insciences journal
open access

Insciences Journal 2011, 1(2), 90-101; doi: 10.5640/insc.010290 ISSN 1664-171X

Review Paper

NO_2-drived NO_3^- metabolism in leaves

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Received: 11 April 2011 / in revised form: 01 June 2011 / Accepted: 10 June 2011 /
Published: 22 June 2011

Abstract: Atmospheric nitrogen dioxide (NO_2) can be dry/wet deposited into plant leaves mainly through stomata, with a small fraction of cuticle deposition. Plants in nature present a wide variation in NO_2 assimilation and the resistance. The ability of leaf NO_2 uptake and efficient remobilization of NO_2-derived NO_3^-/NO_2^- is important for normal plant growth and to withstand nitrogen deprivation. Moreover, leaf- and root-derived NO_3^- metabolisms have both links and differences. This review emphasizes on the fates of NO_2-drived NO_3^-, including i) the assimilation into amino acids, ii) accumulation in vacuoles, and iii) re-emission by NO_x (NO and/or NO_2), and discuss metabolic differences of NO_2-drived and root-derived NO_3^-. Special attention is drawn to NO_x evolution in apoplast and symplast of leaves and its control to stomatal dynamics. Moreover, further progress is proposed to get a better understanding of the dynamic uptake of NO_2 and NO_3^- transporters in leaves.

Keywords: nitrogen dioxide; nitrate reductase; nitrate metabolism; NO_x evolution; photorespiration; stomatal dynamics

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1. Introduction:

Deposition and re-emission of nitrogen dioxide (NO_2) in plant leaves is mainly through stomata. Stomatal traits such as stomatal density, dimension and conductance affect the gas exchange rates across leaf surfaces and the amount of NO_2-N incorporated into overall plant N-assimilation [33,46,17]. Small amounts of atmospheric NO_2 can be deposited on dry cuticles by irreversible adsorption or reaction with cuticle components, and/or deposited in water films of the cuticles [41].

NO_2 can also be removed from the atmosphere by rain and wash-out thereby depositing as NO_3^-/NO_2^- on cuticles. Although little is known about NO_2 flux via cuticles, some evidence has suggested the significance of cuticles in controlling NO_2 deposition and re-emission [22, 16]. In recent years, research has been especially focused on (i) the diffusive processes of leaf NO_2 uptake, involving NO_2 absorption by water films of various thicknesses [12], regulation of NO_2 uptake by stomatal dynamics [11, 46], and the chemical reactions between NO_2 and apoplastic antioxidant [51], etc., and (ii) the metabolic processes of NO_2-derived NO_3^-, including NO_3^-/NO_2^- transporters, accumulation and remobilization of NO_3^- [58, 26], and downstream products of NO_2-N assimilation [32, 56], etc. Currently the NO_3^- assimilation pathway is considered to be the primary NO_2 metabolism path for most plant species. Not much progress has been made in finding alternative NO_2 metabolism paths [29]. Moreover, some of the current research is concentrated on NO_3^--specific signaling pathways; direction and intensity of the signal transmission between leaves and roots depend on ambient NO_2 concentration and N-supply status as well as other factors. In fact, there are obvious differences in physiological ramifications of NO_2-drived and root-derived NO_3^-.

This review focuses on physiological processes of leaf NO_2 uptake and NO_2-drived NO_3^-. The main objectives of the current review are: 1) to summarize the fates of NO_2-drived NO_3^- and 2) to compare the metabolic differences of NO_2-drived and root-derived NO_3^-.

