Novel Metabolism of Nitrogen in Plants

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Our previous study showed that approximately one-third of the nitrogen of ^{15}N-labeled NO_2 taken up into plants was converted to a previously unknown organic nitrogen (hereafter designated UN) that was not recoverable by the Kjeldahl method (Morikawa *et al.*, 2004). In this communication, we discuss metabolic and physiological relevance of the UN based on our newest experimental results. All of the 12 plant species were found to form UN derived from NO_2 (about 10–30% of the total nitrogen derived from NO_2). The UN was formed also from nitrate nitrogen in various plant species. Thus, UN is a common metabolite in plants. The amount of UN derived from NO_2 was greatly increased in the transgenic tobacco clone 271 (Vaucheret *et al.*, 1992) where the activity of nitrite reductase is suppressed less than 5% of that of the wild-type plant. On the other hand, the amount of this UN was significantly decreased by the overexpression of *S*-nitrosoglutathione reductase (GSNOR). These findings strongly suggest that nitrite and other reactive nitrogen species are involved in the formation of the UN, and that the UN-bearing compounds are metabolizable. A metabolic scheme for the formation of UN-bearing compounds was proposed, in which nitric oxide and peroxynitrite derived from NO_2 or endogenous nitrogen oxides are involved for nitrosation and/or nitration of organic compounds in the cells to form nitroso and nitro compounds, including *N*-nitroso and *S*-nitroso ones. Participation of non-symbiotic haemoglobin bearing peroxidase-like activity (Sakamoto *et al.*, 2004) and GSNOR (Sakamoto *et al.*, 2002) in the metabolism of the UN was discussed. The UN-bearing compounds identified to date in the extracts of the leaves of *Arabidopsis thaliana* fumigated with NO_2 include a Δ^2-1,2,3-thiadiazoline derivative (Miyawaki *et al.*, 2004) and 4-nitro-β-carotene.

Key words: Nitrogen Metabolism, Nitrogen Oxides, Reactive Nitrogen Species

Introduction

Atmospheric nitrogen dioxide (NO_2) is a major pollutant in the urban air which is a cause of photochemical oxidants including ozone produced by photochemical reactions (Wellburn *et al.*, 1997). Tropospheric ozone is known to be harmful for plants and animals including humans (Wellburn *et al.*, 1997).

The transactions between plant and NO_x (NO plus NO_2) have so far been investigated on the nutritional role of this gas in plants (Rogers *et al.*, 1979; Yoneyama and Sasakawa, 1979) or on the role of plants as a sink for this air pollutant (Hill, 1971; Rogers *et al.*, 1979; Morikawa *et al.*, 1998). However, the quantitative role of NO_2-nitrogen as the N source remains unclear. Also, quantitative contribution of plants as a sink for the global atmospheric NO_x is yet unknown (Lerdau *et al.*, 2000).

In our attempt to study the mechanism of NO_2 metabolism in the plant-mediated decontamination of this major air pollutant (Morikawa *et al.*, 1998, 2003; Goshima *et al.*, 1999; Takahashi *et al.*, 2001, 2003), we unexpectedly discovered (Morikawa *et al.*, 2004) that about one-third of the total nitrogen derived from NO_2 taken up in the leaves of *Arabidopsis thaliana* was converted to neither inorganic nor Kjeldahl nitrogen, but instead to an as yet unknown nitrogen. We hereafter designate this nitrogen "unidentified nitrogen" (UN).

This finding of the UN raised several questions on the current understanding of the nitrogen metabolism in plants (Morikawa *et al.*, 2004). In this communication we will focus on the following aspects of this novel nitrogen metabolism: (i) Forma-

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tion of UN from NO_2, (ii) formation of UN from nitrate, (iii) UN level in transgenic plants, (iv) mechanism of the formation of UN-bearing compounds, and (v) structures of UN-bearing compounds identified to date. We will also discuss the physiological relevance of this novel nitrogen metabolism and its significance in the phytoremediation implementation.

