frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 30 July 2014
doi: 10.3389/fpls.2014.00360

Nitrate transporters in leaves and their potential roles in foliar uptake of nitrogen dioxide†

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† *This paper was dedicated to Professor Hartmut K. Lichtenthaler on the occasion of his 80th birthday.*

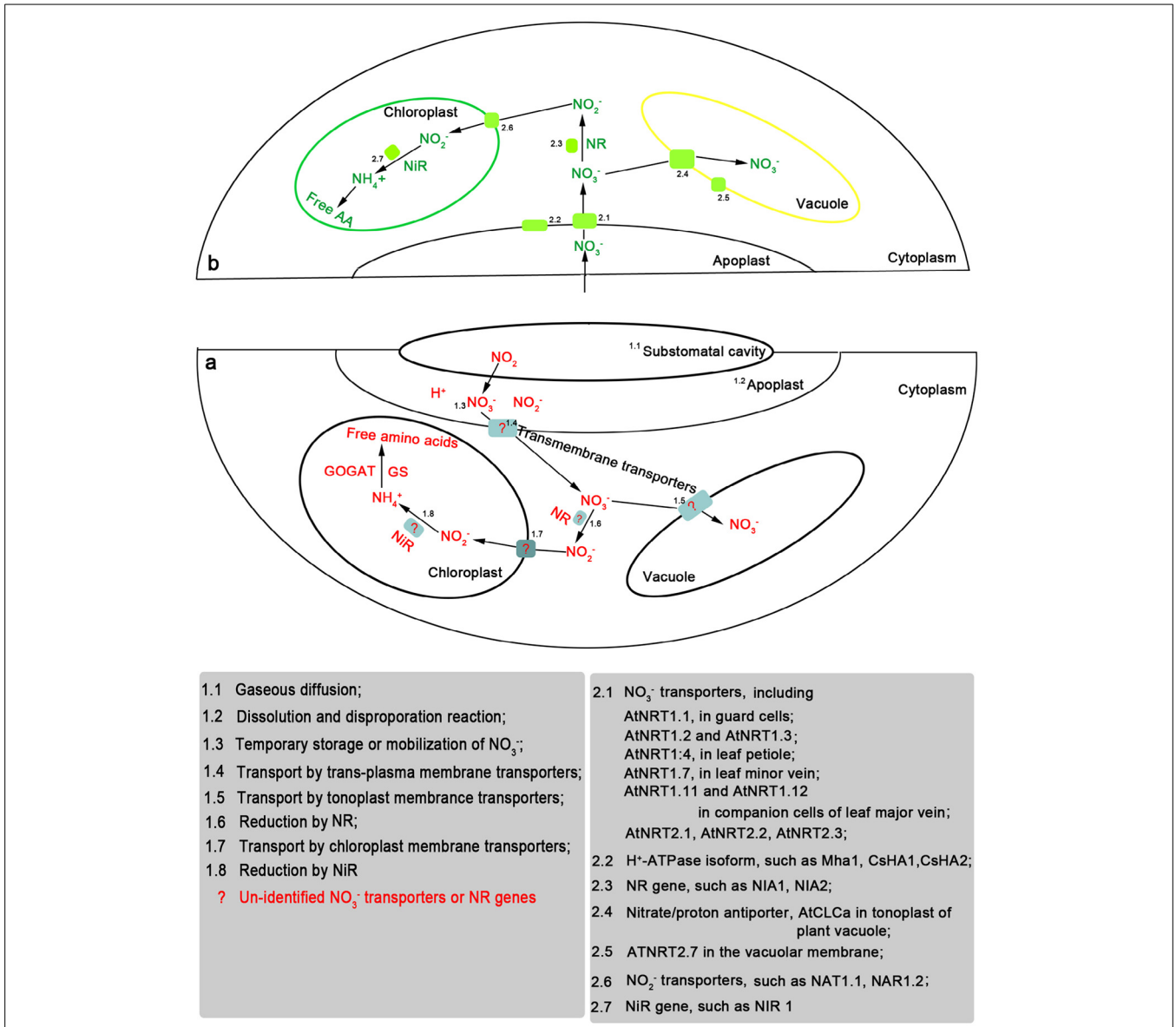
While plant roots are specialized organs for the uptake and transport of water and nutrients, the absorption of gaseous or liquid mineral elements by aerial plant parts has been recognized since more than one century. Nitrogen (N) is an essential macronutrient which generally absorbed either as nitrate (NO_3^-) or ammonium (NH_4^+) by plant roots. Gaseous nitrogen pollutants like N dioxide (NO_2) can also be absorbed by plant surfaces and assimilated via the NO_3^- assimilation pathway. The subsequent NO_3^- flux may induce or repress the expression of various NO_3^--responsive genes encoding for instance, the transmembrane transporters, NO_3^-/NO_2^- (nitrite) reductase, or assimilatory enzymes involved in N metabolism. Based on the existing information, the aim of this review was to theoretically analyze the potential link between foliar NO_2 absorption and N transport and metabolism. For such purpose, an overview of the state of knowledge on the NO_3^- transporter genes identified in leaves or shoots of various species and their roles for NO_3^- transport across the tonoplast and plasma membrane, in addition to the process of phloem loading is briefly provided. It is assumed that a NO_2-induced accumulation of NO_3^-/NO_2^- may alter the expression of such genes, hence linking transmembrane NO_3^- transporters and foliar uptake of NO_2. It is likely that *NRT1/NRT2* gene expression and species-dependent apoplastic buffer capacity may be also related to the species-specific foliar NO_2 uptake process. It is concluded that further work focusing on the expression of *NRT1* (*NRT1.1*, *NRT1.7*, *NRT1.11*, *and NRT1.12*), *NRT2* (*NRT2.1*, *NRT2.4*, and *NRT2.5*) and chloride channel family genes (*CLCa* and *CLCd*) may help us elucidate the physiological and metabolic response of plants fumigated with NO_2.