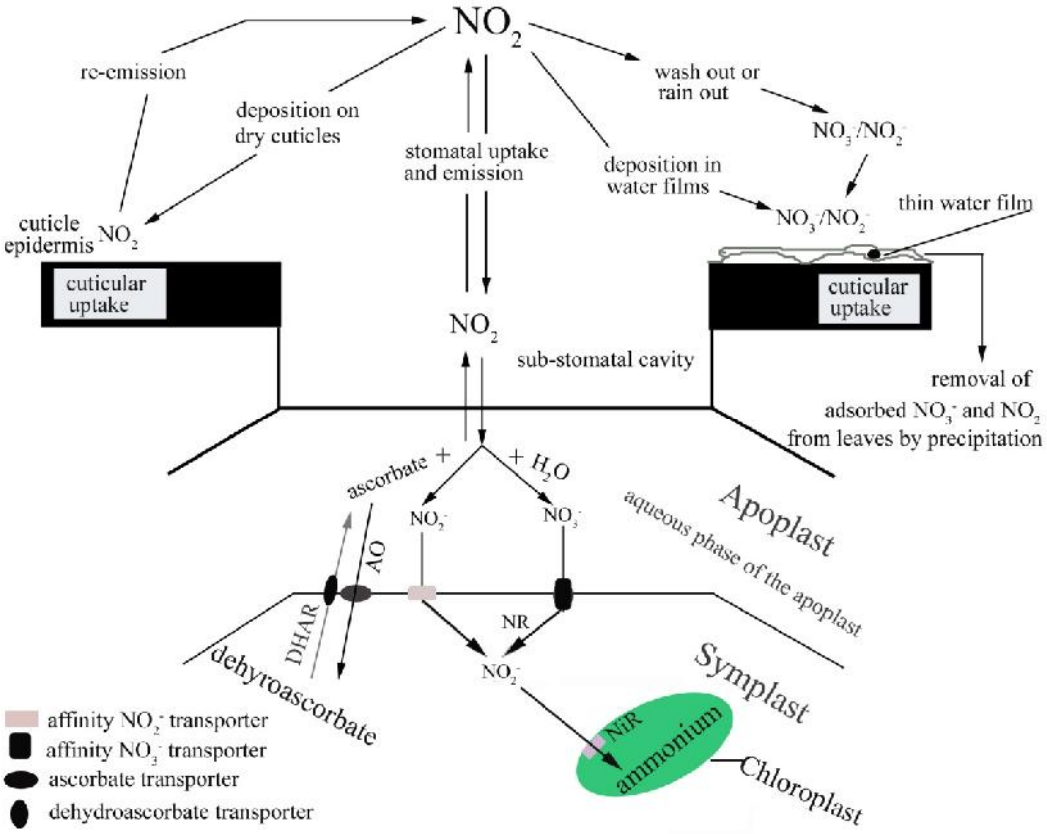
2. Fates of NO_2-drived NO_3^- in leaves

NO_2-drived NO_3^- has at least three fates in leaves: i) assimilation into amino acids, ii) accumulation in vacuoles, and iii) re-emission by NO_x (NO and/or NO_2). In general, the amount of NO_2 emission on leaf surfaces is generally lower than that of NO_2 uptake and assimilation. The three fates can inter-convert at some conditions, which depend on NO_2 concentration, N-supply status and species-specific properties. NO_3^- derived from low concentrations of NO_2 is mainly used for reduction and assimilation. High NO_2 easily results in the imbalance of NO_3^- transport and assimilation, and subsequent NO_3^- accumulation.

2.1. Assimilation into amino acids

In leaves, NO_2-drived NO_3^- is assimilated mainly through NO_3^- assimilation pathway. NO_2 molecules can enter the substomatal cavities directly by gaseous diffusion. In this compartment, NO_2 molecules dissolve in the aqueous phase of the apoplastic space and are transformed by chemical reactions to nitrate (NO_3^-) and/or nitrite (NO_2^-). NO_3^- is rapidly reduced to NO_2^- by the enzyme NO_3^- reductase in the cytosol. NO_2^- is then reduced to NH_4^+ by NO_2^- reductase in chloroplasts (Fig. 1), and eventually assimilated into amino acids (AA). NO_3^-/NO_2^- reduction can induce organic acid (OA) biosynthesis in leaves [7, 1]. In addition to that, NO_3^- per se may also serve as a direct signal triggering production of the OA [31, 52]. Most of the newly formed OA are phloem-translocated to roots where a carboxyl group is released in exchange for a NO_3^- ion [19]. The amino acids generated will be used locally for the synthesis of Chlorophyll, Rubisco, and vegetative storage protein, etc. during rapid vegetative growth, or will be ultimately designated for the filling pods during pod fill. NO_3^- assimilation products (protein/nucleic acids and amino acids/amides) can also be transferred into roots under soil N deficit [58].

Figure 1. Processes of atmospheric NO_2 deposition (and emission) on leaf surfaces. Atmospheric NO_2 can be dry/wet deposited in leaves mainly through stomata, with a small fraction of cuticle deposition. For the route of NO_2 uptake via stomata, the reaction in apoplast is either the disproporation reaction yielding equal amounts of NO_3^- and NO_2^- and/or reaction with apoplastic ascorbate (see for details Rennenberg and Gessler 1999) [41], and in symplast is the assimilation to amino acids. The trans-membrane transport of nitrite and nitrate/ascorbate and dehydroascorbate is dependent on the affinity transporters. AO, ascorbate oxidase; DHAR, dehydroascorbate reductase; NR, nitrate reductase; NiR, nitrite reductase.



