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Review

Exploration of nitrate-to-glutamate assimilation in non-photosynthetic roots of higher plants by studies of ^{15}N-tracing, enzymes involved, reductant supply, and nitrate signaling: A review and synthesis

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ABSTRACT

Roots of the higher plants can assimilate inorganic nitrogen by an enzymatic reduction of the most oxidized form (+6) nitrate to the reduced form (−2) glutamate. For such reactions, the substrates (originated from photosynthates) must be imported to supply energy through the reductant-generating systems within the root cells. Intensive studies over last 70 years (reviewed here) revealed the precise mechanisms of nitrate-to-glutamate transformation in roots with elaborate searches of ^{15}N-tracing, enzymes involved, the reductant-supplying system, and nitrate signaling. In the 1970s, the tracing of ^{15}N-labeled nitrate and ammonia in the roots demonstrated the sequential reduction and assimilation of nitrate to nitrite, ammonia, glutamine amide, and then glutamate. These reactions involve nitrate reductase (NADH-NR, EC 1.7.1.1) in the cytosol, nitrite reductase (ferredoxin [Fd]-NiR, EC 1.7.7.1), glutamine synthetase (GS2, EC 6.3.1.2), and glutamate synthase (Fd-GOGAT, EC 1.4.7.1) in the plastids. NADH for NR is generated by glycolysis in the cytosol, and NADPH for Fd-NIR and Fd-GOGAT are produced by the oxidative pentose phosphate pathway (OPPP). Electrons from NADPH are conveyed to reduce NIR and Fd-GOGAT through Fd-NADP$^+$ reductase (FNR, EC 1.6.7.1) specifically in the roots. Physiological and molecular analyses showed the parallel inductions of NR, NIR, GS2, Fd-GOGAT, OPPP enzymes, FNR, and Fd in response to a short-term nitrate supply. Recent studies proposed a molecular mechanism of nitrate-induction of these genes and proteins. Roots can also assimilate the reduced form of inorganic ammonia by the combination of cytosolic GS1 and plastidic NADH-GOGAT.

1. Introduction

The higher plants use inorganic nitrogen sources for their growth under natural ecosystems and in agricultural fields. Of inorganic nitrogen, nitrate is abundant and the most oxidized form (+6). Therefore, following nitrate absorption, plants must reduce nitrate to carbon-binding organic forms (−2) such as amino acids prior to their incorporation into nucleotides, proteins, and chlorophylls. A series of pioneering works were published in the 1950s regarding the relationship between nitrogen assimilation and respiration in barley roots (Folkes et al., 1952; Yemm and Willis, 1956). These studies reported that the nitrogen supply greatly induced the respiration in a manner specific to N sources (nitrate, nitrite, and ammonium), nitrogen assimilation, and root development. The processes of nitrate assimilation, the enzymes involved, and the supply of reductants have been investigated for the past 70 years (Beevers and Hageman, 1980; Yoneyama et al., 2003).

In the 1970s, ^{15}N-tracing studies provided important evidence about the enzymatic steps and reduction mechanisms of nitrate to ammonium and its assimilation into amino acids in non-photosynthetic roots and photosynthetic shoot tissues. Although the energy-consuming nitrate reduction and assimilation into amino acids depend on the light energy absorbed in the leaf-cell chloroplasts, such activity was largely unknown in the heterotrophic roots to which the photosynthates must be imported.

Beginning in the mid-1980s, the reductant supplying systems to nitrate reduction and assimilation have been extensively examined. Before 2000, physiological studies revealed that nitrate is both the substrate and a signal of nitrate reduction and assimilation in roots, but in just the past 5 years, detailed molecular mechanisms underlying nitrate signaling in nitrate reduction have been established. This review refers to the pioneer works on nitrogen metabolism and synthesizes the

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current knowledge of nitrate reduction and nitrogen assimilation into amino acids in roots, focusing on enzymatic reactions, the reductant supply, and nitrate signaling.

2. ^{15}N tracing studies of nitrate assimilation to amino acids in roots

Nitrate in the culture solution and soil and fertilizer-derived nitrate are taken up by the roots, transported up to the aerial parts, and converted to organic N compounds in both the roots and shoots. However, the nitrate conversion to organic nitrogen is a process that is difficult to identify because numerous N compounds are distributed in different organs, cells, and organelle compartments. In addition, environment conditions such as light, darkness, and temperature also affect the transport and metabolisms of nitrogenous compounds within plants (Beevers and Hageman, 1980; Ireland and Lea, 1999; Yoneyama et al., 2003).

Mendel and Visser (1951) were the first to conduct a nitrogen-15 (^{15}N) tracing experiment to identify the compounds produced through nitrate reduction and assimilation in intact tomato plants. They administered ^{15}N-labeled KNO_3 to 9-week-old tomato plants (46 mg to each group of six plants, 14 atom % excess ^{15}N). The plants, which had been pretreated with N-deficient medium for 1 week, were labeled for 8, 24, and 32 h starting at 7:30 a.m. under dry summer outdoor conditions. The abundances of ^{15}N in different nitrogenous fractions in the roots, stems and leaves were determined by Nier-type mass spectrometry after Kjeldahl digestion to ammonia and the subsequent conversion to nitrogen gas (N_2) by oxidation with alkaline hypobromite. The ^{15}N excess found in whole roots, stems, and leaves were as follows: 0.02, 0.03, and 0.03 atom % excess ^{15}N at 8 h, 0.02, 0.03, and 0.03 atom % excess ^{15}N at 24 h, and 0.05, 0.06, and 0.09 atom % excess ^{15}N at 32 h, respectively. The plant tissues (roots, stems and leaves) were fractionated into alcohol-soluble compounds, ammonia, amino-acid amides, humin, phosphotungstic acid precipitate, glutamic acid, aspartic acid, and the remaining bound N. The ^{15}N abundance in each of these fractions was determined as described for the whole tissue. In the roots, the ^{15}N abundances (atom % excess ^{15}N) of ammonia, amides, and glutamic acid were 0.13, 0.13 and 0.04 at 8 h, 0.18, 0.17 and 0.06 at 24 h, and 0.19, 0.16 and 0.15 at 32 h, respectively. The ^{15}N-labeling levels in the other fractions were lower than those of the above three fractions. The levels of ^{15}N-labeling decreased in the order of ammonia, amides, and glutamic acid in the three tissues, and the levels of total ^{15}N-labeling were higher in the stems and leaves than in the roots.

Thus, the labeling patterns indicate that nitrate is first transformed to ammonia, then amide and glutamic acid. Although the leaves appeared to be active, the exact sites of nitrate reduction to ammonia and its assimilation to amides and glutamic acid could not be determined. It should be noted that Pate (1973) demonstrated that ^{15}N-labeled NO_3^- administered to mature leaves of *Lupinus albus* was transported to primary roots and to a greater extent to growing lateral roots. Therefore, the ^{15}N labeling in the root metabolites as described above for tomato plants might be caused in part by a return of ^{15}N after the reduction and assimilation of shoot $^{15}NO_3^-$.

Mendel and Visser (1951) also fed tomato plants with $(^{15}NH_4)_2SO_4$ (60 mg for each six-plant-group, 15 atom % excess ^{15}N) for 8 h. In contrast to the labeling with ^{15}N-nitrate (above mentioned), the whole roots were more intensively labeled than the stems and leaves. The ^{15}N abundances of ammonia, amino acid amides, and glutamic acid in the roots were 0.78, 0.67 and 0.35 atom % excess ^{15}N, respectively. These results thus indicate that ammonia absorbed by the tomato roots was actively converted to amides and glutamic acid in the roots.