Keywords: chloride channel gene, nitrate reductase, nitrate transporter, nitrogen dioxide, signal transmission

INTRODUCTION

Nitrate (NO_3^-) is the most common form of nitrogen used by plants for growth and development (Bertoni, 2012). Despite the major role of plant roots is absorbing and transporting water and mineral elements, there is abundant evidence showing that nutrients can also be taken up by aerial plant parts (e.g., leaves, fruits and stems) (Eichert and Fernández, 2012; Fernández and Brown, 2013). Foliar applied NO_3^- may be absorbed and assimilated efficiently as shown in several studies carried out with different plant species (e.g., Stiegler et al., 2011; Uscola et al., 2014). Gaseous air pollutants like nitrogen dioxide (NO_2) can also be deposited into plant leaves and be taken up mainly through stomata (Eichert and Fernández, 2012). NO_2 molecules may dissolve in the aqueous phase of the apoplastic space being consequently transformed into nitrate (NO_3^-) and/or nitrite (NO_2^-) by chemical reactions. Thereafter, NO_2-derived NO_3^- may be transported across the plasma membrane by NO_3^- transporters and reach the cytoplasm for further incorporation into cellular N-compounds and/or storage in vacuoles (Hawkesford et al., 2012). The NO_3^- stored in the vacuoles may be exported to compensate for the consumption of NO_3^- in the metabolic pool (De Angeli et al., 2006), which suggests that vacuolar NO_3^- may largely serve as N buffer for transport processes (Hawkesford et al., 2012). NO_3^-/proton antiporters, which may be encoded by chloride channel family (CLC) genes, are responsible for NO_3^- influx into plant vacuoles (Geelen et al., 2000). NO_3^- transmembrane transporters may be expected to play a role after the uptake of exogenous NO_2 or NO_3^- by the foliage. Several NO_3^- transporters identified in leaves have been demonstrated to be closely correlated with e.g., stomatal opening (Guo et al., 2003), NO_3^- reductase activity (Loqué et al., 2003), accumulation and remobilization of NO_3^- (De Angeli et al., 2006; Fan et al., 2009; Lv et al., 2009). Thereby, such physiological processes may significantly influence and also be affected by the foliar uptake of NO_2 or NO_3^-.

Recently some NO_3^- transporter genes were detected in leaves including several members of plant *NRT1* family genes (e.g., *AtNRT1.1*, *AtNRT1.4*, *AtNRT1.7*, *AtNRT1.11*, and *AtNRT1.12*,.), *NRT2* family genes (e.g., *AtNRT2.1*, *AtNRT2.3*, *AtNRT2.4*, *AtNRT2.5*, *AtNRT2.6*, *AtNRT2.7*, *NpNRT2.1* and *ZmNrt2.1*.), and CLC family genes such as *AtClCa* and *AtClCd* (Orsel et al., 2002; Guo et al., 2003; Chopin et al., 2007; Fan et al., 2009; Hsu and Tsay, 2013) (**Figure 1**). These genes show various expression levels



1.1 Gaseous diffusion;
1.2 Dissolution and disproporation reaction;
1.3 Temporary storage or mobilization of NO_3^-;
1.4 Transport by trans-plasma membrane transporters;
1.5 Transport by tonoplast membrance transporters;
1.6 Reduction by NR;
1.7 Transport by chloroplast membrane transporters;
1.8 Reduction by NiR
? Un-identified NO_3^- transporters or NR genes