Materials and Methods

Plant materials

Arabidopsis thaliana (L.) Heynh. ecotype C24, *Glycine max* (L.) Merr. cv. Okuhara-wase, *Hordeum vulgare* L. cv. Uzuakashinri, *Spinacia oleracea* L. cv. Taiheiyo were grown as described previously (Kawamura *et al.*, 1996). Briefly, after germination seedlings were grown in pots containing vermiculite and perlite (1:1, v/v) that were placed in a growth chamber (model ER-20-A; Nippon Medical and Chemical Instruments Co., Osaka, Japan). Plants were grown for 5 weeks under continuous light (70 μmol photons m^{-2} s^{-1}) at 25.0 ± 0.3 °C (22.0 ± 0.3 °C for *Arabidopsis*) at a relative humidity of 70 ± 4% with irrigation at 4-day intervals with a half-strength solution of the inorganic salts of Murashige and Skoog's medium (Murashige and Skoog, 1962) that contained 19.7 mM nitrate and 10.3 mM ammonium salts. *Eucalyptus viminalis* Labill. was grown as described previously (Morikawa *et al.*, 1998). After germination, seedlings were cultured in a greenhouse under natural light for about 2 months by using a weekly supply of 0.1% (v/v) Hyponex (Hyponex Japan, Osaka) that contained 0.4 mM nitrate and 0.07 mM ammonium salts. Other plants were grown as reported elsewhere (Morikawa *et al.*, 2004).

Fumigation of plants with ^{15}N-labeled NO_2; treatment with ^{15}N-labeled nitrate

Fumigation of plants with ^{15}N-labeled NO_2 was performed as reported previously. For treatment with ^{15}N-labeled nitrate, two-month-old plants of *Eucalyptus viminalis* and 5-week-old plants of other species were used. Plants were supplied at the roots with a half-strength solution of the inorganic salts of Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 20 mM $K^{15}NO_3$ (10.6 atomic percentage ^{15}N) as the sole nitrogen source for one week under the conditions described above. Then, leaves were harvested, washed with distilled water, lyophilized, ground to powder, and stored until use.

The total, Kjeldahl and inorganic nitrogen content in each sample was analyzed as reported elsewhere (Morikawa *et al.*, 2004). UN was calculated from the following equation: N = total nitrogen – (Kjeldahl nitrogen + inorganic nitrogen).

Hereafter, the total, Kjeldahl and inorganic nitrogen and UN derived from NO_2 was designated TNNO2, RNNO2, INNO2 and UNNO2, respectively, and the total, Kjeldahl and inorganic nitrogen and UN derived from nitrate was designated TNNIT, RNNIT, INNIT and UNNIT, respectively.

Results and Discussion

Formation of UN from nitrogen dioxide

Table I summarizes the UNNO2 in the leaves of 12 plant species that were fumigated with 4 ppm ^{15}N-labeled NO_2 for 4–8 h. Clearly, all the plant species were found to contain UNNO2 (10.2–31.1% of the TNNO2). Thus, the formation of UNNO2 in plants appears to be common. We therefore conclude that there must be a novel metabolism to form UN from NO_2 in plants. It is noteworthy that the mean UNNO2 contents markedly varied among plant species from 22.8 to 281 ng per mg dry weight. This suggests that the UN-bearing compound(s) among plant species or that the composition of UN-bearing compounds differs, or both. It should be noted, however, that since NO and NO_2 enter the leaves through the cuticle and stomata (Larcher, 1995) the different level of UN in the tested plants might simply reflect different uptake of nitrogen oxides rather than different metabolism.

Table I also shows the results for the transgenic tobacco clone 271 (Vaucheret *et al.*, 1992) whose nitrite reductase (NiR) activity is almost completely removed by the expression of NiR cDNA in an antisense orientation (Vaucheret *et al.*, 1992; Takahashi *et al.*, 2001). The mean UNNO2 content of this transgenic tobacco was very similar to that of the RNNO2, and its percentage UNNO2 was about 2.5 times higher than that of the wild type (Table I). Because the nitrite content in this transgenic tobacco is about five times higher than that in the wild type (Vaucheret *et al.*, 1992; Goshima *et al.*, 1999), high levels of nitrite ion in the cells seem to have enhanced the formation of UN (see below). This interpretation was further supported by the fact that although the uptake of

Table I. Kjeldahl nitrogen, inorganic nitrogen and unidentified nitrogen (UN), designated RNNO2, INNO2 and UNNO2, respectively, in the leaves of 12 plant species after fumigation with ^{15}N-labeled NO_2 (from Morikawa *et al.*, 2004)[a].