Yemm and Willis (1956) fed barley roots with ^{15}N-labeled ammonium phosphate (approx. 29.7 atom % ^{15}N) for 5.5, 10.5 and 15.5 h. The ^{15}N abundance of both free ammonia (extracted by distillation *in vacuo* after the addition of borate-hydroxide buffer) and glutamine amide-N (obtained by hydrolysis at pH 6.5 for 2 h in a boiling water bath) in the root extracts showed similar ^{15}N labeling patterns by increasing during the first 5.5 h and leveling off thereafter. However, the asparagine amide-N (obtained by hydrolysis with 2-N H_2SO_4 at 100 °C for 3 h) displayed a distinct increasing ^{15}N abundance at lower values than those of glutamine amide-N throughout the $^{15}NH_4^+$ feeding. In addition, Cocking and Yemm (1961) conducted a short-time $^{15}NH_4^+$ feeding (1 and 2 h) to barley roots, and they determined the ^{15}N-labeling in each amino acid by separating amino acids with an ion-exchange column-chromatography. Following the 2-hr feeding of $^{15}NH_4^+$ (29.7 atom % excess ^{15}N), the ^{15}N abundance (atom % excess ^{15}N) was 24.3 in ammonia-N (including amino-acid amide-N), 26.1 in glutamine amide-N, 19.2 in the glutamine amino-N, and 7.6 in glutamic acid. These results confirmed that ammonium absorbed by the roots was rapidly assimilated to the glutamine amide-N.

As examined previously, the $^{15}NO_3^-$ feeding over long periods (8, 24, and 32 h; Medel and Visser, 1951) caused the long-distance xylem transport of ^{15}N from the roots to the stems and leaves and the return of ^{15}N-metabolites via the phloem. This complex transport system makes it difficult to determine the specific reduction and assimilation of nitrate within the roots.

Yoneyama and Kumazawa (1975) reported the ^{15}N-labeling patterns of ammonia and amino acids in rice (*Oryza sativa* L.) seedlings treated with short-term $^{15}NO_3^-$-pulses (5, 15, 30, 60, 90 and 120 min) and then ^{14}N-chase (5, 15, 30, 60, 120 and 180 min) (Fig. 1A). The ^{15}N labeling was measured in ammonia (collected by microdiffusion) and in the amino acids, which were separated on two-dimensional thin-layer chromatograms first with phenol:water (4:1, v/v) and then with butanol:acetic acid:water (4:1:1, v/v/v), and identified with ninhydrin spray. The ^{15}N abundance (atom % excess ^{15}N), determined by optical emission spectrometry using an N-15 analyzer (NIA-1, JASCO, Tokyo) in Fig. 1A showed that with the 5-min $^{15}NO_3^-$ feeding, the value was the highest in ammonia, followed by glutamine and glutamic acid. The highest ^{15}N abundance during the ^{15}N-pulse phase was associated with glutamine and then glutamic acid. When the $^{15}NO_3^-$ in the medium was replaced with $^{14}NO_3^-$ at 120 min of the ^{15}N-pulse, the ^{15}N abundances of ammonia and glutamine were quickly decreased without apparent delays, whereas a lag of 5–15 min were observed for glutamic acid before the decline.

In contrast, the ^{15}N labeling in the shoots started 30 min after a $^{15}NO_3^-$ pulse in alanine, aspartic acid, glutamic acid, and glutamine (Yoneyama and Kumazawa, 1975) and thereafter increased slowly. Despite the increased ^{15}N abundances, the labeling levels in the shoots remained lower than in the roots throughout the ^{15}N-pulse phase. In the $^{15}NO_3^-$ feeding experiment, the treatment of rice seedlings with 1-methionine-*dl*-sulfoxine (MSO), a glutamine synthetase inhibitor, caused a $^{15}NH_4^+$ accumulation and reduced the ^{15}N-labeling in amino acids including glutamic acid and glutamine in the roots (Arima, 1979).

A similar pulse-chase experiment was conducted by feeding rice seedlings $^{15}NH_4^+$ instead of $^{15}NO_3^-$ (Yoneyama and Kumazawa, 1974). The results in Fig. 1B indicated a rapid uptake of $^{15}NH_4^+$ into the whole roots and active ^{15}N incorporation to glutamine and glutamic acid.

Table 1 shows the ^{15}N labeling values of the glutamine amide-N and amino-N and glutamic acid in corn roots fed $^{15}NO_3^-$ or ^{15}N-labeled nitrite ($^{15}NO_2^-$) (Yoneyama et al., 1977, 1980) and in barley roots fed $^{15}NH_4^+$ (Cocking and Yemm, 1961). By a short-term ^{15}N-feeding (30 min), $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation occurred first to the glutamine amide-N and then to glutamic acid. The labeling patterns of amino acids in the roots fed $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ were in line with a rapid ^{15}N assimilation into the ^{15}N glutamine amide-N by glutamine synthetase (GS). This labeled glutamine served as the substrate of glutamate synthase (GOGAT), forming one molecule each of labeled and non-labeled glutamate. The feeding of a large amount of $K^{15}NO_3$ (400 μg ml^{-1}) to *Datura stranonium* L. roots for 5 min resulted in the highest ^{15}N abundance in ammonia, followed by glutamine and then

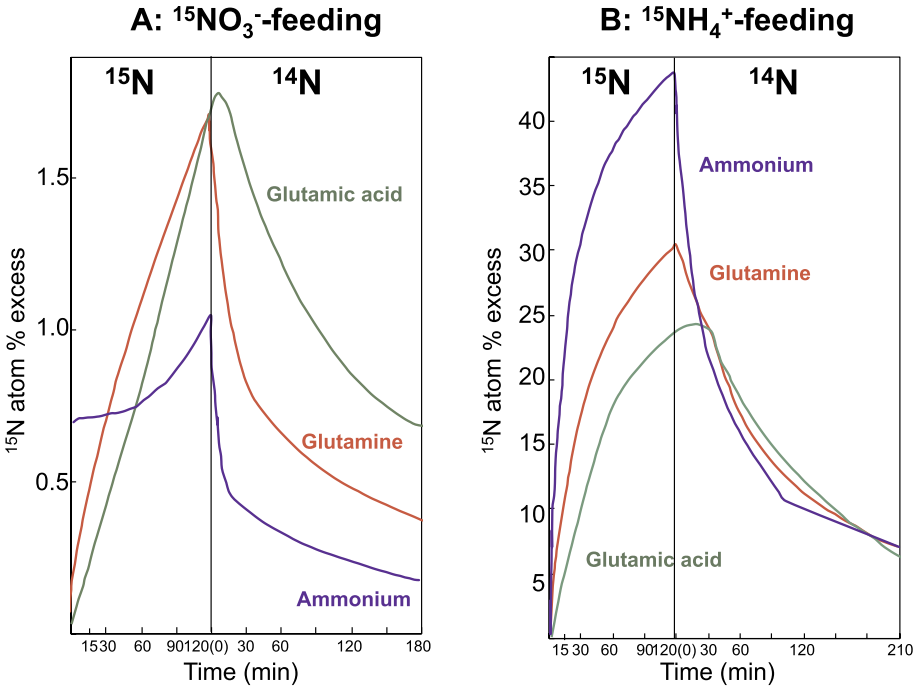

Fig. 1. Kinetics of the ^{15}N labeling of ammonium, glutamine, and glutamic acid in roots of rice seedlings fed $^{15}NO_3^-$ (A) or $^{15}NH_4^+$ (B). Panels A and B are based on the data presented in Table 2 of Yoneyama and Kumazawa (1975) and Table 2 of Yoneyama and Kumazawa (1974), respectively.

Table 1
^{15}N incorporation (atom % ^{15}N excess) from $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ into the amide-N and amino-N of glutamine and glutamic acid in corn and barley roots.

