In contrast, *AtNPF7.2* is mainly expressed in parenchyma cells near the root xylem, where it functions in the unloading of NO_3^- from the xylem to regulate

the distribution ratio of NO_3^- between the roots and shoots (Li et al., 2010). The concentration of NO_3^- in plants is thus affected by multiple processes, among which there is a close interdependency that facilitates the coordinated regulation of NO_3^- accumulation in plants.

As an important source of inorganic nitrogen, the NO_3^- content of food products may originate from soil, water resources, chemical fertilizers, or food additives, consequently affecting the human food chain (Quijano et al., 2017). To date, numerous studies have been conducted on the NO_3^- content of plants and have revealed high concentrations of NO_3^- in different vegetables (Bahadoran et al., 2016), particularly leafy vegetables, which are the most important source of NO_3^- in the human diet, accounting for more than 80% of the NO_3^- intake (Anjana and Iqbal, 2007; Bondonno et al., 2018; Chetty et al., 2018). However, during the storage and processing of plants, excess NO_3^- that remains unassimilated in plant tissues can be enzymatically converted to NO_2^- . The NO_3^- ingested by humans can also be reduced to NO_2^- *via* the activity of gut microorganisms. NO_2^- is a strong carcinogen and causes the accumulation of methemoglobin, a compound with potentially toxic effects on human health (Salehzadeh et al., 2020). The content of NO_3^- in vegetables depends on its distribution and utilization and has become established as one of the indices used to assess the quality of vegetables and their processed products. Consequently, measures that can be used to reduce the concentration of NO_3^- in leafy vegetables, by regulating its distribution and utilization, are of importance with respect to reducing environmental pollution and ensuring a healthy diet.

SHORT-DISTANCE (VACUOLE TO CYTOPLASM) NO_3^- DISTRIBUTION IN VEGETABLES

The evolutionary success of higher plants is largely attributable to their unique cellular architecture (Kriegel et al., 2015). Vacuoles, as the largest organelles in mature plant cells, account for approximately 90% of the cell volume and are generally utilized for the storage of nutrients (Liao et al., 2002; Shen et al., 2003; Krebs et al., 2010; Liao et al., 2018). Having been absorbed by plants, NO_3^- , an important plant nutrient, is allocated to the metabolic pool (cytoplasm) or the storage pool (vacuole) (Miller et al., 2007). The concentrations of NO_3^- in the vacuole and cytoplasm are typically within the ranges of 30 to 50 mol m^{-3} and 3 to 5 mol m^{-3} , respectively (Martinoia et al., 2000), and the fraction stored in the vacuole cannot be metabolized by plants unless it is initially allocated to the surrounding cytoplasm. The vacuole is accordingly the main storage site of NO_3^- , and thus the role of this vacuolar fraction in NO_3^- distribution should not be overlooked in studies on plant NO_3^- contents (Martinoia et al., 1981; Huang et al., 2012; Han et al., 2015).

In general, the assimilatory power of leaf cell cytoplasm is sufficient with respect to reducing NO_3^- concentrations. As long

as NO_3^- remains distributed outside the vacuole, it can be rapidly assimilated, and therefore the content of NO_3^- in plants is largely determined by the ratio of the distribution of NO_3^- within and outside the vacuole (Shen et al., 2003; Huang et al., 2012; Han et al., 2016). Changes in the relative size of these two NO_3^- reservoirs are mediated by the $2\text{NO}_3^-/\text{H}^+$ exchange channel CLCa in the tonoplast (Krebs et al., 2010; Liao et al., 2019) and is dependent on the activity of the vacuolar H^+ -ATPase (V-ATPase) and the vacuolar H^+ -pyrophosphatase (V-PPase) proton pumps. The combined activity of these vacuolar proton pumps, utilizing MgATP and MgPPi as substrates, respectively, establishes an electrochemical H^+ gradient across the tonoplast that drives the transport of NO_3^- against its electrochemical potential (Martinoia et al., 2007; Lin et al., 2012) (**Figure 1A**).