Plant species	RNNO2 [ng mg^{-1} DW]	INNO2 [ng mg^{-1} DW]	UNNO2 [ng mg^{-1} DW]	
Wild-type plants				
Pittosporum tobira	50.2 ± 3.8	N. D.[b]	22.8 ± 5.2	(31.1)[c]
Arabidopsis thaliana	358 ± 75	11.6 ± 1.2	164 ± 52	(30.9)
Hordeum vulgare	785 ± 95	19.6 ± 3.1	272 ± 5	(25.3)
Cucurbita maxima	253 ± 13	7.5 ± 1.6	81.6 ± 13.0	(23.8)
Oryza sativa	951 ± 150	14.8 ± 2.0	281 ± 149	(22.5)
Erechtites hieracifolia	786 ± 51	22.1 ± 0.7	202 ± 51	(20.0)
Rhododendron mucronatum	114 ± 3	N. D.	28.5 ± 2.5	(19.9)
Nicotiana tabacum	798 ± 142	0.1 ± 0.2	182 ± 42	(18.8)
Triticum aestivum	573 ± 63	16.8 ± 4.9	108 ± 64	(15.5)
Glycine max	477 ± 88	34.3 ± 9.4	91.4 ± 12.9	(15.2)
Eucalyptus viminalis	741 ± 52	23.1 ± 2.9	134 ± 53	(14.9)
Spinacia oleracea	2320 ± 69	46.4 ± 2.0	270 ± 69	(10.2)
Transgenic plant				
Nicotiana tabacum (clone 271)[d]	238 ± 43	10.5 ± 3.8	214 ± 40	(46.3)

[a] Plants were fumigated with 4 μl l^{-1} ^{15}N-labeled NO_2 for 4 h except for *Pittosporum tobira* which was fumigated for 8 h. Each value represents the mean ± SD (n = 3 to 5).
[b] Not detected.
[c] Each value in parenthesis corresponds to the amount of UNNO2 as a percentage of TNNO2.
[d] Provided by Dr. Michel Caboch, Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Versailles, France.

NO_2-nitrogen by the transgenic tobacco (reflected in TNNO2) was less than twice that by the wild type, the mean UNNO2 contents of these two types of tobacco did not largely differ (see Table I). This suggests that the conversion efficiency of NO_2-nitrogen to UNNO2 is much higher in the transgenic than in the wild type.

Formation of UN from nitrate

Table II summarizes the UNNIT in the leaves of 6 plants species that were grown with a medium containing 20 mM or 50 mM KNO_3 as the sole nitrogen source for 1 week. It appeared that all but *Hordeum vulgare* formed a significant amount of UNNIT. Clearly, all but barley exhibited the formation of the UNNIT. Thus, the UN can be formed from the nitrate nitrogen. Our present findings of the formation of the UN from both NO_2 and nitrate collectively indicate that there is a novel nitrogen metabolism for the formation of UN in plants.

The UNNIT of the four plant species such as *Arabidopsis thaliana*, *Spinacia oleracea*, *Glycine max* and *Eucalyptus viminalis* was 0.2 to 1.4 μg per mg dry weight, which comprised 7 to 19% of the TNNIT. Note that these values were somewhat smaller than the value observed with tobacco (see Table II), but that they are orders of magnitude greater than UNNO2 shown in Table I. Upon fumigation with nitrogen dioxide (NO_2), *Hordeum vulgare* converted approximately 25% of TNNO2 into UNNO2 as reported previously (Morikawa *et al.*, 2004). The reason why this species did not form significant level of UNNIT is unclear.