Plant species	Corn roots		Barley roots
^{15}N treatment	$^{15}NO_3^-$ 30 min	$^{15}NO_2^-$ 30 min	$^{15}NH_4^+$ 30 min
Glutamine			
Amide-N	7.81	3.42	25.3
Amino-N	1.29	0.24	22.4
Glutamic acid	4.52	2.07	5.5
Reference	Yoneyama et al. (1977)	Yoneyama et al. (1980)	Cocking and Yemm (1961)

glutamic acid (Probyn and Lewis, 1979). The addition of 7 mM MSO to the culture solution inhibited the ^{15}N incorporation into amino acids but caused a ^{15}N accumulation in the ammonia pool.

Thus, these results are consistent with the idea that the nitrate assimilation in the roots occurs by a nitrate reduction to nitrite and then to ammonia, which is incorporated into the glutamine amide-N and then to glutamic acid and other amino acids. The feeding of roots with $^{15}NO_3^-$ (Arima, 1979; Probyn and Lewis, 1979) in the presence of MSO inhibited the ^{15}N incorporation into amino acids including glutamic acid and glutamine, indicating that the primary nitrogen assimilation takes place predominantly through GS but not by glutamate dehydrogenase (GDH).

To investigate the reversibility of GDH reaction, *in situ* ^{15}N NMR assays were performed by monitoring the isotope exchange between glutamate and $^{15}NH_4^+$ in isolated mitochondria from potato (*Solanum tuberosum* L.) tubers, and the results showed that GDH catalyzes mainly the oxidative deamination of glutamate (Aubert et al., 2001). These ^{15}N labeling experiments provided strong evidence that the primary nitrogen assimilation to amino acids in the roots occurs through the GS-GOGAT pathway, and that the contribution of direct ammonia binding (amination) to keto acids (2-OG) by GDH is insignificant. Thus, we could infer that the sequential reactions of nitrogen assimilation into amino acids from either nitrate or ammonia occurs via nitrate reductase (NR), nitrite reductase (NiR), GS, and GOGAT.

Organic nitrogenous compounds such as amino acids and ureides in the xylem exudates from the stem may be derived largely from absorbed nitrate in the roots. Glutamine and amino acids are highly labeled compounds in the xylem sap of $^{15}NO_3^-$-grown barley seedlings (Joy et al., 1992) and $^{15}NH_4^+$-grown rice seedlings (Yoneyama et al., 2003).

3. Investigations of enzymes' roles in nitrate assimilation in roots

3.1. Nitrate reduction to ammonia

The enzymatic reduction of nitrate to ammonia in roots shown in Fig. 2 is catalyzed by the coupled reactions of nitrate reductase in the cytosol (Eq. (1)) and ferredoxin (Fd)-dependent nitrite reductase in the plastid (NiR, EC 1.7.7.1) (Eq. (2)).

Nitrate reductase (NADH-NR, EC 1.6.6.1)

$$NO_3^- + NADH \text{ or } NAD(P)H + H^+ \rightarrow NO_2^- + NAD^+ \text{ or } NADP^+ + H_2O \quad (1)$$

Nitrite reductase (NiR, EC 1.7.7.1)

$$NO_2^- + 6\ Fd_{red} + 7\ H^+ \rightarrow NH_4^+ + 6\ Fd_{ox} + 2\ H_2O \quad (2)$$

NADH serves as a major electron donor to nitrate reductase (NADH-NR, EC 1.6.6.1) in roots. This enzyme is a homodimer composed of two identical approx. 110-kDa subunits and localized in the cytosol (Dalling et al., 1972; Crawford and Ford, 2002). NR transfers two electrons from NAD(P)H to nitrate via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor, which is a complex of molybdate and pterin, and two metal ions (Fe and Mo) in each subunit (Privalle et al., 1989).

Pyridine nucleotide [NAD(P)H]-dependent NR activity in plant roots was first detected by Evans and Nason (1953) by assaying nitrite formation in nitrate-treated extracts of the root tissues of potato, barley, muskmelon (*Cumcumis melo* L.), wheat (*Triticum vulgare* Vill.), tomato (*Lycopersicum esculentum* Mill.), corn (*Zea mays* L.), and soybean. The induction of NADH-dependent NR activity in the roots, presumably via a *de novo* synthesis of protein, was detected in barley after a 6-hr exposure to 10 μM nitrate (Smith and Thompson, 1971) and in

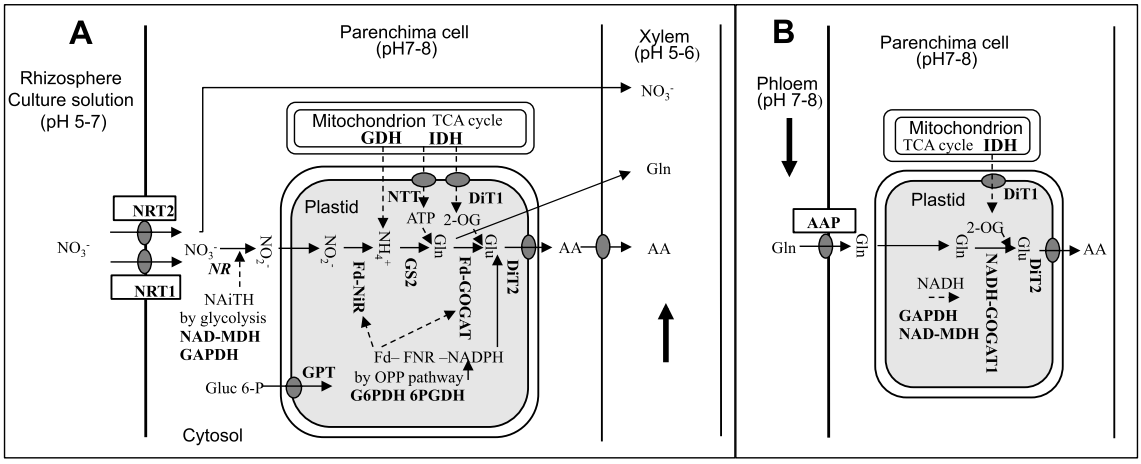

Fig. 2. Schematic presentation of the uptake of nitrate (A) and phloem-derived glutamine (B) and their assimilation into amino acids in roots grown with nitrate. 6PGDH, 6-phosphogluconate dehydrogenase; AA, amino acids; AAP, amino acid permease; DiT1, 2-oxoglutarate/malate translocator; DiT2, glutamate/malate translocator; Fd, ferredoxin; Fd-GOGAT, Fd-dependent glutamate synthase; FNR, ferredoxin-NADP$^+$ reductase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phoshate dehydrogenase, GDH, glutamate dehydrogenase; GPT; glucose phosphate translocator; GS2, plastidial glutamine synthetase; IDH, isocitrate dehydrogenase; NAD-MDH, NAD-dependent malate dehydrogenase; NiR, nitrite reductase; NR nitrate reductase; NRT1, low-affinity nitrate transporter; NRT2, high-affinity nitrate transporter; NTT, ATP/ADP translocator; OPP pathway, oxidative pentose phosphate pathway.

Arabidopsis (Wang et al., 2003). The nitrate induction of NR activity in the roots was repressed in barley (Smith and Thompson, 1971) or remained unchanged in tomato (Rufty et al., 1986) by the addition of ammonium and amino acids, which are products of the primary nitrate assimilation. The NR is largely localized in the cytosol (the supernatant by cell homogenate centrifugation) of root cells (Miflin, 1970; Dalling et al., 1972; Suzuki et al., 1981). Although NR proteins (assayed by enzyme-linked immunology) were located in all cell types of corn roots fed with nitrate, the NR protein was located predominantly in the epidermal cells and to lesser extents in the cortex and stele cells in the vicinity of the radial transport route of nitrate (Rufty et al., 1986).