2.1 NO_3^- transporters, including
AtNRT1.1, in guard cells;
AtNRT1.2 and AtNRT1.3;
AtNRT1:4, in leaf petiole;
AtNRT1.7, in leaf minor vein;
AtNRT1.11 and AtNRT1.12
in companion cells of leaf major vein;
AtNRT2.1, AtNRT2.2, AtNRT2.3;
2.2 H^+-ATPase isoform, such as Mha1, CsHA1,CsHA2;
2.3 NR gene, such as NIA1, NIA2;
2.4 Nitrate/proton antiporter, AtCLCa in tonoplast of plant vacuole;
2.5 ATNRT2.7 in the vacuolar membrane;
2.6 NO_2^- transporters, such as NAT1.1, NAR1.2;
2.7 NiR gene, such as NIR 1

FIGURE 1 | Nitrate (NO_3^-) transporters and NR/NiR genes potentially involved after foliar uptake and assimilation of exogenous nitrogen dioxide (NO_2) (a)/ NO_3^- (b).

in leaves and play diverse roles in regulating NO_3^- metabolism (Hawkesford et al., 2012). For example, *AtNRT1.1*, a dual-affinity NO_3^--inducible transporter, showed a strong expression in guard cells and supports the stomatal function in the presence of NO_3^- (Guo et al., 2003). *McNRT1*, *LeNRT1.1*, and *NpNRT1.1* were detected in the leaves of *M. crystallinum*, tomato and *Nicotiana plumbaginifolia*, respectively. *McNRT1*, sharing 60% homology with the *AtNRT1.1*, was expressed in the mesophyll cells and cells adjacent to metaxylem vessels in the leaves (Popova et al., 2003). The gene plays potential roles in NO_3^- uptake in the mesophyll cells, distribution and partitioning of NO_3^- within the leaves. Moreover, *AtNRT1.4* and *AtNRT1.7* are of pure low-affinity transporters. *AtNRT1.4* was expressed primarily in the leaf petiole (Chiu et al., 2004). *NRT1.7* mRNA was detected in the distal lamina of older leaves, but not in the roots (Fan et al., 2009). The two transporter genes participate in the process of leaf NO_3^- storage and remobilization. For the members of the *NRT2* family, the amounts of *AtNRT2.4* transcripts were predominant in leaves of the adult plants, followed by *AtNRT2.5*; the expression of *AtNRT2.1*, *AtNRT2.6*, and *AtNRT2.7* were at low levels (Orsel et al., 2002). *AtNRT2.1*, *AtNRT2.3*, *AtNRT2.4*, and *AtNRT2.5* are NO_3^--responsive genes, whereas *AtNRT2.6* and *AtNRT2.7* appear to be constitutive genes (Loqué et al., 2003). Particularly, *AtNRT2.7* showed a strong leaf- and seed-specific expression pattern (Orsel et al., 2002; Chopin et al., 2007), while *AtNRT2.3* was specifically expressed in leaves at a reproductive stage.

Previous studies on foliar uptake of NO_2 mainly focused on the deposition pathways, metabolic processes associated with NO_2-derived NO_3^- (Hu, 2011; Hu et al., 2014), and downstream products of NO_2-N assimilation (Nussbaum et al., 1993; Weber et al., 1995). The current state of knowledge on the potential plant responses to NO_2 exposure is summarized in **Table 1**. However, the relationship between NO_3^- transmembrane transporters and foliar NO_2 uptake has received only limited scientific attention so far. Foliar uptake of NO_2 seems to be species-specific and concentration-dependent (Hu and Sun, 2010). Expression of the genes encoding leaf NO_3^- transporters also appears to be species-specific (Ono et al., 2000; Orsel et al., 2002). The contribution of various expression patterns of transporter genes to species-specific NO_2 uptake is currently unknown. Given the N transport mechanisms described above, a potential relationship between foliar NO_2 uptake (substomatal build-up of NO_2 and the subsequent reduction, storage, and remobilization of NO_3^-) and NO_3^--responsive genes encoding the transmembrane transporters and NO_3^-/NO_2^- reductases may be hypothesized. For validating such hypothesis, future work focusing on the relationship between organ-specific expression of NRT1/NRT2 genes and species-specific NO_2 uptake should be carried out.