The capacity of NO_2-drived NO_3^- assimilation presents a wide variation between species and between individuals of a species [48]. This variation is regulated by genotype-environmental interactions. For genetic foundation, variation of genotypes in individual species tomatoes significantly affected foliar NO_3^- assimilation [45]. NO_2-N assimilation rate is controlled by key enzymes of the primary NO_3^- assimilation pathway, including NO_3^- reductase (NaR), NO_2^- reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT), etc. NO_3^- reductase is considered as an important rate-limiting enzyme of NO_2-N assimilation. A linear correlation was found between NaR activity, NO_2 concentration and amount of N incorporated into amino acids. However, NaR transformants of *Arabidopsis* did not show a significant increase in amount of NO_2-N incorporated into total plant N, although NaR activity of the transformants was significantly higher than that of the non-transformed control [49]. Yet the NiR transformants showed a significant increase in NO_2 assimilation capacity. This implies the rate-limiting role of NiR in foliar NO_2 assimilation. Moreover, metabolic and storage pools of NO_3^- in higher plants vary with species and physiological variables [20]. For

environmental factors, NO_2 concentration and N-supply status in the environment play roles in regulating assimilation of NO_2-drived NO_3^- and/or redistribution of the assimilation products. Research has shown that, at the same level of NO_2, NO_3^- reductase activity was lower in needles of the trees grown on nitrogen-phosphorus-potassium (NPK)-fertilized soil than that of grown on non-fertilized soil [55]. Meanwhile, under sufficient N-supply, NO_2-drived N-compounds were utilized locally, and it did not effect the roots. However, the products of the NO_3^- assimilation were redistributed into roots when soil N supply becomes limiting [58]. Takahashi *et al.* (2005) [48] have investigated assimilation of NO_2-drived NO_3^- of 70 taxa of woody plants. They found that NO_2 assimilation values at 4 $\mu l \cdot l^{-1}$ NO_2 were higher by one or two orders of magnitude than that at 0.1 $\mu l \cdot l^{-1}$ NO_2. Several woody species, such as *Robinia pseudoacacia* and *Sophora japonica* had high NO_2 assimilation. On the other hand, other species such as *Cryptomeria japonica* and *Thea sinensis* had the low NO_2 assimilation and low NO_2 tolerance. Different responses of plant species to various concentrations of NO_2 may be ascribed partially to differences in the tolerance to NO_2^- accumulated in leaves and the ability to metabolize NO_2^- [61]. Moreover, NO_2 concentration might affect the selection of NO_3^- assimilation pathway. It is known that NO_2-derived NO_3^- is assimilated to organic nitrogenous compounds via nitrite and ammonium. The results of Srivastava and Ormrod (1984) [47] indicated that NO_3^- derived from low concentrations of NO_2 is assimilated primarily through GS/GOGAT pathway at low-level N supplies, while the assimilation of high NO_2-derived NO_3^- is mainly through glutamate dehydrogenase (GDH) pathway at high levels of nutrient N. Alternation of the two pathways partially depends on energy status of the cells. However, it is worthy of note that the main function of GDH is to deaminate glutamate [2, 9, 28]. Dubois et al. (2003) [9] concluded that physiological function of the enzyme still remains largely speculative.

2.2. Accumulation in vacuoles

NO_2-drived NO_3^- mainly accumulates in leaf vacuoles. The mechanisms of NO_3^- transport across tonoplast consist of H^+/NO_3^- antiport for NO_3^- influx and H^+/NO_3^- symport for NO_3^- effluxes. De Angeli *et al*. (2006) [8] have demonstrated that the NO_3^-/proton antiporter *AtCLCa* mediated NO_3^- accumulation in plant vacuoles. Accumulated NO_3^- in vacuoles can be exported to compensate the consumption of NO_3^- in the metabolic pool and to maintain NO_3^- reductase activity. Therefore, NO_3^- accumulation has significance in maintaining the balance of NO_3^- metabolism. Leaf NO_3^- accumulation is regulated to a great extent by NO_3^- supply, light intensity, temperature, water and oxidation conditions in the soil, etc. [3, 40, 43].