These two proton pumps are among the most abundant tonoplast proteins, thereby indicating the considerable amounts of energy expended in vacuolar transport (Carter et al., 2004; Jaquinod et al., 2007). V-ATPases are highly conserved, multi-subunit proton pumps that consist of two sub-complexes, the peripheral V_1 and membrane-integral V_0 complexes. The peripheral V_1 complex comprises eight subunits (VHA-A to -H), which are exposed on the cytoplasmic side of the vacuolar membrane and are responsible for ATP hydrolysis. The membrane-integral V_0 complex, which consists of six subunits (VHA-a, -c, -c', c'', -d, and -e), is integrated within the membrane and functions as a channel for the translocation of protons from the cytoplasm into the lumen of endomembrane compartments and also serves as the binding site for the polymerization and assembly of V_1 subunits (Cipriano et al., 2008; Luo, 2012). Compared with tonoplast H^+ -ATPase, the H^+ -PPase proton pump, which is widely distributed in plants, a few algae, protozoa, and bacteria, has a simple structure comprising an oligopeptide chain and accounts for approximately 1 to 10% of membrane proteins (Zhao and Liu, 1999; Maeshima, 2000).

V-ATPase and V-PPase not only serve as key tonoplast proton pump enzymes but also function as determinants of NO_3^- re-utilization (Shen et al., 2003). From the perspective of reducing NO_3^- accumulation in plant vacuoles and further improving the nitrogen-utilization efficiency of crops, it is of considerable significance to elucidate the short-distance distribution mechanism of NO_3^- associated with tonoplast proton pump activity (Han et al., 2014). In a study examining two varieties of rapeseed seedlings with high and low nitrogen-use efficiency, respectively, Han et al. (2015) revealed that V-ATPase and V-PPase activities and NO_3^- internal flow rate into the vacuole in the highly efficient variety were lower compared with those in the variety with low nitrogen-use efficiency. This indicates that in the former, larger amounts of NO_3^- are assigned to the cytoplasm for subsequent metabolism, thereby reducing the vacuolar content of NO_3^- and consequently enhancing the nitrogen-use efficiency of rapeseed (Han et al., 2016). The findings of a further study have indicated that the storage capacity for NO_3^- is limited in the absence of the *AtVHA-a2* and *AtVHA-a3* genes, with the *vha-a2 vha-a3* double mutant being found to contain 80% less NO_3^- , whereas the total NO_3^- reductase activity had increased by 90% (Krebs et al., 2010). Similarly, it has been observed that the vacuoles of *atvha-a2*, *atvha-a3*, and *atavp1* mutants contain less NO_3^- (Liao et al., 2019).

Driven by vacuolar proton pumps, NO_3^- enters the vacuole *via* chloride channel proteins (Gaxiola et al., 2001; De Angeli et al., 2006; Brüx et al., 2008). Chloride and malic acid channels, two main types of anion channels, are inward rectifying channels located in the tonoplast (Qu and Yu, 2008). CLC proteins facilitate the flux of anions such as Cl^- and NO_3^- , whereas glutamate transport is limited. CLC proteins are activated only in the presence of cytoplasmic Ca^{2+} (Berechi et al., 1999). This activation is promoted by calmodulin domain protein kinase