Root NR mRNA was detected within 40 min and peaked at 2 h after a nitrate supply to barley roots. In nitrate-free medium, NR mRNA showed a half-life decline of 20 min, and that of NR activity ranged from 2 to 3 h in corn roots (Oaks et al., 1972). NADH-NR proteins of high specific activity were isolated from corn and barley roots with Michaelis constant (*K*m) values of 10–70 μM for NADH because of the technical advancement of protein stabilization and purification on blue (detran)-Sepharose affinity chromatography (Beevers and Hageman, 1980). The response of NR gene expression to nitrate is rapid. The steady-state mRNA levels of *NIA1*, one of two NR genes in Arabidopsis, increased within 15–30 min in the roots by the nitrate addition (Wang et al., 2003). The rapid increase of NR mRNA in response to nitrate is due at least in part to increased transcription (Privalle et al., 1989). Using a non-differentiated cell type of maize suspension cells, Privalle et al. (1989) demonstrated the nitrate induction of NR by *de novo* synthesis sensitive to the cycloheximide in response to exogenous nitrate but not by the activation/inactivation of NR protein.

Ferredoxin-nitrite reductase (NiR, EC 1.7.7.1) catalyzes the six-electron reduction of nitrite to ammonia using reduced ferredoxin (Fd) as the electron donor. NiR has a molecular mass of 61–64 kDa in plants, and all Fd-dependent NiR contain two prosthetic groups, a siroheme and an iron-sulfur cluster. Sirohemes are axially coordinated through a cysteine residue that also serves as one of the ligands for the iron-sulfur cluster (Beevers and Hageman, 1980; Lahners et al., 1988). High NiR activity, assayed by the disappearance of nitrite or the formation of ammonia, was detected in the presence of strong reducing dyes such as dithionite-reduced methyl viologen or benzyl viologen in the root extracts of tomato and barley (Beevers and Hageman, 1980). However, NAD(P)H-dependent NiR activity was absent in the root-tissue extracts from tomato and barley (Beevers and Hageman, 1980). The subcellular localization of NiR in roots was determined in particulate plastids (Dalling et al., 1972; Miflin, 1970; Suzuki et al., 1981). It has been reported that the root particle plastids contained NADPH-supplying glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) or 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), which functions in the oxidative pentose phosphate pathway (OPPP), in addition to NADPH diaphorase (Dalling et al., 1972; Emes and Fowler, 1983; Bowsher et al., 1993).

In the early 1970s, root-type Fd and NADPH diaphorase were not known although the NiR activity was measured in the roots with chemically reduced leaf Fd. NiR proteins were purified from the roots of barley and pea roots, and the pea root NiR had a specific activity of 28 μmol NO_2^--reduced min^{-1} mg^{-1} of protein at the optimum pH of 7.5 (Ida et al., 1974). In roots and cultured cells, the nitrate induction of NiR synthesis has been studied extensively (Lahners et al., 1988; Privalle et al., 1989). In *Nicotiana plumbagifolia*, four genes encode NiR: *NII1, NII2*, *NII3*, and *NII4*, and nitrate treatment increased *NII2* and *NII4* mRNA in the roots (Kronenberger et al., 1993). In maize roots, one of the NiR genes, probed with spinach NiR cDNA clone pCIB400, was induced by nitrate to its maximum within 2 h (Lahners et al., 1988). Cycloheximide had no effect on the induction of NiR mRNA, whereas NR was synthesized *de novo* as cycloheximide completely blocked its induction (Privalle et al., 1989).

3.2. Ammonia assimilation to glutamate

In 1973–4, when one of this review's authors (Tadakatsu Yoneyama) was preparing the manuscripts on the ^{15}N-tracing of nitrate and ammonia assimilation in the roots of rice seedlings, published later by Yoneyama and Kumazawa (1974, 1975), it was generally accepted that the ammonia assimilation in rice roots occurs by ammonia-inducible glutamate dehydrogenase (GDH, EC 1.4.1.2), which transfers ammonia to 2-oxoglutarate, forming L-glutamate (Kanamori et al., 1972) (Eq. (3)), and that by using this glutamate, glutamine synthetase [GS or L-glutamate:ammonia ligase (ADP), EC 6.3.1.2] detoxifies a large amount of ammonia taken up in the roots and temporarily stores it as glutamine (Kanamori and Matsumoto, 1972) (Eq. (4)).

Shortly after that manuscript's submission, a microbiologist (Professor Tasuku Takahashi) gave a special lecture at Tokyo and introduced an alternative pathway of ammonia assimilation that operates in bacterial species (Tempest et al., 1970). In this pathway, the ammonium assimilation is catalyzed by the coupled reactions of GS and NADPH-dependent glutamate synthase [L-glutamine (amide):2-

oxoglutarate aminotransferase, NADPH-GOGAT, EC 1.4.1.13] (Tempest et al., 1970). Lea and Miflin (1974) reported glutamate synthase activity dependent on ferredoxin (Fd-GOGAT, EC 1.4.7.1) (Eq. (5)) in the illuminated chloroplasts of higher plants. This new route of ammonium raised a debate as to how ammonium is assimilated into glutamate in the roots where Fd and Fd-$NADP^+$ reductase (FNR, EC 1.6.7.1) responsible for Fd oxidoreduction were not known yet.

Nevertheless, the ^{15}N labeling data from $^{15}NH_4^+$ uptake (Yoneyama and Kumazawa, 1974) or primary $^{15}NO_3^-$ reduction (Yoneyama and Kumazawa, 1975) in rice seedlings would be consistent with the sequential labeling of the glutamine amide-N by GS and then glutamate by GOGAT, which transfers the glutamine amide-N to 2-oxoglutarate. In addition, $^{15}NH_4^+$ tracing experiments of the roots, treated with the GS inhibitor MSO, indicated that the ammonia assimilation by GDH was insignificant, even when a high level of ammonia was fed to the roots, as noted above in Section 2 (^{15}N tracing studies of root nitrate assimilation).

Since the discoveries of NADPH-GOGAT in bacteria (Tempest et al., 1970) and Fd-GOGAT in plant leaves (Lea and Miflin, 1974), the properties and roles of GDH (Eq. (3)), GS (Eq. (4)), Fd-GOGAT (Eq. (5)), and NADH-GOGAT (Eq. (6)) in the roots have been investigated for almost 50 years. Evidence based on labeling kinetics, genetics, gene and cDNA cloning, gene expression and localization analyses, and the use of mutants and inhibitors has established that the major route of primary and photorespiratory nitrogen assimilation in leaves occurs via the GS/Fd-GOGOAT cycle (Ireland and Lea, 1999; Suzuki and Knaff, 2005).

NADH-glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2)

$$\text{2-Oxoglutarate} + NH_3 + \text{NADH} + H+ \leftrightarrow \text{L-Glutamate} + NAD^+ + H_2O \quad (3)$$

Glutamine synthetase (GS, EC 6.3.1.2)

$$\text{L-Glutamate} + NH_3 + ATP \rightarrow \text{L-Glutamine} + ADP + Pi \quad (4)$$

Fd-glutamate synthase (Fd-GOGAT, EC 1.4.7.1)

$$\text{L-Glutamine} + \text{2-Oxoglutarate} + Fd_{red} \rightarrow 2\ \text{L-Glutamate} + Fd_{ox} \quad (5)$$

NADH-glutamate synthase (NADH-GOGAT, EC 1.4.1.14)

$$\text{L-Glutamine} + \text{2-Oxoglutarate} + \text{NADH} \leftrightarrow 2\ \text{L-Glutamate} + NAD^+ \quad (6)$$

Strikingly, Fd-GOGAT activity was also present in non-photosynthetic roots when assayed with chemically reduced Fd prepared from leaves (Miflin and Lea, 1975; Arima, 1978; Oaks et al., 1980) and was fivefold more active than NADH-GOGAT in rice (Suzuki et al., 1982). Without any report of Fd or FNR in roots, the group of one of the authors (Akira Suzuki) focused on and characterized the Fd-like electron carrier and the electron donor system to GOGAT and NiR in maize roots (Suzuki et al., 1985) in a close collaboration with Dr. Ann Oaks (McMaster University, Canada). This study led to further investigations of the significant role of the GS/Fd-GOGAT cycle in primary nitrate assimilation and the ammonia assimilation into amino acids in non-photosynthetic roots.