It should be noted that the nitrogen data in Table I reflect the nitrogen derived from the nitrate taken up "freshly" during the one-week feeding of ^{15}N-labeled nitrate. Thus, the UNNIT in Table I derives from those compounds that were formed *de novo* during this feeding period. Among the six plant species including tobacco, the TNNIT varied from about 2 to 20 μg per mg dry weight. *Hordeum vulgare* exhibited close to the maximum TNNIT, and thus the dependence of the UNNIT level on the TNNIT, as envisaged in our previous report (Morikawa *et al.*, 2004), was not observed in the present study.

Four species shown in Table II except for *Eucalyptus viminalis* and tobacco were grown with a half-strength MS salt for 5 weeks, while *Eucalyp-*

Table II. Total nitrogen, Kjeldahl nitrogen, inorganic nitrogen and unidentified nitrogen (UN), designated TNNIT, RNNIT, INNIT and UNNIT, respectively, in the leaves of 6 plant species after being fed with ^{15}N-labeled KNO_3.

Plant species	TNNIT[a] [μg mg^{-1} DW]	RNNIT[a] [μg mg^{-1} DW]	INNIT[a] [μg mg^{-1} DW]	UNNIT[a] [μg mg^{-1} DW]
Eucalyptus viminalis	7.15 ± 1.31	5.25 ± 1.00	0.54 ± 0.17	1.37 ± 0.34 (19)[c]
Glycine max	2.25 ± 0.29	1.97 ± 0.21	0.06 ± 0.02	0.23 ± 0.08 (10)
Spinacia oleracea	14.9 ± 3.9	9.20 ± 2.49	4.45 ± 0.97	1.23 ± 0.53 (8)
Arabidopsis thaliana	20.6 ± 1.8	10.9 ± 2.2	8.25 ± 0.71	1.43 ± 1.36 (7)
Hordeum vulgare	19.0 ± 2.6	11.5 ± 1.8	7.32 ± 1.51	0.23 ± 0.53 (2)
Nicotiana tabacum[b]	20.2 ± 2.6	4.86 ± 0.57	9.79 ± 1.92	5.58 ± 2.57 (28)

[a] All plants except tobacco were fed with 20 mM $K^{15}NO_3$ for 1 week. Data except for tobacco represent mean of 3 to 4 replicates with SD.
[b] Data for tobacco was taken from Morikawa *et al.* (2004), where plants were fed with 50 mM $K^{15}NO_3$ for 1 week.
[c] Each value in parenthesis corresponds to the amount of UNNIT as a percentage of TNNIT.

tus viminalis was grown with a 1/80 strength of MS salts for 2 months (see Materials and Methods). Tobacco was cultured in B-medium (Bourgin *et al.*, 1979) which contained 20 mM KNO_3 as the sole nitrogen source for the first two weeks followed by a further 4-week culture with B-medium supplemented with 10 mM ammonium succinate as the sole nitrogen source as reported elsewhere (Morikawa *et al.*, 2004). It is likely that the state and way of nitrogen supply (concentration and compounds) is an important factor for the formation of UN in plants. This factor deserves further study.

UN level in transgenic plants

Sakamoto *et al.* (2002) have shown that an enzyme formerly known as glutathione dependent formaldehyde dehydrogenase (GS-FDH) from *Arabidopsis thaliana* possesses the activity to reduce *S*-nitrosoglutathione (GSNO) and to convert the nitroso group of GSNO to ammonia. This enzyme, GSNO reductase (GSNOR), is involved in the metabolism of reactive nitrogen species (RNS) in *Arabidopsis thaliana* plants. More recently, mammalian GS-FDH/NOR metabolizes GSNO to glutathione sulfinamide (Höög *et al.*, 2003).

We therefore produced transgenic plants of *Arabidopsis thaliana* which bear a cDNA of GSNOR gene from *Arabidopsis thaliana* under the control of cauliflower 35S promoter and nopaline sythase terminator. A transgenic line (line TR 4-3-2) that had a ten-fold higher GSNOR activity than the wild-type plants was obtained (our unpublished results). Plants of this line were fumigated with 4 ppm ^{15}N-labeled NO_2 for 4 h. The amount of UNNO2 in the transgenic plants with comparison with that of the wild-type (WT) was analyzed.