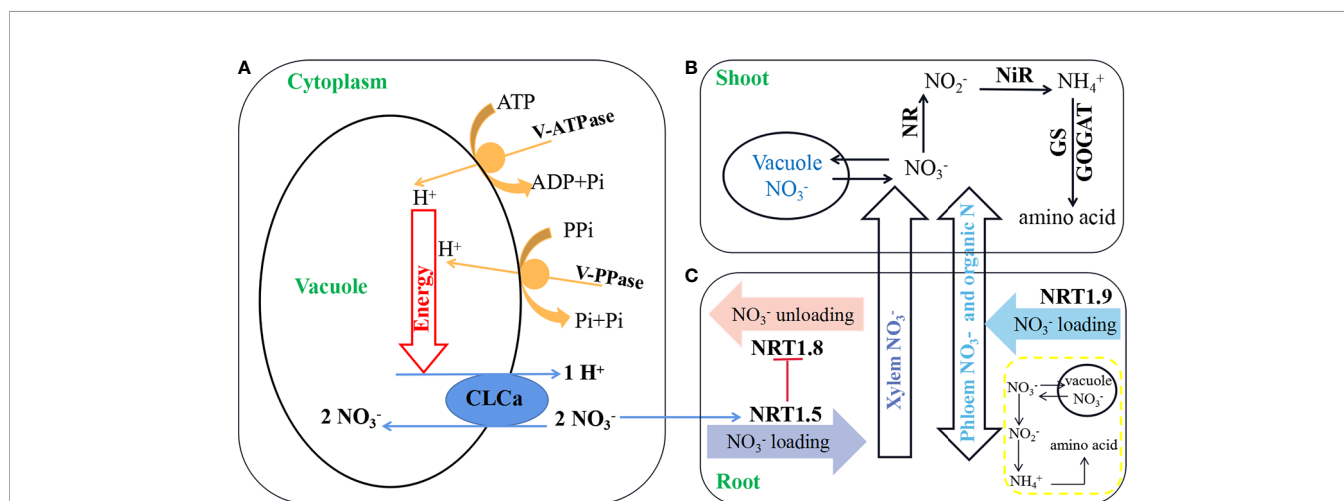


FIGURE 1 | A model of short-distance distribution and long-distance transport of NO_3^- in plants. Modified from Zhang (2017). **(A)** Accumulation of NO_3^- in the vacuole is regulated by chloride channels (CLCa) and relies on an H^+ gradient established by V-ATPase and V-PPase. **(B)** Short-distance distribution of NO_3^- and reactions catalyzed by NO_3^- reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) in shoots. **(C)** Long-distance transport of NO_3^- from roots to shoots is mainly regulated by *NPF7.3*, *NPF7.2*, and *NPF2.9* genes.

(CDPK) in the presence of ATP and inhibited by niflumic acid. Unlike general anion channels, CLCs are not inhibited by 4,4'-dinitrostilbene-2,2'-disulfonic acid (White and Broadley, 2001; Yu and Liu, 2004; Qu et al., 2009).

In *Arabidopsis*, NRT2.7 and CLCa are two transporters that facilitate the transfer of NO_3^- into the vacuole (Geelen et al., 2000; Chopin et al., 2007). Other transporters with potentially similar functions include CLCc and NPF6.2 (Chiu et al., 2004; Harada et al., 2004). Seven CLC family members have been identified in *Arabidopsis thaliana*, and De Angeli and colleagues (2006) were the first to demonstrate that AtCLCa is a tonoplast-localized $2\text{NO}_3^-/\text{H}^+$ antiporter involved in the regulation of NO_3^- sequestration into vacuoles. It has been found that the accumulation of NO_3^- in the vacuole is reduced by approximately 50% and that the contents of NO_3^- in both shoots and roots are reduced in *atclca* mutant plants (Geelen et al., 2000; De Angeli et al., 2006; Monachello et al., 2009). Similar results were obtained in a study by Liao et al. (2019). AtCLCc is also assumed to participate in vacuolar NO_3^- storage, given that it is tonoplast localized, and plants with a mutation in this proton are characterized by lower NO_3^- contents (Harada et al., 2004). Among the NPF proteins in *Arabidopsis*, AtNPF6.2 is specifically expressed in NO_3^- -rich petioles, thereby indicating that it might participate in the accumulation of NO_3^- in petiole cell vacuoles. However, the function of AtNPF6.2 has yet to be conclusively established owing to a lack of evidence to indicate whether this protein is located in the vacuolar membrane (Chiu et al., 2004). AtNRT2.7, which is located in the tonoplast and expressed exclusively in seeds, is, to date, the only protein in the NRT family confirmed to participate in the vacuolar storage of NO_3^- . Within seeds, this protein is responsible for the transport of NO_3^- from the cytoplasm into the vacuole for storage, thereby contributing to seed nitrogen accumulation (Chopin et al., 2007). Compared with NPF and NRT2 family proteins, CLC proteins are primarily responsible for vacuolar NO_3^- accumulation during plant growth, and a reduction in their activity enhances the utilization of NO_3^- , thereby improving the nitrogen-use efficiency of crops (Geelen et al., 2000; De Angeli et al., 2006; Monachello et al., 2009).