SUBSTOMATAL BUILD-UP OF NO_2 MAY DISTURB APOPLASTIC pH AND NO_3^- TRANSPORTERS

The apoplast is defined as the area within the plant tissues which is beyond the cell plasma membrane, and includes the cell wall, middle lamella, xylem and gas and water filled intercellular spaces (Sattelmacher, 2001). The leaf apoplastic space plays a role in ion exchange and as a diffusion barrier (Sattelmacher, 2001). Estimates of the volume of leaf water in the apoplast vary from 10 to 35% of total leaf water (Speer and Kaiser, 1991; Wardlaw, 2005). Dissolution of NO_2 in the apoplast may produce H^+ and NO_3^-/NO_2^-. Foliar NO_2 uptake is calculated to yield at most 0.22 mol excess H^+ per mol N (Raven, 1988). Therefore, a build-up of NO_2 in the leaf substomatal cavities may lead to apoplastic pH disturbances. Among other factors, the resulting apoplastic pH changes will depend on NO_2 concentration, root N supply and plant N status. The supply of NO_3^- via the root system significantly increased the leaf apoplastic pH of *Phaseolus vulgaris* and *Helianthus annuus*, whereas the depletion of NO_3^- in nutrient solution led to lower leaf apoplastic pH values in *Zea mays* (Mühling and Lauchli, 2001). NH_4NO_3 nutrition did not change the leaf apoplastic pH in sunflower (Kosegarten et al., 1999). Moreover, foliar NH_4^+ fertilization may either lead to apoplastic alkalinization (Felle and Hanstein, 2002) or acidification (Mühling and Lauchli, 2001). When supplying NH_4C1 (1 mM) via the root to soybean plants, low concentrations of NO_2 (0.2–0.25 $\mu L \cdot L^{-1}$) significantly increased the leaf apoplastic pH (Qiao and Murray, 1997), whereas under a higher root NO_3^- dose (5 mM), high concentrations of NO_2 (1.1 $\mu l \cdot l^{-1}$) increased the acidity of the leaves (Qiao and Murray, 1998). Apoplastic pH is an important factor affecting plasmalemma proton pumps (Hoffmann et al., 1992; Sattelmacher, 2001). Apoplastic alkalinization or acidification may induce plasma membrane depolarization or hyperpolarization (Hedrich et al., 2001). This may further modulate the deactivation or activation of membrane-bound proton-transporting enzymes, and the corresponding ion channel regulation for co-transport of anions (Savchenko et al., 2000). Wippel et al. (2010) found that the fluctuation of apoplastic pH had a regulatory effect on plant sucrose transporters. Based on the above information, it can be reckoned that the apoplastic pH changes caused by NO_2 may repress or induce NO_3^--responsive genes encoding the transmembrane transporters.

Some NO_3^- transporter genes (such as *AtNRT1.1* and *ZmNrt2.1*) in leaves are NO_3^--inducible, while others such as *AtNRT2.5* are NO_3^--repressible (Okamoto et al., 2003). Low-affinity transporter systems (*NRT1* family) may significantly contribute to NO_3^- uptake at external NO_3^- concentrations above 250 μM. However, high-affinity transporters (*NRT2* family) including the constitutive (cHATS Km = 6–20 mM) and inducible HATS (Km = 20–100 mM), are active at low external concentrations of 0–0.5 mM (Crawford and Glass, 1998; Quaggiotti et al., 2003; Hawkesford et al., 2012). When analyzing the *AtNRT1.7* NO_3^- transporter gene *in Arabidopsis*, Fan et al. (2009) applied 50 mM $K^{15}NO_3$ to carry out measurements on distal parts of the rosette leaf. The ^{15}N-NO_3^- tracing assay showed that the percentage of total ^{15}N in the leaves ranged from 0 to 10% for wild-type plants, and between 5 and 15% for the *nrt1.7* mutants. The percentage of NO_3^-–^{15}N was in the range of the NO_2-derived reduced N of wild herbaceous plants (from 0.98 to 10.1%) and woody plants (0.15–12.7%) for the 217 taxa fumigated with 4.0 ± 0.1 $\mu mol \cdot mol^{-1}$ NO_2 (Morikawa et al., 1998). Accordingly, the content of NO_2-derived reduced N ranged from 0.25 to 5.72 mg $N \cdot g^{-1}$ dry weight for wild herbaceous plants, and 0.04–6.57 mg $N \cdot g^{-1}$ dry weight for woody plants. This comparison suggests that the amounts of NO_2-derived NO_3^- in leaves are in the range of the NO_3^- concentrations which may induce the two types of transporter systems (i.e., high and low affinity).

From the reasoning provided above, it can be reckoned that substomatal build-up of NO_2 may lead to concentration-dependent changes of apoplastic pH and NO_3^- concentration. Such pH fluctuations may influence NO_3^- transmembrane transport by the induction or repression of the transporters and transporter gene expression, and may provide some sort of feedback regulation on the uptake of NO_2 by the foliage. For example, apoplastic mesophyll signals have been recently found to induce rapid stomatal responses in *Commelina communis* (Fujita et al., 2013). In response to NO_2 fumigation, multiple physiological and metabolic responses may occur which could either ultimately favor or inhibit the process of symplastic N uptake (**Table 1**). The multi-responses of NO_3^- transporters to the substomatal build-up of NO_2 may partially contribute to species-specific NO_2 uptake, but future studies with different plant species shall be carried out for clarifying this complex issue.