The amount of UNNO2 comprised 12% on average of TNNO2 in WT whereas it comprised 9% of TNNO2 in the transgenic line TR 4-3-2. This difference was significant at $P < 0.01$ level. This indicates that the level of the UN was significantly reduced by the overexpression of the transgene. This provides the first direct evidence that the UN-bearing compounds are metabolizable. Which type of the UN-bearing compounds are metabolized in the transgenic plant line is not known yet (see also below).

Mechanism of the formation of UN-bearing compounds

Fig. 1 depicts a scheme (modified from Morikawa *et al.*, 2004) for the formation of UN-bearing compounds. The reaction of NO_2 with water (pathway 1) to form nitric acid and NO is similar to the one in the Ostwald process for manufacturing nitrate from ammonia produced by the Habor process. Formation of nitrate from NO_2 taken up by plant cells has been reported (Amman *et al.*, 1995). Nitrate formed is metabolized via the primary nitrate reduction pathway and glutamine-glutamate cycle to organic nitrogenous compounds (Lea, 1993; pathway 2), the nitrogen of which is stoichiometrically recoverable by the Kjeldahl method.

NO formed in the pathway 1 as well as the one formed via endogenous enzymatic and nonenzymatic processes (Yamasaki *et al.*, 1999; Chandok *et al.*, 2003 and references therein) may nitrosate and/or nitrate organic compounds in the cells to form nitroso and nitro compounds, including *N*-nitroso and *S*-nitroso ones (Beckman, 1996). Or it may be transformed in combination with the su-

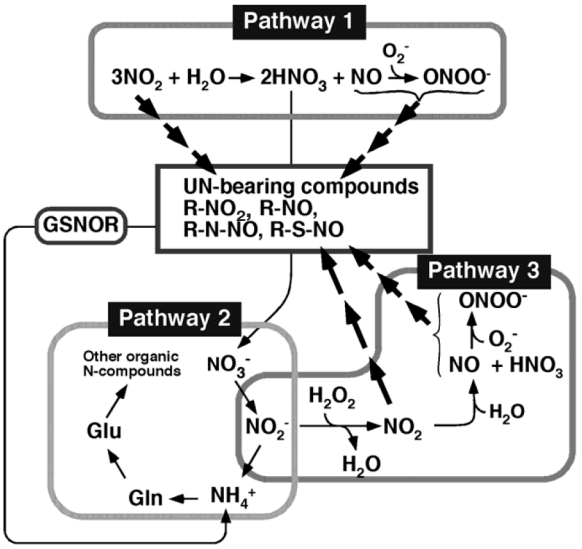

Fig. 1. A proposed metabolic pathway to form the UN-bearing compounds in plants (modified from Morikawa *et al*., 2004).

peroxide anion (O_2^-) to peroxynitrite ($ONOO^-$), a strong nitrating reagent (Nonoyama *et al.*, 1999).

As described above (see Table I), the maximal ratio of the Kjeldahl nitrogen to UN observed is close to two to one in 12 different plant species fumigated with NO_2. Direct nitration of cellular organic compounds may also occur by the reaction with either exogenous or endogenous NO_2 (see pathway 1 and 3, respectively). The nitrate taken up through roots and that produced from NO_2 gas taken up through leaf stomata is principally metabolized through pathway 2 (Lea, 1993).

Pathway 3 depicts a novel one to form UN-bearing compounds through NO and $ONOO^-$ formed from nitrite produced in pathway 2. This mechanism is based on the fact that an antisense tobacco clone 271, in which nitrite reductase activity is suppressed to less than 5% of the wild-type plant (Vaucheret *et al.*, 1992), had a high nitrite content and formed a UN more than two times greater than the wild-type plant (see Table I; Morikawa *et al.*, 2004). The conversion of nitrite to NO_2 shown in pathway 3 may occur by the peroxidase-like activity of non-symbiotic haemoglobin in plants (Sakamoto *et al.*, 2004). Or at low pH values, nitrite can form NO through formation of nitrous acid and nitrosonium ion (NO^+) in the cells (Morikawa *et al.*, 2004). NO emission from plants has been measured directly (Rockel *et al.*, 2002) and the amount of UN produced by plants cannot be accounted for by the maximal activity of NR. Therefore, an alternative route depicted in pathway 3 must exist in plant cells. The GSNOR that converts the nitrogen of GSNO to ammonia is the only enzyme that is known to date to be involved in the metabolism of the UN-bearing compounds (see above).