With respect to the efflux of NO_3^- from vacuoles, it has been established that the tonoplast-localized proteins AtCLCb and OsNPF7.2 could transport NO_3^- , although there is currently no direct evidence to indicate whether these proteins are involved in vacuolar NO_3^- influx or efflux (von der Fecht-Bartenbach et al., 2010; Hu et al., 2016). AtNPF5.11, AtNPF5.12, and AtNPF5.16, regulate NO_3^- re-allocation between roots and shoots by mediating NO_3^- efflux from the vacuole to the cytosol. In triple mutants characterized by disruption of *AtNPF5.11*, *AtNPF5.12*, and *AtNPF5.16*, it has been found that larger amounts of root-fed $^{15}\text{NO}_3^-$ are translocated to the shoots (He et al., 2017). Moreover, it is speculated that the tonoplast-localized OsNPF7.2 could also be involved in NO_3^- efflux from the vacuoles, and heterologous expression in *Xenopus laevis* oocytes has indicated that it mediates NO_3^- absorption, although, to date, it has yet to be demonstrated whether functional disruption of *OsNPF7.2* leads to an accumulation of

NO_3^- within the vacuoles (von der Fecht-Bartenbach et al., 2010; Hu et al., 2016). Furthermore, NO_3^- supply has a significant effect on NO_3^- distribution, both in the metabolic pool and the storage pool of leaf blades in three leafy vegetables (rapeseed, Chinese cabbage, and spinach) (Chen et al., 2004).

The $2\text{NO}_3^-/\text{H}^+$ antiporter located in the tonoplast assists in the transfer of NO_3^- into the vacuole from the cytoplasm (De Angeli et al., 2006; Krebs et al., 2010). From the perspective of nitrogen-use efficiency, the role of vacuolar NO_3^- in maintaining an osmotic balance within the vacuole is considered wasteful (Shen et al., 2003). However, whether it would be feasible to replace vacuolar NO_3^- with Cl^- to maintain cell osmotic pressure within a certain range, and thereby reduce the level of NO_3^- accumulated in the vacuole, remains to be ascertained.

LONG-DISTANCE (ROOT TO SHOOT) NO_3^- TRANSPORT IN VEGETABLES

A proportion of the NO_3^- absorbed by plant roots is stored within vacuoles or assimilated into organic nitrogen through a series of reactions catalyzed by NO_3^- reductase, nitrite reductase, glutamine synthetase, and glutamate synthase (Williams et al., 1987) (Figure 1C). However, most of the NO_3^- taken up by roots is transported through cortical tissues into xylem conduits for long-distance transport to different shoot tissues and organs for subsequent assimilation and utilization in plant growth and development (Han et al., 2016; Zhang, 2017) (Figure 1B).

NO_3^- is primarily transported upward by the xylem and downward by the phloem (Dechorgnat et al., 2011). The long-distance transport and distribution of NO_3^- between roots and shoots in *Arabidopsis* are mainly co-regulated by *NPF7.3* and *NPF7.2*, two members of the PTR family, the expression of which is strongly induced by NO_3^- (Lin et al., 2008; Li et al., 2010; Chen et al., 2012; Leran et al., 2014; Zhang Z. H. et al., 2014) (Figure 1C). *AtNPF7.3* is predominantly expressed in the columnar sheath cells surrounding the protoxylem in the root and is responsible for the loading of NO_3^- from the root cytoplasm into the xylem for transport to the shoots (Lin et al., 2008). *AtNPF7.2* is expressed in xylem parenchyma cells and affects NO_3^- transport from roots to shoots by regulating NO_3^- unloading from the xylem (Li et al., 2010). Thus, although playing opposing roles in the loading and unloading of NO_3^- into and out of the xylem, *AtNPF7.3* and *AtNPF7.2*, respectively, cooperatively contribute to the long-distance transport of NO_3^- from the roots to the shoots. Furthermore, *OsNPF2.2* has been shown to be involved in unloading NO_3^- from the xylem, thereby facilitating root to shoot NO_3^- transport and plant development. *osnfp2.2* mutant plants have been found to maintain high levels of NO_3^- in the roots and have a low shoot:root NO_3^- ratio (Li et al., 2015). Moreover, it has been observed that *AtNPF2.3* plays an important role in root to shoot NO_3^- translocation in plants subjected to saline conditions (Taochy et al., 2015).