High NO_2 resulted in increased accumulation of NO_3^- in *B. campestris* leaves [26]. The increasing accumulation might be partially attributed to the imbalance of NO_3^- uptake and assimilation. On one hand, leaf NO_2 deposition via stomata is driven by the gradient of NO_2 concentration between leaf interior and ambient air. Foliar NO_2 deposition rate increased with increasing NO_2 concentration, thus, high NO_2 concentrations could lead to the increase of NO_2-drived NO_3^- in leaves. On the other hand, high concentrations of the pollutant resulted in a loss of NO_3^- reductase activity in leaves. Decrease in NO_3^- reductase activity might be ascribed to the following two aspects: first, high NO_2 inhibits the activities of glutamine synthetase and glutamate synthase, which results in ammonium accumulation and subsequently brings about a loss of NaR activity [34, 36]; second, high NO_2 -caused stomatal closure may lead to a rapid decrease of NO_3^- reductase (NaR) due to a decrease of CO_2 availability. A possible reason is that high NO_2 significantly decreases apoplastic pH, which readily results in

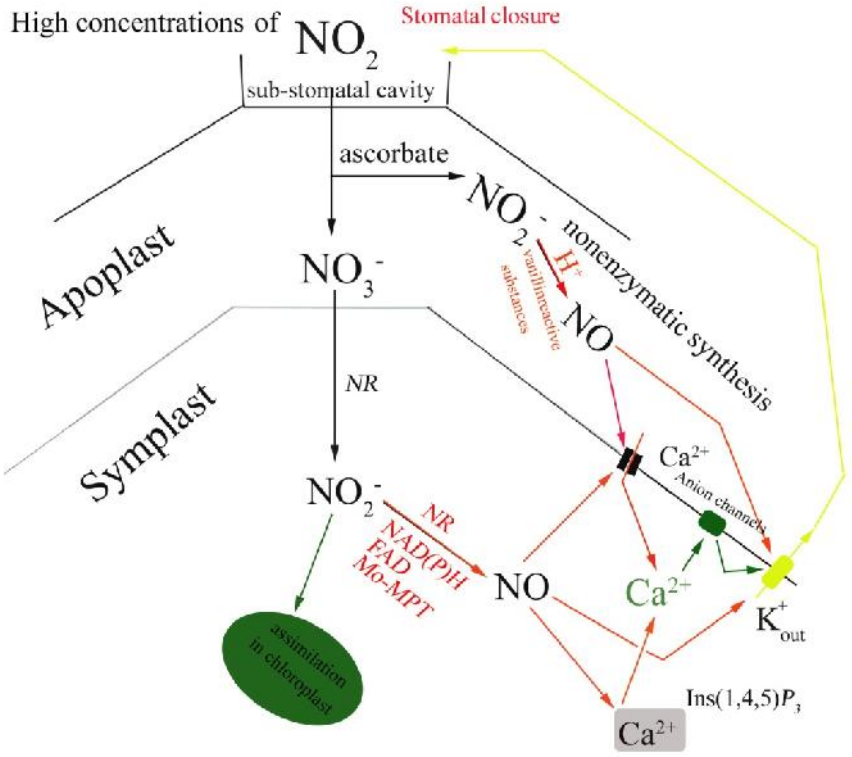
stomatal closure [38]. Stomatal closure may trigger a chain reaction wherein external CO_2 availability decreased and activity of NO_3^- reductase decreased rapidly in leaves [21]. Moreover, the results of Hisamatsu *et al*. (1988) [15] study indicated that NO_2 put an inhibitory effect on the light-induced NaR synthesis. In accordance with these results, Takeuchi *et al.* (1985) [50] found that NO_2 fumigation inhibited NO_3^- reductase activity in squash cotyledons, but the inhibition was recovered when the seedlings were transferred to NO_2-free conditions.