NITRATE TRANSPORTERS ARE POSSIBLY INVOLVED IN THE REDUCTION AND ACCUMULATION OF NO_2-DERIVED NO_3^-

In leaf cytoplasm, NO_2-derived NO_3^- has at least two fates: (i) assimilation into amino acids, and (ii) accumulation in vacuole (Hawkesford et al., 2012). The metabolic pathway will depend on the external NO_3^- concentration and leaf N demand (Stulen et al., 1998). NO_2-derived NO_3^- will be assimilated mainly through the NO_3^- assimilation pathway (Morikawa et al., 1998). NO_3^-

Table 1 | Physiological and metabolic responses of plant organs to nitrogen dioxide (NO_2) exposure.

Plant organ	Action site	Physiological function of exogenous NO_2 on plants		References
		Low NO_2 concentration (e.g., 40–60 nl.l^{-1})	**High NO_2 concentration (e.g., 1–4 μl.l^{-1})**	
Leaf	Stomata	Stimulation on stomatal aperture and stomatal conductance[1.3]; Reduced stomatal density[1.2]	Stomatal closure and declined stomatal conductance[1.1]	[1.1]Qiao and Murray, 1998; [1.2]Siegwolf et al., 2001; [1.3]Takagi and Gyokusen, 2004
	Apoplast	Increase in the malondialdehyde (MDA) level and superoxide dismutase (SOD) at 0.5 μL.L^{-1} NO_2[2.1]	Acidity of apoplast[1.1]; Induced expression of germin-like proteins (RmGLP2) [2.2]; Decline in MDA content and SOD activity[2.3]; Decline in ASA[2.1]	[2.1]Ma et al., 2007; [2.2]Kondo et al., 2008; [2.3]Chen et al., 2010
	Chloroplast	Increase in NR, NiR[3.4], photosynthetic rate[3.5], and chlorophyll content, etc.	Decline in chlorophyll content, ratio of Fv/Fm[2.3], and apparent photosynthesis[3.1]; Accumulation of NO_3^- and NO_2^-[3.2] as well as increase in NR and NiR[3.2]; Inhibition of NR[3.3]	[3.1]Srivastava et al., 1974; [3.2]Yoneyama et al., 1979; [3.3]Hisamatsu et al., 1988; [3.4]Weber et al., 1995 [3.5]Schmutz et al., 1995
	Mitochondria/ Peroxisome		Inhibition of dark respiration and apparent photorespiration[3.1,4.2]; Protrusions from both plastids and mitochondria of *Phaseolus vulgaris* exposed to NO_2 (10 ml.l^{-1})[4.1]	[4.1]Dolzmann and Ullrich, 1966; [4.2]Carlson, 1983
	In developing or maturing leaves	Increased leaf area[1.2]; NO_2-N incorporation into free amino acids such as Glu, Asp and Gln[3.4;5.3]; Stimulation on cell proliferation and enlargement as well as up-regulation of the related genes, such as ARGOS, GRF5, and KLU[5.4]	NO_2-N incorporation into free amino acids such as glutamine, glutamic acid, γ-amino butyric acid and aspartic acid[5.1]; NO_2 led to swollen thylakoids and a reduction in the number of grana stacks[5.2]	[5.1]Yoneyama and Sasakawa, 1979; [5.2]Schiffgens-Gruber and Lutz, 1992 [5.3]Nussbaum et al., 1993; [5.4]Takahashi et al., 2014
Stems	Xylem	Enlarged width of xylem in the main stem of Poplar trees[3.5]	stem growth significantly decreased by NO_2 at 1.0 μl.l^{-1} [6.1]	[6.1]Eastham and Ormrod, 1986
	Phloem	NO_2-N incorporation into free amino acids of bark of Norway spruce[5.3]	NO_2-N incorporation into free amino acids such as serine, asparagine and glutamine[6.2]	[6.2]Wellburn, 1990
Roots		NO_2-N incorporation into free amino acids in Norway spruce roots[5.3]	Decrease in root/shoot ratio, dry matter production, concentration of soluble sugars in roots, root respiration of kidney bean plants [7.1] Decrease in root nitrate uptake in sunflower plants[7.2] and soybean plants[1.1], increase in the ammonium concentration in roots of soybean plants at 1.1 μl.l^{-1} NO_2[1.1]	[7.1]Ito et al., 1985; [7.2]Okano et al., 1985
Flowers		Acceleration of flowering time and increase in flower number[5.4;8.1]		[8.1]Takahashi et al., 2011;
Fruits		Increased fruit yield[8.1] or grain yield (the number and weight of grain) and protein stored (at NO_2 of 170 nl.1^{-1})[9.1]		[9.1]Murray et al., 1994