As shown in Eq. (3), GDH catalyzes reversible reactions *in vitro*: the NADH-dependent reductive amination of 2-oxoglutarate forming L-glutamate, and the NAD-dependent oxidative deamination of glutamate releasing ammonia and 2-OG. The physiological function of GDH can be defined by its *in vivo* reaction. GDH located in the mitochondria of roots was more active in the mature regions than in apical root tips where higher activities of NR, GS, and GOGAT were found (Oaks et al., 1980). The root GDH activity was induced by NH_4^+ feeding, possibly through a *de novo* synthesis of the protein (Kanamori et al., 1972; Oaks et al., 1980). GDH purified from a variety of plant organs exhibit high Km values for ammonia (> 1 mM), which argues its role of ammonia assimilation *in vivo* (Ireland and Lea, 1999).

By [^{15}N]glutamate feeding to the roots, [^{15}N]ammonia was first released by GDH and then incorporated into the glutamine amide-N by cytosolic GS1 in maize (Stewart et al., 1995). The root analysis of transgenic plants expressing GDH β-subunit (and *in vivo* nuclear magnetic resonance spectroscopy (Purnell and Botella, 2007) demonstrated that GDH deaminates glutamate *in vivo* and fuels the carbon skeleton (2-OG) to the mitochondrial TCA cycle, and that GDH does not assimilate ammonia even under N-rich and C-deficient conditions. A single gene apparently codes for the α-subunit, and the β-subunit is encoded by at least two genes in dicots. The reversible GDH reactions may be associated with seven isoenzymes, which are derived from the combination of the anodic α-subunit and cathodic β-subunit in the hexamer (Purnell and Botella, 2007).

Initial studies showed that GS of higher plants occurs in two forms (Mann et al., 1980): cytosolic GS1 encoded by a small gene family, and plastidial GS2 encoded by a single gene in most of higher plants (Ireland and Lea, 1999). GS is an octameric protein and may be either homomeric or heteromeric composed of the subunits of 40–45 kDa (Ireland and Lea, 1999). Rice roots contained abundant mRNA for OsGLN1;1 and OsGLN1;2, both of which had high affinity to ammonia (Km = 27–73 μM for ammonia) (Ishiyama et al., 2003). Therefore, the higher affinity of cytosolic GS1 toward NH_4^+ in rice seedlings grown with ammonia (Kanamori and Matsumoto, 1972) compared to mitochondrial GDH matches the efficient NH_4^+ assimilation by GS1 in the growing root sections.

In Arabidopsis, five genes (*GLN1;1* to *GLN1;5*) encoding cytosolic GS1 were all expressed in the roots whereas only a faint signal of GS2 mRNA was detected (Ishiyama et al., 2004).). Cytosolic GS1 activity in pea roots was induced by infection with *Rhizobium,* coupled with NH_4^+ release from N_2 fixation (Tingey et al., 1987). In maize roots, two of five putative cytosolic GS1 genes (*GS1a and GS1b*) were significantly expressed, and *GS1c* and *GS1d* mRNA started to accumulate within 1 h after the addition of ammonia (Sakakibara et al., 1992). Among four GS1 isoenzymes tested from Arabidopsis roots (GLN1.1, GLN1.2, GLN1.3 and GLN1.4), the abundant GLN1.2 isoenzyme (Km = 2.5 mM for NH_4^+) in the vasculature was up-regulated by NH_4^+ at the mRNA and protein levels, whereas the GLN1.1 and GLN1.4 isoenzymes (Km = < 50 μM for NH_4^+) in the root epidermis were down-regulated by surplus NH_4^+ (Ishiyama et al., 2004). Overall, glutamine synthesis in response to the NH_4^+ supply seems to be controlled by a marked increase of GLN1.2 isoenzyme by NH_4^+ (Ishiyama et al., 2004).

When higher plants were treated with NO_3^-, mRNA induction occurred for NiR, plastidic GS2, and Fd-GOGAT in the roots of Arabidopsis (Wang et al., 2003) and maize (Redinbaugh and Campbell, 1993). This induction of GS2 mRNA in maize roots was rapidly detected (within 30 min) by the application of nitrate as low as 10 μM (Redinbaugh and Campbell, 1993), suggesting that plastidial GS2 assimilates NH_4^+ generated by nitrite reduction in the plastids of nitrate-grown roots.

Two forms of glutamate synthase (GOGAT) are distinguished in higher plants: Fd-GOGAT (EC 1.4.7.1) and NADH-GOGAT (EC 1.4.14). GOGAT forms two molecules of glutamate from glutamine and 2-OG by the combined intramolecular reactions of glutaminase releasing NH_2 and the reductive amination of 2-OG with $-NH_2$ using reduced Fd (Eq. (5)) or NADH (Eq. (6)) (Suzuki and Knaff, 2005). In the roots, two types of GOGAT activity were identified by either chemically reduced leaf Fd or NADH in pea (Miflin and Lea, 1975; Matoh and Takahashi, 1982) and rice (Suzuki et al., 1982). Fd-GOGAT and NADH-GOGAT are distinct proteins located in the plastid or chloroplast, and a small gene family encodes Fd-GOGAT and/or NADH-GOGAT according to the plant species (Suzuki and Knaff, 2005).

Fd-GOGAT protein in higher plants has a molecular mass of approx. 165 kDa deduced from the cDNA nucleotide sequence (Suzuki and Rothstein, 1997). It is notable that Fd-GOGAT genes (*GLU, Fd-gltS*) are homologous to *gltB*, which encodes the α-large subunit of bacterial NADPH-GOGAT (approx. 150 kDa). In contrast, *GLU* lacks homology to *gltD*, encoding the β-small subunit (approx. 50 kDa) to which NADPH binds in the αβ protomer of bacterial NADPH-GOGAT (Suzuki and

Rothstein, 1997). Fd-GOGAT purified from maize leaves had the *K*m values of 1100 μM (L-glutamine), 240 μM (2-OG), and 1.7 μM (Fd), whereas the rice root Fd-GOGAT showed a *K*m of 20 μM for Fd (see Table 1 from Suzuki and Gadal, 1984). Plant NADH-GOGAT encoded by *GLT* or *NADH-gltS* had a molecular mass of approx. 220 kDa, sharing a conserved sequence of fused α- and β-subunits (Suzuki and Knaff, 2005). NADH-GOGAT purified from rice suspension cells had a molecular mass of 194 kDa and the *K*m values of 811 μM (L-glutamine), 76 μM (2-OG), and 3 μM (NADH) (Hayakawa et al., 1992). Both proteins of Fd-GOGAT and NADH-GOGAT were exclusively located in the plastid fraction of root cells (Suzuki and Knaff, 2005).

Developmental and cell-specific studies showed that NADH-GOGAT activity in roots was active in the apical region of the pea (Matoh and Takahashi, 1982) and maize seedlings (Oaks et al., 1980), whereas Fd-GOGAT activity in the roots was enhanced in the mature section. The activity ratio of NADH-GOGAT to Fd-GOGAT in the roots was higher at an early period (3 and 5 days) when glutamine was the major mobilized amino acid in the phloem from the embryo, and with aging the Fd-GOGAT activity increased in the elongating roots (Matoh and Takahashi, 1982). It is noteworthy that the monomeric NADH-GOGAT (220 kDa) in plants resembles monomeric NADH-GOGAT (190–195 kDa) in animal cells where Fd is lacking (silkworm gland and midgut) (Suzuki and Knaff, 2005).