2.3. NO_2 and NO emission on leaf surfaces

A phenomenon of NO_2 emission on leaf surfaces has been observed in many plant species, such as Scots pine [42], wheat (*Triticum aestivum* L.) [57], and spruce [12], when atmospheric NO_2 concentration is below NO_2 compensation point of species. NO_2 emission rate is closely correlated with ambient NO_2 concentration, UV irradiance, leaf nitrogen content [46], and/or NO_3^- reductase activity [41]. In the present review, our concern is whether or not NO_2-N incorporated into NO_3^-/ NO_2^- can be re-emitted by NO and/or NO_2 and what are the possible mechanisms. It is known that NO_x emission on leaf surfaces mainly involves two mechanisms: 1) an enzymic conversion of NO_2^- by NO_3^- reductase to NO_x ($NO+NO_2$); 2) nonenzymic, chemical reactions between plant metabolites and accumulated NO_2^- and/or decomposition of nitrous acid. NO_2^- accumulation appears to be the key and the foundation of leaf NO_x evolution. A comparison between NO_x evolution mechanisms of wild-type and nr1 mutant soybean leaves showed that high NO_2^- accumulation led to enzymic NO_x evolution of wide type leaves, with small amounts of nonenzymic NO_x evolution [24], and the nr1 soybean mutant nonenzymically formed and evolved NO_x under dark, anaerobic conditions. Moreover, NO_2 evolution of wild type accounted for only 1% to 2% of total NO_x evolution, whereas the nr_1 mutant evolved 15% to 30% NO_2. High concentrations of NO_2 are expected to evolve NO_x on leaf surfaces. This assumption is supported by the following facts. First, high NO_2 resulted in NO_2^- accumulation in leaves in the light [61] or in the dark [60]. A low concentration of NO_2 (0.3 $\mu l \cdot l^{-1}$) also led to NO_2^- accumulation of greening bean seedling leaves with nutrient NO_3^- supply. Second, high NO_2 fumigation can result in a loss of NO_3^- reductase activity in leaves, which might be partially due to the depletion of excessive NO_2^-. Third, reduction of NO_2^- in apoplast can nonenzymically evolve NO (Fig. 2), which the NO generation required an acid apoplast. By coincidence, NO_2 can react with apoplastic antioxidant to produce NO_2^-. This reaction significantly increased the acidity in apoplast. This reaction may provide benefit in two ways. First, NO_2^--dependent NO synthesis can inhibit an accumulation of the toxic NO_2^- in mesophyll cells. Secondly, NO synthesis contributes to abscisic acid-induced stomatal closure, which finally controls excessive NO_2 influx into a leaf.

Zhou *et al*. (2003) [62] have reported that photolysis of deposited NO_3^- or HNO_3 produces NO_2, with some HONO (nitrous acid) and NO on snow and glass surfaces. Kesselmeier *et al*. (2005) [23] found that NO_2 emission of beech was occurred in the dark when stomata were basically closed. This implies that there are other pathways of NO_2 emission occurred on leaf surfaces. Unfortunately, so far this topic has received very little attention. The related mechanisms are still unknown as well. Therefore, this topic requires further research.

Figure 2. Nitrite-dependent NO synthesis in the apoplast and/or symplast mediates stomatal closure at high concentrations of NO_2. Atmospheric NO_2 can be reduced to nitrite in the apoplast by the reaction with ascorbate or in the symplast by the reduction of nitrate. Excess nitrite will produce NO by nonenzymatic synthesis in the apoplast and by reaction with nitrate reductase in the symplast (Klepper, 1990; Bethke et al., 2004) [24, 3]. The formed NO regulates stomatal closure directly or indirectly by mediating K+ channels (Sokolovski and Blatt, 2004) [45]. FAD, flavin adenine dinucleotide; Mo-MPT, molybdene-molybdopterin; Ins(1,4,5)P3, inositol (1,4,5)-trisphosphate.



3. Metabolic differences of NO_2-drived and root-derived nitrate

Nitrate required for plant growth is derived mainly from roots for most plant species. At the current atmospheric NO_x concentrations, small accounts of NO_3^- can also be obtained via foliar uptake of atmosphere NO_2 [51, 46, 25]. Under various concentrations of NO_2, only a few plant species studied incorporated more than 10% of NO_2-N into total plant N [30, 54]. A special case is that *phyllospadix torreyi* (surfgrass), one of very few seagrass species grown on rocks, obtains NO_3^- mainly through leaves but not roots. NO_2-drived and root-derived NO_3^-, in general, depend on NO_3^- metabolic pathway. However, metabolic processes of NO_2-drived and root-derived NO_3^- have significant differences. This is supported by at least the following four aspects (Fig. 3):