reductase (NR) is considered as a key rate-limiting enzyme of NO_2-N assimilation (Hawkesford et al., 2012). A linear correlation was found between NR activity, NO_2 concentration and amounts of N incorporated into amino acids (Sparks et al., 2001). However, high levels of NO_2 fumigation resulted into a loss of NR activity or a rapid inactivation of the leaf NR (Takeuchi et al., 1985; Hisamatsu et al., 1988), and NO_3^- accumulation (Ma et al., 2007). This down-regulation of the NR may be ascribed to at least one of the following phenomena: (i) a high NO_2 concentration will inhibit the activities of glutamine synthetase and glutamate synthase, which leads to NH_4^+ accumulation and subsequently brings about a loss of NR activity (Orebamjo and Stewart, 1975; Padidam et al., 1991), and (ii) a high NO_2-induced stomatal closure may lead to a rapid NR inactivation due to a low CO_2 availability. High NO_2 rapidly induced stomatal closure (Qiao and Murray, 1998). Stomatal closure may trigger a chain reaction wherein the lower CO_2 availability will lead to the subsequent leaf NR activity decrease (Kaiser and Forster, 1989). *NIA1* and *NIA2* genes encode the two isoforms of the NR apoprotein (Wilkinson and Crawford, 1993). Recent reports show a close relationship between the expression of *NIA1/NIA2* genes and *NRT1/NRT2* genes. Addition of external NO_3^- strongly induced the expression of genes encoding the NR (*AtNIA1* and *AtNIA2*) and the transmembrane transporters (*AtNRT1.1*, *AtNRT2.1*, *NpNRT2.1*; Fraisier et al., 2001; Jonassen et al., 2009). In contrast, high levels of external NO_3^- caused a down-regulation of *AtNRT1.1* and *AtNIA1* through a pathway of NO_2^--induced repression (Loqué et al., 2003). This down-regulation of the *AtNRT1.1* gene is associated with a decrease in the NO_3^- influx. Earlier studies showed that high NO_2 concentration fumigation under light or dark conditions resulted in leaf NO_2^- accumulation (Yoneyama and Sasakawa, 1979; Yu et al., 1988). Thus, we may assume that a high NO_2-caused NO_2^- accumulation may lead to a negative feedback regulation on leaf NO_2 uptake through the down-regulation of the *NRT1.1* gene and the subsequent repression of the NO_3^- influx. Moreover, studies on NR mutants showed that *AtNRT1.1*, *AtNRT1.7*, *NpNRT2.1*, and *AtNIA1* are up-regulated in NR-deficient mutants (*NIA2*- and/or *MoCo* biosynthesis-deficient mutants) (Lejay et al., 1999; Vidmar et al., 2000; Fan et al., 2009). Under NR-repressible or -deficient conditions, this up-regulation of the transporter genes may be beneficial to an exportation of excess NO_3^- in the leaf (Fan et al., 2009). Moreover, Jonassen et al. (2009) demonstrated that the bZIP transcription factors *HY5* and *HYH* regulate positively *NIA2* gene and negatively *NRT1.1* gene. However, *HY5* and *HYH* appear to be mediated by light but not by external NO_3^-.

Excess NO_3^- may be accumulated in leaf vacuole (Hawkesford et al., 2012). The H^+/NO_3^- antiport across tonoplast is responsible for NO_3^- influx and H^+/NO_3^- symport for NO_3^- efflux (**Figure 1**). The flux direction will depend on the requirements and conditions of the cell (Schumaker and Sze, 1987). Three members of the chloride channel family (CLC) genes *AtClCa* (De Angeli et al., 2006), *AtClCc* (Harada et al., 2004) and *AtClCd* (Lv et al., 2009) have been identified in the leaf tonoplast. De Angeli et al. (2009) demonstrated that adenosine triphosphate (ATP) induces a negative regulation on *AtCLCa* activity. NO_2 fumigation significantly increased ATP amounts of *Lolium perenne* and *Phleum pratense,* the amounts increasing with raising NO_2 concentrations (Wellburn et al., 1981). This may be due to the formation of free radicals in response to NO_2 fumigation, which may damage photosynthetic membranes and hence alter the proton gradients to which ATP formation is linked (Wellburn et al., 1981). Yoneyama and Sasakawa (1979) found that 8 ppm NO_2 fumigation under dark conditions resulted in NO_2^- accumulation in spinach leaves. High doses of NO_2^- resulted in the peroxidation of lipid constituents of chloroplastic membrane (Ezzine and Ghorbel, 2006). Chen et al. (2010) found that leaf uptake of NO_2 reduced the rate of photosynthesis and increased the malondialdehyde (MDA) concentration, may be due to a competition for nicotinamide adenine dinucleotide phosphate (NADPH) between the processes of NO_2^- reduction vs. carbon assimilation, and the generation of reactive oxygen species (ROS) (Sabaratnam and Gupat, 1988; Shimazaki et al., 1992).