Transcripts and proteins of plastidic GS2 and Fd-GOGAT accumulated in the roots in response to nitrate supply (Redinbaugh and Campbell, 1993), whereas the induction of cytosolic GS1 and NADH-GOGAT occurred by ammonium, either supplied in the medium (Yamaya et al., 1995) or derived from N fixation in the nodule (Tingey et al., 1987).

Fig. 2 depicts the schema of nitrate assimilation into amino acids. Nitrate taken up is reduced to NO_2^- in the cytosol by NR using NADH generated by glycolysis. Nitrite is diffused into the plastid and transformed to ammonia by Fd-NiR. The Ammonia is assimilated to glutamine by plastidic GS2 and then to glutamate by Fd-GOGAT using 2-OG (probably derived from mitochondria). Schematic pathways of ammonia assimilation into amino acids are illustrated in Fig. 3. Ammonium taken up from the culture solution and also from intracellular production (such as by GDH) may be assimilated to glutamine by cytosolic GS1 using ATP (probably supplied from mitochondria; Hodges, 2002) and then glutamate by plastidic NADH-GOGAT.

In NH_4^+-fed rice seedlings, Fd-GOGAT was immunochemically detected in the meristem zone, stele, and cortex cells and sclerenchyma cells, whereas NH_4^+ feeding induced cytosolic GS1 and plastidic NADH-GOGAT proteins in the three surface cell layers of the apical roots (Ishiyama et al., 2003). In addition, glutamine remobilized via phloem may be taken up into the plastid of the central cylinders and apical meristems of the growing apical root where glutamine and 2-OG are metabolized by NADH-GOGAT to glutamate for use in the transamination to other amino acids (Ishiyama et al., 2004).

4. Reductant-supplying systems for nitrogen assimilation in roots

4.1. Reductant supply for nitrate reductase and nitrite reductase

NADH-requiring nitrate reductase (Eq. (1)) is located in the cytosol of root cells. The midpoint redox potential (Em_7) of spinach NR is −60 mV, and the Em_0 of the NR-reducing NADH is −320 mV. NADH is generated by glycolytic enzymes such as malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) using carbohydrates. The electron flow in the NR reaction is believed to be NADH → FAD → cytochrome b_{557} → MoCo (molybdenum cofactor) → NO_3^- (Beevers and Hageman, 1980).

Nitrite in the cytosol is transferred to the plastids by passive diffusion (Bowsher et al., 2007) and reduced to ammonia by the reducing activity of NiR (Eq. (2)), which involves a six-electron flow from Fd_{red} → [(4Fe–4S) → siroheme] → NO_2^-. The *E*m values of the NO_2^--binding siroheme and [4Fe-4S] cluster of spinach NiR were revealed to be −290 mV and −365 mV, respectively (Hirasawa et al., 1994). NiR requires reduced Fd, which had *E*m values between −300 mV and −460 mV (Hase et al., 1991a).

Ferredoxins were discovered in 1963 in the photosynthetic tissues of *Cucurbita pepo* (Hewitt and Betts, 1963) and other plant species. Twenty years later, the Fd-like proteins were identified and purified from non-photosynthetic tissues of maize roots (Suzuki et al., 1985), the plastid fraction of barley roots (Oji et al., 1985), and the storage fraction of radish roots (Wada et al., 1986). The root Fd was tested in a NO_2^- reduction assay by reducing Fd with dithionite. The structure of the root-type Fd was different from that of leaf-type Fd in maize and spinach (Hase et al., 1991b).

In maize, three Fds were identified by NiR assays showing tissue-specific molecular properties: FdI (*E*m = −423 mV) and FdII in leaves, and FdIII (*E*m = −345 mV) in roots (Hase et al., 1991b; Yonekura-Sakakibara et al., 2000). The root-type FdIII, incubated with purified NiR and reduced with dithionite (Em_7 = approx. −660 mV), showed a higher activity of $^{15}NO_2^-$ reduction to $^{15}NH_4^+$ than the leaf-type FdI (Yoneyama et al., 2015). The primary electron donor to Fds in the roots may be NADPH (*E*m = −320 mV), which is regenerated by G6PDH or 6PGDH of the OPPP (Dalling et al., 1972; Oji et al., 1985; Bowsher et al., 2007) at the expense of glucose 6-phoshate imported from the cytosol to the plastids. To supply reduced Fd for the enzymatic reactions, plant-type FNR (*E*m = approx. −320 mV) may catalyze the reversible electron transfer between NADPH and Fd as shown in Eq. (8).

$$2Fd{\bullet}Fe^{3+} + NADPH \leftrightarrow 2Fd{\bullet}Fe^{2+} + NADP^+ + H^+ \quad (8)$$

The presence of FNR-like proteins was suggested in maize roots

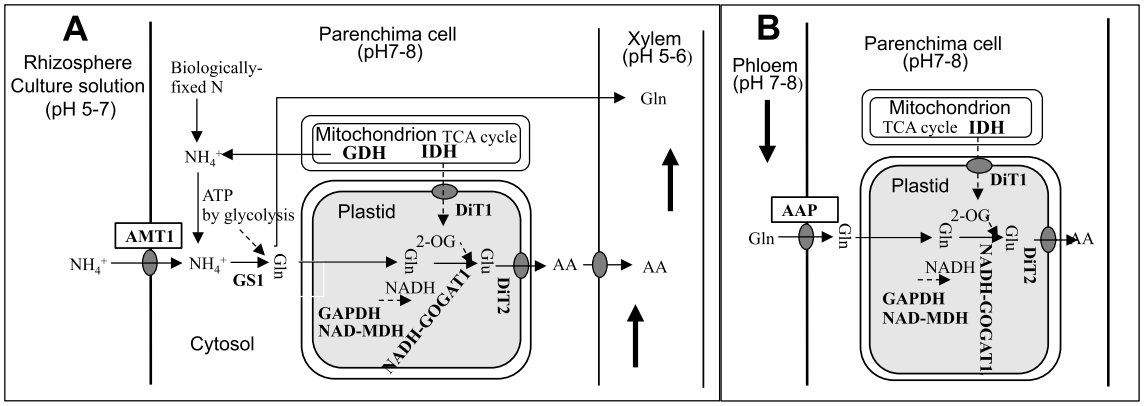

Fig. 3. Schematic presentation of the uptake of ammonium (A) and phloem-derived glutamine (B) and their assimilation into amino acids in roots grown with ammonia. Abbreviations are explained in Fig. 2 legend.

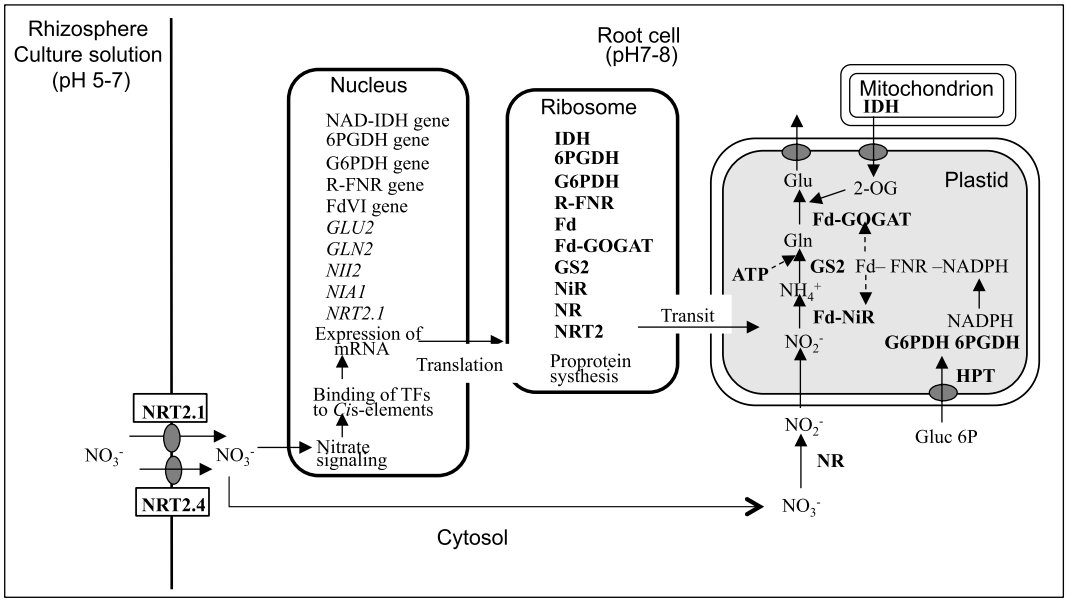

Fig. 4. The nitrate signaling pathway and the induction of the expression of genes for NRT (*NRT2.1*), NR (*NIA1*), NiR (*NII2*), GS2 (*GLN2*), Fd-GOGAT (*GLU2*), Fd, R-FNR, G6PDH, 6PGDH, and NAD-IDH in the roots. TF, transcription factor.