1) NO_3^- assimilation products (amino acids): $^{15}NO_2$ fumigation increased total content of free amino acids in leaves, shoots and roots, which the highest ^{15}N-enrichment was detected in the α-amino group of free amino acids in the leaves and the lowest enrichment in the roots. $^{15}NO_2$-N is incorporated into various amino compounds. Nussbaum *et al.* (1993) [32] found that 13 kinds of amino acids of Norway spruce needles were labeled in the α-amino N with ^{15}N from $^{15}NO_2$, of which ^{15}N content of free glutamate was the highest in all the detected amino acids. Similarly Weber *et al.* (1995) [56] observed

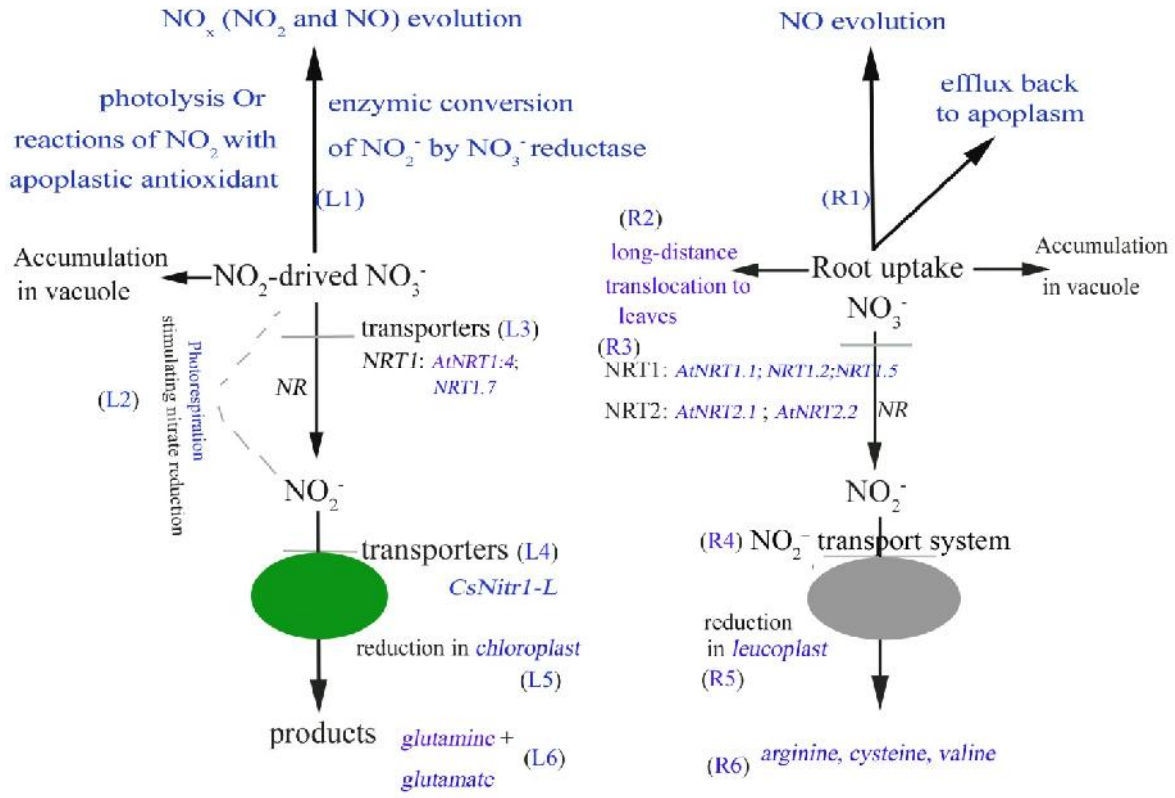
that free glutamate in wheat (*Triticum aestivum*) was the pool with the most labeled $^{15}NO_2$-N. Pearson and Steer (1977) [37] results agreed that glutamine was the most primary one of amino acids assimilated from NO_3^- in leaves whereas alanine was a more important constituent of roots than of leaves. Moreover, NO_2 can produce a pronounced effect on amino acid constituent in the xylem of plants growing on low levels of NO_3^-. Higher amounts of serine, asparagine and glutamine instead of arginine, cysteine, valine and lysine were found in the xylem of plants [58].