Structure and function of UN-bearing compounds identified to date

Fig. 2 depicts the structure of two compounds identified to date as the UN-bearing compounds in the extracts of the leaves of *Arabidopsis thaliana* fumigated with 4 ppm NO_2 for 4–8 h. As reported previously (Morikawa *et al.*, 2004), the major part of the UN of NO_2-fumigated leaves of *Arabidopsis thaliana* was water-soluble. After close examination of HPLC fractions from the water extract on the basis of ^1H, ^{13}C and ^{15}N NMR spectra (1D/2D NMR) as well as MS fragmentation patterns (Miyawaki *et al.*, 2004), a Δ^2-1,2,3-thiadiazoline derivative (Fig. 2A) has come out as one of the UN candidates (Miyawaki *et al.*, 2004). The literature search revealed that there was very little description about Δ^2-1,2,3-thiadiazolines, and thus chemical synthesis of this compound to quantify its amount in the leaves remains to be determined.

An acetone extract from NO_2-fumigated leaves of *Arabidopsis thaliana* was analyzed by reverse-phase HPLC, and a peak corresponding to 4-nitro-β-carotene (Fig. 2B) was found (Hirata *et al.*, unpublished results). Authentic β-carotene was reacted with tetranitromethane to obtain 4-nitro-β-

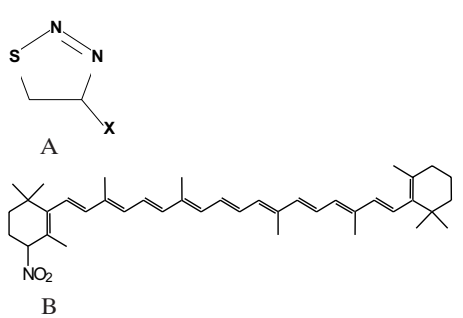

Fig. 2. UN-bearing compounds identified in the extracts of the leaves of *Arabidopsis thaliana* fumigated with 4 ppm NO_2 for 4–8 h: a Δ^2-1,2,3-thiadiazoline derivative (A) and 4-nitro-β-carotene (B) from the water- and acetone-soluble fraction, respectively.

carotene to confirm this interpretation, and the amount of this compound was estimated to be < 1% of the UNNO2 in the leaves.

The fluorometric method (Marzinzig *et al.*, 1997) for the analysis of *S*-nitrosothiols (RSNO) indicated that the level of RSNO was increased by about 5 times upon fumigation of *Arabidopsis thaliana* leaves with NO_2, which accounted for about 5% of the UNNO2 (Morikawa *et al.*, 2004). Nitrated protein tyrosines were found to form by fumigation with NO_2 (Morikawa *et al.*, 2004), which accounted for about 0.1–1% of the UNNO2. Kjeldahl digestion of these UN-bearing compounds or their derivatives confirmed that they contain Kjeldahl-unrecoverable nitrogen (our unpublished results). Whether these UN-bearing compounds are formed from nitrate-nitrogen is to be investigated in future.

The structures of the UN-bearing compounds identified to date strongly suggest in turn the involvement of reactive nitrogen species (RNS) such as NO and NO_2 in the formation of the UN-bearing compounds (Miyawaki *et al.*, 2004; see Fig. 1). Conceivably, there must exist a cross talk between the UN metabolism and RNS metabolism. It is thus likely that some UN-bearing compounds, if not all, should provide a chemical basis for understanding the metabolic fate and physiological function of RNS in the organism. Consistent with this speculation is the report by Sakamoto *et al.*, (2003) that 2-Cys peroxiredoxin (2CPRX) from *Arabidopsis thaliana* has a peroxynitrite-scavenging activity and thus detoxifies the cytotoxicity of RNS. More recently the formation of *N*-nitroso compounds from NO and its involvement in mammalian signal transduction has been reported (Bryan *et al.*, 2004). The relevance of the UN-bearing compounds in the air pollution control and in the quality control of crops deserves further investigation.

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