(Suzuki et al., 1985) and barley root plastids (Oji et al., 1985). Later, the root-specific FNR (different from the leaf-type FNR) was identified in radish root plastids (Morigasaki et al., 1990) and in nitrate-supplied roots of rice (Aoki et al., 1995).

4.2. Reductant supply for Fd-GOGAT and NADH- GOGAT

Ammonia that is derived from uptake, the primary nitrate reduction by NiR in root plastids, and internal production is assimilated into glutamate to form glutamine by GS using energy (ATP) from mitochondria (Mann et al., 1980; Ireland and Lea, 1999). The subsequent transfer of the glutamine amide-N to 2-OG to yield glutamate takes place in the root plastid by Fd-GOGAT under nitrate medium (Fig. 2) or NADH-GOGAT under ammonium medium (Fig. 3) (Suzuki and Knaff, 2005). Fd-GOGAT is a flavin and iron-sulfur-containing protein. Originally, the *E*m values of FMN and [3Fe-4S] cluster of spinach Fd-GOGAT were estimated as −180 ± 10 and −170 ± 15 mV, respectively, and later, the isopotential of the chromophore and cluster was reported to have the *E*m of −225 ± 10 mV (see the chapter "Redox properties" of Suzuki and Knaff, 2005).

NADPH is a primary reductant of Fd-GOGAT as well as Fd-NiR in the root plastids and generated by the OPPP (Bowsher et al., 2007). An *in vitro* electron donation system from NADPH to R-FNR and to FdIII resulted in efficient Fd-GOGAT activity using [^{15}N-aminde]glutamine (ratio of mol NADPH used to mol [^{15}N]glutamate formed = 1.02), whereas the electron donation from NADPH to L-FNR and to FdI was less efficient with a ratio of 0.68 (Yoneyama et al., 2015), confirming that the affinity between Fd and FNR is important for electron transfer.

Ammonium induction of NADH-GOGAT was observed in rice roots, and its activity was high in the vascular parenchyma cells close to the phloem (Yamaya et al., 1995). It was also suggested that NADH may be generated by the malate-oxalate valves, implicating plastidic NAD-MDH and GAPDH (Scheibe, 2004). It remains to be determined whether the rate of NADH generation in the plastids is sufficient to meet the operation of ammonia-induced NADH-GOGAT activity. Alternatively, constitutively present Fd-GOGAT (Yamaya et al., 1995) may form glutamate from the glutamine produced by cytosolic GS1 by the generation of NADPH via the OPPP. Ammonia assimilation by a combination of GS1 and Fd-GOGAT was suggested in conifer leaf cells, which lack plastidic GS (Suárez et al., 2002). The availability of NADH or NADPH may be an important controlling factor of the operation of NADH-GOGAT and Fd-GOGAT, respectively.

5. Molecular response to nitrate and ammonia nutrition in roots

5.1. Induction of nitrate uptake and nitrogen-assimilating enzymes by nitrate nutrition and N starvation

Nitrate is one of the major nitrogen sources in arable lands, and it is absorbed by different types of nitrate transporters located in the membranes to synthesize organic N compounds for growth (Fig. 2). Two types of nitrate transport systems (low-affinity NRT1 and high-affinity NRT2) co-exist in different organs (roots, stem, leaves, seeds) and coordinate to take up nitrate and distribute it to different tissues and cells (Crawford and Ford, 2002). A high-affinity nitrate transporter, NRT2.1, in the roots is active at low nitrate concentrations (< 1 mM). NRT2.1 mRNA was induced by feeding with nitrate and repressed by high plants' N status (endogenous N assimilates) in the roots of Arabidopsis (Gansel et al., 2001).

An important finding is that another high-affinity nitrate transporter (NRT2.4) mRNA was induced in N-starved plants in the epidermis of lateral roots (Kiba et al., 2012), whereas in adult plants, NRT2.5 mRNA was most abundantly expressed in the epidermis and the cortex of roots under N starvation (Lezhneva et al., 2014).

Under nitrate nutrition, nitrate is the substrate of nitrate reductase and also a regulatory signal (Crawford and Ford, 2002). Nitrate as a signal regulates the levels of mRNA and proteins of the enzymes involved in the nitrogen assimilation and carbon metabolism (Fig. 4). Nitrate induced NR activity and protein in corn roots (Oaks et al., 1972), and *NIA1* mRNA in *Arabidopsis* roots (Wang et al., 2003). Nitrate induced mRNA, protein, and the activity of several nitrate-assimilating enzymes in the roots: NiR in pea root plastids (Emes and Fowler, 1983), GS2 in Arabidopsis (Wang et al., 2003) as well as maize (Redinbaugh and Campbell, 1993), and Fd-GOGAT in maize (Redinbaugh and Campbell, 1993). *GLU2*-encoded Fd-GOGAT isoenzyme was preferentially expressed in the roots of Arabidopsis (Redinbaugh and Campbell, 1993).

In addition, nitrate induced the components of the electron donation system NADPH-FNR-Fd in roots such as Fd in pea plastids (Bowsher et al., 1993), FdVI in maize (Matsumura et al., 1997), FNR in pea root

plastids (Bowsher et al., 1993) as well as rice (Aoki et al., 1995), and Arabidopsis (Wang et al., 2003). To enhance the NADPH generation in root plastids, nitrate also induced G6PDH and 6-PGDH activities of the OPPP in barley (Dalling et al., 1972), and pea (Emes and Fowler, 1983).

In tobacco roots, NO_3^- induced the mitochondrial NADH-dependent isocitrate dehydrogenase (NAD-IDH, EC 1.1.1.41), which can supply 2-OG to the Fd-GOGAT reaction (Hodges, 2002). The reduction system NADPH-FNR-Fd (Fig. 2) may be operated essentially without a nitrate supply, since in the assimilatory process of sulfate, an essential nutrient, reductant supply via NADPH-FNR-Fd is prerequisite (Yonekura-Sakakibara et al., 2000). In addition, nitrate feeding may specifically induce this entire assimilation system through nitrate signaling by the substrate and plant's N starvation (Fig. 4).

5.2. Responses of ammonia uptake and nitrogen-assimilating enzymes to ammonia

Ammonia is one of the major nitrogen sources in flooded soil such as rice paddy fields, and it can be derived from nitrogen fertilizers and endogenously from nitrogen fixation in root nodules (Fig. 3). In ammonium nutrition, a high-affinity ammonia transporter (AMT1.1) in roots is active in ammonium uptake to the steady-state level, and *AMT1*.1 mRNA is highly induced in severely N-starved roots (Gansel et al., 2001). The level of *AMT1*.1 mRNA was negatively correlated with the glutamine level in the roots (Gansel et al., 2001). Following the uptake, a large part of ammonia is assimilated into amino acids in an organelle-specific manner.