2) NO_3^- (or NO_2^-) transporters: three types of NO_3^- transports, NRT1, NRT2, and CLC family genes have been identified in roots. NO_3^- transporters in roots play diverse roles in NO_3^- balance in higher plants [10, 35, 53]. Various NO_3^- transporters cooperate to regulate NO_3^- uptake, accumulation and remobilization, which ensure roots efficient uptake and utilize NO_3^-. In contrast, only two members of NRT1 family, AtNRT1:4 and NRT1.7, have been identified in leaves for contribution to the NO_3^- transport. Currently, the effects of NO_3^- transporters on NO_2-drived NO_3^- metabolism are unavailable. Moreover, NO_2^- transporters, Nar1;1 in lower plant chloroplasts and CsNitr1-L in higher plant chloroplasts, have been identified, whereas the identified nitrite affinity transporters in roots and the related regulatory genes are less reported.

3) NO_x generation: NO_x can be evolved in both plant leaves and roots by enzymatic and nonenzymatic pathways [27], which the pathway selection depends on plant species, physiological state of the plant and environmental conditions, etc. There are significant differences in the regulation of NO_x generation between the two plant organs. For the enzymatic pathway, arginine- and nitrite-dependent NO production in plants has been well demonstrated by recent documents [13, 27, 5]. It is concluded that mitochondria are an important source of arginine- and nitrite-dependent NO production in plants. Gupta et al. (2005) [13] revealed that only root mitochondria, but not leaf mitochondria are able to reduce NO_2^- to NO, both in vitro (isolated mitochondria) and in situ. In NaR-containing roots, the reduction of NO_2^- to NO was mainly dependent on the catalysation of mitochondria, and less on NaR. In contrast, NaR-free leaf slices were not able to reduce NO_2^- to NO. Moreover, NO_3^- supply affects the distribution of NO_3^- reductase in cytosol (cNaR) in plant organs. High external NO_3^- decreases cNaR activity in roots, but may increase the enzymic activity in leaves. Thus the cNaR appears to play a more important role for NO formation in leaves than in roots at sufficient NO_3^-.

4) Photorespiration: NO_3^- assimilation in roots and leaves is closely associated with respiration process. Reductant required for root NO_3^- reduction is supplied from stored carbohydrates at considerable energetic cost [59, 14]. Excess photoreductant, in turn, can supply electrons for NO_3^- reduction in light-saturated leaves, minimizing the energy cost of leaf NO_3^- reduction. Recently photorespiration is coupled into the process of leaf NO_3^- assimilation. Rachmilevitch et al. (2004) [39] proposed that NO_3^- assimilation in shoots depends on photorespiration, and the effects of photorespiration on NO_3^- assimilation increase with mesophyll cell and chloroplast development. A recent review article by Hu and Sun (2010) [18] discussed the relationship between photorespiration and leaf NO_3^- assimilation, involving malate shuttle-caused chain reactions, generation and reassimilation of photorespiratory ammonia, and coupling reaction with PEPc activity. Moreover, the result of Carlson (1983) [6] showed that NO_2 fumigation reduced leaf apparent photorespiration and dark respiration. Therefore, photorespiration is assumed to have close relations with NO_2-N metabolism, although the direct evidence is unavailable.

Figure 3. Differences in NO_2-drived (L1-6) and root uptake (R1-6) nitrate metabolisms. The assimilation of NO_3^-derived from ambient NO_2 and root uptake has obvious differences in the nitrate/nitrite transporters, the location of nitrite reduction, and the assimilation products, etc.



4. Conclusion

NO_2-derived NO_3^- is assimilated mainly by NO_3^- metabolic pathway; the assimilation rate depends on plant growth status and environmental conditions such as atmospheric NO_2 concentration, light, and root nitrogen supply, etc. The imbalance between leaf uptake rate of NO_2 and the assimilation rate will lead to accumulation of NO_3^-/NO_2^- and NO_x reemission. Recently, more work is focused on root NO_3^- metabolism such as root nitrogen uptake, identification of NO_3^- transporters, and nitrogen-fixing bacteria, etc. as compared with NO_2-derived NO_3^- metabolism. Available information indicates that leaf- and root-derived NO_3^- metabolism have significant differences in at least four aspects, including the assimilation products, NO_3^- transporters, and NO_x generation, etc. Further work is proposed to investigate the relationship between gene expression of leaf NO_3^- transporter and species-specific NO_2 uptake and the metabolic coupling of NO_2-N, carbon, and sulphur.

Acknowledgements

This work was financially supported by Fundamental Research Funds for the Central Universities (DL10BB24). The author is grateful to Dr. A. Arivin Rivaie, and Dr. V.P. Tewari to go through the manuscript for language correction.

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