TRANSMEMBRANE TRANSPORTERS IN LEAVES MEDIATING NO_3^- SIGNALING

NO_2 fumigation may significantly disrupt plant morphology and physiology by, for instance, changing the shoot to root ratio, stomatal, and gas exchange dynamics, or modifying root N uptake (Qiao and Murray, 1997, 1998; **Table 1**). The exposure of plants to NO_2 increased the total content of soluble free amino acids in leaves and shoots (Nussbaum et al., 1993). Most of the amino acids may be used locally for the synthesis of e.g., Chlorophyll and Rubisco during rapid vegetative growth, or be ultimately designated for e.g., filling pods (Imsande and Touraine, 1994). NO_3^- assimilation products (protein/nucleic acids and amino acids/amides) can also be transferred into roots under soil N deficit (Wellburn, 1990). Under a low NO_3^- supply, gaseous NO_2 may change the amino acid ratio of the xylem. For example, the amount of serine, asparagine and glutamine were high in the xylem of plants exposed to atmospheric NO_2, whereas arginine, cysteine, valine and lysine were high in the control plants (Wellburn, 1990; **Table 1**). Moreover, NO_2 treatment increased phloem transport of organic N and inhibited the rate of xylem N translocation.

NO_2-N metabolism and the mobilization of metabolic products will trigger various signaling pathways that regulate the physiological and metabolic processes. The dissolution of NO_2 and subsequent reduction can result in root NO_3^- uptake changes. The xylem is part of leaf apoplast (Felle and Hanstein, 2002). Thus, NO_2-caused apoplastic pH changes may serve as a signal to modify the uptake of NO_3^- via the root system. This NO_3^- signaling pathway has been explained by Qiao and Murray (1998). Moreover, NO_3^- reduction can produce malate (Touraine et al., 1988); the organic acid needs to be membrane transported to be loaded into the phloem. A transport of malate from leaves to roots can serve as another signal to control root uptake of NO_3^- (Touraine et al., 1992). This signaling pathway has been reported by Imsande and Touraine (1994). Tonoplast transport of malate plays an important role in physiological regulation in NO_3^- nutrition (Hawkesford et al., 2012). At the cellular level, NO_3^- accumulation led to increased expression of genes encoding organic acid synthesis (*PPC*, cytosolic PK, CS, *ICDH-1*) and accumulation of malate and a-oxoglutarat. In contrast, leaf

malate supply can inhibit *NIA* expression, affecting both the *NIA* transcript level and the activity (Müller et al., 2001).

NO_3^- itself may serve as a signaling molecule (Scheible et al., 1997). NO_3^- addition to the growing media can induce or repress the expression of various genes encoding e.g., NO_3^- transporters, NO_3^-/NO_2^- reductase, ferredoxin reductase, and the enzymes in the pentose phosphate pathway, or iron or sulfate transport and metabolism (Wang et al., 2003; Marschner, 2012). The expression of NO_3^--responsive genes [such as NADH-specific and NAD(P)H-bispecific NR genes] is dependent upon NO_3^- flux but not on the NO_3^- amount stored in the tissue (Gojon et al., 1991). Excess external NO_3^- may be stored in several vacuoles and recirculated after storage (Hawkesford et al., 2012). NO_3^- remobilization may occur among different organs, for instance, from older leaves to younger leaves during the vegetative stage or from leaves to seeds during the reproductive stage (Schiltz et al., 2005; Hawkesford et al., 2012). N remobilization is rate-limited by the transport of NO_3^- across tonoplast of vacuole, plasma membrane of mesophyll cell, plasma membrane of companion cell and sieve element, and phloem loading (Fan et al., 2009). CLC genes (*AtClCa*, *AtClCc*, and *AtClCd*) are required for the transport of NO_3^- across tonoplast in vacuole. Disruption of one of these genes will influence the flux of NO_3^- in vacuoles (De Angeli et al., 2006). *AtNRT2.4* showed a strong induction in a low NO_3^- provision. Orsel et al. (2004) suggested that *AtNRT2.4* and *AtNRT2.5* participate in the transport of NO_3^- from stored pools (vacuoles) to cytoplasm. Moreover, *AtNRT2.7* was also involved in this type of NO_3^- flux; this gene could play roles in leaf balance between the amount of NO_3^- used for assimilation and that re-absorbed for further transport (Orsel et al., 2002). Four NRT1 family genes (*AtNRT1.4, AtNRT1.7*, *AtNRT1.11*, and *AtNRT1.12*) participate in the phloem- and/or xylem-loading of NO_3^- (**Figure 1**). *AtNRT1.4* was expressed predominantly in the leaf petiole and involved in petiole NO_3^- accumulation (Chiu et al., 2004). The mutation of the *AtNRT1:4* resulted in significant changes of NO_3^- content in leaf petiole and the lamina. Furthermore, the deficiency of *AtNRT1.4* can alter leaf development. *NRT1.7* was expressed in the phloem of the leaf minor vein and mediated the remobilization of excess NO_3^- from older leaves to younger leaves (Fan et al., 2009). Compared with the wild-type plants, the *nrt1.7* null mutants accumulated a higher amount of NO_3^- in the older leaves and decreased the NO_3^- content of phloem exudates from older leaves. The newly identified NO_3^- transporters (NRT1.11 and *NRT1.12*) were expressed in the companion cells of the major vein (Hsu and Tsay, 2013). They play roles in xylem-to-phloem transfer for redistributing NO_3^- into developing leaves. Moreover, several NRT2 genes may also be involved in the N remobilization. For example, *ZmNrt2.1* plays a potential role in NO_3^- loading from the xylem or in its compartmentation (Quaggiotti et al., 2003).