In ammonium nutrition, cytosolic GS1 and plastidic NADH-GOGAT are affected by the NH_4^+ supply (Fig. 5). In maize roots, the enzyme activity and the protein and mRNA accumulation for cytosolic GS were enhanced by the ammonium addition to a growth medium (Sakakibara et al., 1992). The induction of GS1 (*OsGLN1*) and NADH-GOGAT (*OsNADH-GOGAT1*) by the NH_4^+ supply was observed at the mRNA and protein levels in rice roots (Yamaya et al., 1995). However, Fd-GOGAT was not affected by NH_4^+ feeding in terms of mRNA, protein, and activity (Yamaya et al., 1995). It should be noted that Fd-GOGAT protein was localized in the sclerenchyma cells, whereas NH_4^+ induced NADH-GOGAT in the epidermis and exodermis, in line with a rapidly coordinated expression of two GOGAT isoforms in NH_4^+ assimilation (Ishiyama et al., 2003).

An ammonia supply induced mRNA and corresponding subunits of mitochondrial NAD-IDH (OsIDHa and OsIDHc;1), which can increase the NH_4^+ assimilation by providing 2-OG, a substrate of GOGAT (Abiko et al., 2005). Ammonium nutrition also induced plastidic glucose 6-phosphate dehydrogenase (Scheibe, 2004). Glutamine or its metabolites serve as a signal to regulate the interaction of nitrogen and carbon metabolisms in roots (Fig. 5). The signal transduction of NH_4^+/glutamine essentially occurs even without an exogenous NH_4^+ supply, because glutamine is formed from endogenous NH_4^+ produced by the deamination and deamidation of amino acids and biological N_2 fixation. Glutamine and its metabolites produced in the shoot are transported via the phloem to the growing non-photosynthetic tissues, and they likely function as long-distance signals (Miller et al., 2007).

5.3. Molecular mechanism of nitrate signaling for the primary nitrogen assimilation to amino acids

As indicated above in Section 5.1 (Induction of nitrate uptake and nitrogen-assimilating enzymes by nitrate nutrition and N starvation), nitrate serves as a signal for the induction of mRNA accumulation of genes for nitrate uptake, nitrate assimilation, and the generation of reductants for nitrate transformation to glutamate. Evidence obtained by molecular and genetic analyses indicates that nitrate itself without its metabolism initiates its signal transduction mediated by a constitutively pre-existing transcription factor (TF) without *de novo* synthesis (Redinbaugh and Campbell, 1993; Yoneyama et al., 2011).

Recent studies by Liu et al. (2017) and Maeda et al. (2018) provided evidence regarding the mechanisms of nitrate signaling and the induction of genes for nitrate transporters, nitrate and nitrite reduction, and reductant-generating enzymes. Nitrate taken up into the root cells by NRTs increased the intercellular concentration of Ca^{2+}, which activated Ca^{2+}-dependent protein kinases (CPK10, CPK30, CPK32) and phosphorylated NIN-like protein (NLP) transcription factors. NLPs bound to the promoters of specific genes (Liu et al., 2017) and induced the expression of the genes for nitrate transporters (NRT2.1, NRT2.2, NRT2.4, NAR2.1, NRT1.1), nitrate reductase (NIA1), nitrite transporter (NIRT2.2), nitrite reductase (NIR1), and NADPH-generating enzymes of the OPPP (G6PD1, G6PD3) (Maeda et al., 2018).

Maeda et al. (2018) provided further evidence that the NLPs induce the negative regulator NIGT1, which down-regulated the aforementioned 10 genes tested. This NLPs-NIGT1 cascade system may function for the transcriptional regulation of these genes. It remains to be determined whether this nitrate signaling pathway controls other nitrate-inducible proteins such as GS2, Fd-GOGAT, and FNR. One of the most intriguing questions is the role of NRT1.1/CHL1, likely a nitrate sensor (Ho et al., 2009). The mutant study identified that an *npf6.3* nitrate transporter mutant is impaired for transducing the nitrate signal, while functional for nitrate transport (Ho et al., 2009). Pertinent data issued from the recent discovery of nitrate-CPK-NLP signaling (Liu et al., 2017) or involved in other undefined signaling systems.

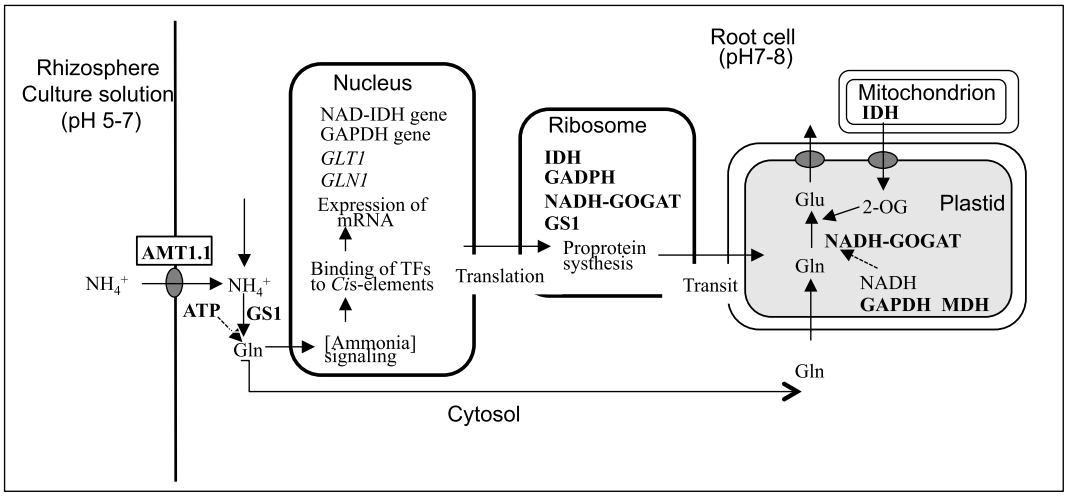

Fig. 5. The ammonia signaling pathway and the induction of the expression of genes for GS1 (*GLN1*), NADH-GOGAT (*GLT1*), GAPDH, and NAD-IDH in the roots.

Interestingly, the transient induction of NADH-GOGAT gene by NH_4^+ feeding in rice cell culture was mimicked specifically by okadaic acid, which inhibits serine/threonine phosphatases 1 and 2A, and resulted in a continuous accumulation of NADH-GOGAT mRNA for at least 24 h (Hirose and Yamaya, 1999). It can be hypothesized that ammonium signaling involves the phosphorylation of transcription factors. Further studies are required to elucidate the ammonium signaling in the expression of genes for GS1 and NADH-GOGAT (Fig. 5).

5.4. Shoot-to-root signaling essential for NRT2.1 induction

The split-root experiments established that the shoot-to-root systemic signals which are caused by root N starvation (or by a high N supply), induce (or repress) the mRNA accumulation of *NRT2.1* in the nitrate (or nitrate-free) compartment by the control of the shoot signal of N demand (Gansel et al., 2001). A recent study by Ohkubo et al. (2017) showed that N-starved roots produced a root-to-shoot mobile peptide, C-terminally encoded peptide (CEP), which was transported via the xylem to the leaves and concentrated in the phloem. The recognition of CEP by C-terminally encoded peptide receptor (CEPR) in the phloem induced the production of CEP downstream1 (CEPD1) polypeptide, and the CEPD1 polypeptide was transported via the phloem to the roots.

It is suspected that the shoot-derived CEPD1 signal up-regulates the *NRT2.1* expression in the roots within the nitrate-free rhizosphere. However, the molecular interactions between the local nitrate signaling and the N-starvation-induced systemic signaling pathways are not yet clear. It is also of interest to characterize the N-starvation factor and molecular mechanisms that may induce the local expression of *AMT1.1* (Gansel et al., 2001) and *NRT2.4* (Kiba et al., 2012).

CRediT authorship contribution statement

Tadakatsu Yoneyama: Writing - review & editing. **Akira Suzuki:** Writing - review & editing.

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