Currently, *AtNPF2.9* is the only gene that has been confirmed to play a role in the loading of NO_3^- into root phloem and

negatively regulates the root to shoot transport of NO_3^- (Fan et al., 2009; Wang and Tsay, 2011). It has also been established that *AtNPF5.11*, *AtNPF5.12*, and *AtNPF5.16* play roles in the uptake of NO_3^- from the vacuole to the cytoplasm in *Arabidopsis* to regulate the shoot:root ratio, and in a triple mutant for these genes, larger amounts of NO_3^- were found to be translocated to the shoots (He et al., 2017). In *Arabidopsis*, *AtNPF2.9*, *AtNPF7.2*, and *AtNPF7.3* are the main NO_3^- transporters contributing to the regulation of the long-distance transport and redistribution of NO_3^- from roots to shoots (Figure 1C). In this regard, it is worth noting that the concentration of NO_3^- transported in the xylem is typically several tens to hundreds of times higher than that carried in the phloem, which accordingly explains why studies on NO_3^- distribution in shoots and roots have focused primarily on NO_3^- transport in the xylem rather than in the phloem (Lin et al., 2008; Li et al., 2010; Wang and Tsay, 2011). To date, research on the relationships between NPF genes and NO_3^- long-distance transport in crop plants has mainly been conducted on wheat, rice, and rapeseed. However, only the responses of the *NPF7.3* and *NPF7.2* genes to nitrogen stress have been studied in wheat (Xuan et al., 2014), whereas the physiological characteristics of nitrogen have been analyzed in rice lines overexpressing *OsNPF7.3* (Ma, 2011). In our studies, we have analyzed the response of *NPF7.3* and *NPF7.2* to NO_3^- deficiency and constructed a co-expression network to identify key genes involved in NO_3^- transport from root to shoot in rapeseed seedlings (Hua et al., 2018; Liang et al., 2019). Evidence that has accumulated to date essentially confirms that *NPF7.3* and *NPF7.2* regulate NO_3^- transport and distribution in a range of crop plants, in which they play roles similar to those observed in *A. thaliana*. We thus believe that the relationships between NO_3^- long-distance transport and NPF genes described in *Arabidopsis* would also be applicable to cultivated crops or vegetables.

Xylem flow is known to be strongly influenced by transpiration, which in turn has a considerable effect on the long-distance transport of NO_3^- . NO_3^- loaded into the xylem is continuously transported to the above-ground parts of plants, mediated through the influence of the transpirational pull, thereby promoting the transport of NO_3^- from the roots to shoots (Li et al., 2010; Chen et al., 2012). Consistently, it has been demonstrated that the inhibition of transpiration reduces the transport of NO_3^- to the shoots in pea plants, thus resulting in an increase in the accumulation of NO_3^- in the roots and a reduction in contents in the edible parts of plants (Hernandez et al., 1997). The long-distance transport of NO_3^- not only determines the distribution and assimilation in different tissues, but also represents an important physiological mechanism whereby plants respond to environmental change (Zhang G. B. et al., 2014). Chen et al. (2012) and Li et al. (2010) have shown that transpiration regulates the transport of nutrient elements from roots to shoots under normal conditions, whereas NO_3^- accumulation in roots, which is also mediated by *AtNPF7.3* and *AtNPF7.2*, might be the dominant regulatory factor in NO_3^- distribution in plants exposed to stress. For example, under conditions of cadmium stress, wild-type *Arabidopsis*

plants have been observed to maintain a higher root to shoot NO_3^- ratio than *atnfp7.2* mutant plants, indicating that this ratio is actively regulated by *NPF7.2* and other transporters, rather than passively by transpiration (Li et al., 2010). Therefore, by inhibiting the expression of the *NPF7.3* gene and enhancing that of *NPF7.2* in plants, it might be possible to control the long-distance transport of NO_3^- from roots to shoots, which would represent a useful approach for reducing the content of NO_3^- in leafy vegetables.

CONCLUSIONS AND FUTURE DEVELOPMENTS

In plants, NO_3^- accumulation depends on its absorption and metabolism. Plants actively take up NO_3^- from the environment through a proton/nitrate-coupled mechanism (Wang et al., 2018). The NO_3^- absorbed by plants can be directly assimilated into organic nitrogen, secreted into the rhizosphere, accumulated in the vacuole, or transported from roots to shoots (Zhang, 2017). The concentrations of NO_3^- in plants are thus affected by multiple processes, among which there is a close interdependency that facilitates the coordinated regulation of NO_3^- accumulation in plants.