CONCLUSION

The substomatal build-up of NO_2 and subsequent NO_3^- metabolism may lead to apoplastic alkalinization or acidification and to NO_3^-/NO_2^- concentration fluctuations in the leaf apoplast and symplast (e.g., cytoplasm and vacuole), which depend on NO_2 concentration and root N supply. These changes will cause complex responses of NO_3^--responsive genes encoding NO_3^- transporters and NO_3^-/NO_2^- reductase. For example, addition of external NO_3^- produced a strong induction on NR genes (such as *AtNIA1* and *AtNIA2*) and the transporter genes (such as *AtNRT1.1*, *AtNRT2.1*, and *NpNRT2.1*). However, excess NO_2^- significant inhibited the expression of *AtNRT1.1* and *AtNIA1*, and disturbed CLC family genes by regulating the generation of ATP. This down-regulation of the *NRT1.1* gene is associated with a decrease in NO_3^- influx. Moreover, *AtNRT2.4*, *AtNRT2.5*, and *AtNRT2.7* may participate in the transfer of NO_3^- from stored pools (vacuoles) to cytoplasm. *AtNRT1.4*, *AtNRT1.7*, *AtNRT1.11*, and *AtNRT1.12* are involved in the phloem- and/or xylem-loading of NO_3^-. Thus, these genes are suggested to play rate-limiting roles in foliar uptake of NO_2. Further work is proposed to investigate the relationship between organ specificity of NRT1/NRT2 gene expression and species-specific NO_2 uptake.

In practical terms, a high rate of low concentration NO_2 absorption by the foliage may be positive for preserving an adequate plant N status. However, a high NO_2 concentration may alter leaf apoplast chemistry, leading to the accumulation of NO_3^- and NO_2^-, and providing signals which may negatively affect plant N nutrition. These factors are however closely linked with leaf NO_3^- transporters and may also interact with the foliar uptake processes (e.g., by promoting stomatal closure). Thereby, a low NO_2 concentration may act as a positive regulation signal (Takahashi et al., 2014) by stimulating the leaf NO_3^- transporters, and enhancing NO_3^- transport and distribution. In contrast, a high NO_2 concentration in relation to a high rate of foliar NO_2 absorption, may repress the expression of NO_3^- transporters and enzymes, which may protect the cells or organelles from NO_2 damage.

AUTHOR CONTRIBUTIONS

Yanbo Hu organized and wrote the original manuscript; Yanbo Hu and Victoria Fernandez discussed and revised the revising manuscript and approved the final version; Ling Ma collected the information for **Table 1**, and went through the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from "Fundamental Research Funds for the Central Universities" (Grant No. 2572014CA24, DL10BB24) and "National Natural Science Foundation" (Grant No. 31300506).

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Conflict of Interest Statement: 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.

Received: 28 March 2014; accepted: 04 July 2014; published online: 30 July 2014.
*Citation: Hu Y, Fernández V and Ma L (2014) Nitrate transporters in leaves and their potential roles in foliar uptake of nitrogen dioxide. Front. Plant Sci. **5**:360. doi: 10.3389/fpls.2014.00360*
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science.