Vacuolar NO_3^- accumulation and release play important roles in regulating the concentration and re-allocation of NO_3^- . The influx of vacuolar NO_3^- is mainly affected by the tonoplast-localized proteins, such as *AtCLCa*, *AtCLCc*, *AtNPF6.2*, and *AtNRT2.7*, whereas *AtCLCb* and *OsNPF7.2* are believed to play transporting roles, although there is currently no direct evidence to indicate whether these proteins are involved in vacuolar NO_3^- influx or efflux (von der Fecht-Bartenbach et al., 2010; Hu et al., 2016). Furthermore, *AtNPF5.11*, *AtNPF5.12*, and *AtNPF5.16* regulate NO_3^- re-allocation between roots and shoots by mediating NO_3^- efflux from the vacuole to the cytosol (He et al., 2017). However, the main factors regulating the short-distance of NO_3^- are the tonoplast CLC proteins and proton pump transport systems (Chiu et al., 2004; Chopin et al., 2007). The NO_3^- transport system is present in the vacuolar membrane in all plant tissues and represents the main process whereby short-distance NO_3^- distribution is regulated (De Angeli et al., 2006; Krebs et al., 2010). By inhibiting the activity of both tonoplast proton pumps and the CLC proteins, a larger proportion of plant NO_3^- could be re-distributed in the cytoplasm, wherein it can subsequently be metabolized, thereby contributing to a reduction in the NO_3^- content of vegetables.

The vacuolar-cytoplasmic distribution of NO_3^- affects not only the distribution and utilization of NO_3^- (Gaxiola et al., 2001; Brüx et al., 2008; Krebs et al., 2010), but also affects the expression and function of genes induced by NO_3^- (Glass et al., 2002; Huang et al., 2013). *NPF2.9*, *NPF7.3*, and *NPF7.2*, which are responsible for NO_3^- loading in the phloem, loading in the xylem, and unloading from the xylem, respectively, are the main regulators of long-distance NO_3^- transport. However, the phloem is primarily involved in the transport of organic nitrogen, whereas the concentration of inorganic nitrogen

carried in the phloem is typically in the order of tens to hundreds of times smaller than that in the xylem (Lin et al., 2008; Fan et al., 2009; Li et al., 2010; Wang and Tsay, 2011). Therefore, NPF7.3 and NPF7.2 are the main regulators of the long-distance transport of NO_3^- . Enhancing the activity of the NPF7.2 protein in conjunction with a reduction in the activity of the NPF7.3 protein would contribute to favorably regulating the long-distance transport of NO_3^- , thus reducing the transport of NO_3^- from roots to shoots, and thereby the contents of NO_3^- in leafy vegetables.

Research on mechanisms underlying the short- and long-distance translocation of NO_3^- needs to be further expanded to address the following questions (Li et al., 2010; Schroeder et al., 2013). (1) In addition to regulating osmotic functions, how does the storage of NO_3^- in vacuoles affect the expression and function of its inducible genes? (2) What is the difference between the contribution of chloride channels (CLC) and proton pumps with respect to the distribution of NO_3^- in vacuoles? (3) Given that the transport of NO_3^- from cytoplasm to vacuoles is primarily regulated by CLCa, how does this process affect the further assimilation of NO_3^- ? On the basis of the findings of previous studies, the vacuolar NO_3^- distribution system, which plays an important role in the short-distance NO_3^- distribution, and the vascular bundle long-distance transportation system, which is important in long-distance

NO_3^- transportation, are identified as primary targets to further studies on mechanisms underlying the short-distance distribution and long-distance transport of NO_3^- . Gaining a better understanding of these mechanisms will contribute to facilitating a more effective control of NO_3^- contents in plants and provide important guidance for the breeding and cultivation of leafy vegetables with low NO_3^- concentrations.

AUTHOR CONTRIBUTIONS

GL organized and wrote the original manuscript. GL and ZZ discussed and revised the manuscript and approved the final version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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