Novel Metabolism of Nitrogen in Plants
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Our previous study showed that approximately one-third of the nitrogen of \textsuperscript{15}N-labeled NO\textsubscript{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 \textit{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\textsubscript{2} (about 10–30\% of the total nitrogen derived from NO\textsubscript{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\textsubscript{2} was greatly increased in the transgenic tobacco clone 271 (Vaucheret \textit{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\textsubscript{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 \textit{et al.}, 2004) and GSNOR (Sakamoto \textit{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 \textit{Arabidopsis thaliana} fumigated with NO\textsubscript{2} include a \textit{\Delta}2-1,2,3-thiadiazoline derivative (Miyawaki \textit{et al.}, 2004) and 4-nitro-\beta-carotene.

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

Introduction

Atmospheric nitrogen dioxide (NO\textsubscript{2}) is a major pollutant in the urban air which is a cause of photochemical oxidants including ozone produced by photochemical reactions (Wellburn \textit{et al.}, 1997). Tropospheric ozone is known to be harmful for plants and animals including humans (Wellburn \textit{et al.}, 1997).

The transactions between plant and NO\textsubscript{x} (NO plus NO\textsubscript{2}) have so far been investigated on the nutritional role of this gas in plants (Rogers \textit{et al.}, 1979; Yoneyama and Sasakawa, 1979) or on the role of plants as a sink for this air pollutant (Hill, 1971; Rogers \textit{et al.}, 1979; Morikawa \textit{et al.}, 1998). However, the quantitative role of NO\textsubscript{2}-nitrogen as the N source remains unclear. Also, quantitative contribution of plants as a sink for the global atmospheric NO\textsubscript{x} is yet unknown (Lerdau \textit{et al.}, 2000).

In our attempt to study the mechanism of NO\textsubscript{2} metabolism in the plant-mediated decontamination of this major air pollutant (Morikawa \textit{et al.}, 1998, 2003; Goshima \textit{et al.}, 1999; Takahashi \textit{et al.}, 2001, 2003), we unexpectedly discovered (Morikawa \textit{et al.}, 2004) that about one-third of the total nitrogen derived from NO\textsubscript{2} taken up in the leaves of \textit{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 \textit{et al.}, 2004). In this communication we will focus on the following aspects of this novel nitrogen metabolism: (i) Forma-
tion of UN from NO₂, (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 ¹⁵N-labeled NO₂; treatment with ¹⁵N-labeled nitrate

Fumigation of plants with ¹⁵N-labeled NO₂ was performed as reported previously. For treatment with ¹⁵N-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 K¹⁵NO₃ (10.6 atomic percentage ¹⁵N) 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 NO₂ 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 ¹⁵N-labeled NO₂ 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 NO₂ 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 NO₂ 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 15N-labeled NO2 (from Morikawa et al., 2004)*.

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

* Plants were fumigated with 4 µl l⁻¹ 15N-labeled NO2 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.

NO2-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 NO2-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 KNO3 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 NO2 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 (NO2), 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 15N-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 Eucalypt-
4 ppm results. Plants of this line were fumigated with wild-type plants was obtained (our unpublished had a ten-fold higher GSNOR activity than the terminator. A transgenic line (line TR 4-3-2) that of cauliflower 35S promoter and nopaline synthase gene from Arabidopsis thaliana glutathione sulfinamide (Höög mammalian GS-FDH/NOR metabolizes GSNO to in the metabolism of reactive nitrogen species (RNS) enzyme, GSNO reductase (GSNOR), is involved in the nitroso group of GSNO to ammonia. This enzyme formerly known as glutathione dependent formaldehyde dehydrogenase (GS-FDH) from Arabidopsis thaliana formaldehyde dehydrogenase (GS-FDH) from Arabidopsis thaliana glutathione sulfinamide (Höög 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 synthase 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 15N-labeled NO2 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 NO2 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 Haber process. Formation of nitrate from NO2 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-

### 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 15N-labeled KNO3.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>TNNITa [µg mg⁻¹ DW]</th>
<th>RNNITa [µg mg⁻¹ DW]</th>
<th>INNITa [µg mg⁻¹ DW]</th>
<th>UNNITa [µg mg⁻¹ DW]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus viminalis</td>
<td>7.15 ± 1.31</td>
<td>5.25 ± 1.00</td>
<td>0.54 ± 0.17</td>
<td>1.37 ± 0.34 (19)c</td>
</tr>
<tr>
<td>Glycine max</td>
<td>2.25 ± 0.29</td>
<td>1.97 ± 0.21</td>
<td>0.06 ± 0.02</td>
<td>0.23 ± 0.08 (10)</td>
</tr>
<tr>
<td>Spinacia oleracea</td>
<td>14.9 ± 3.9</td>
<td>9.20 ± 2.49</td>
<td>4.45 ± 0.97</td>
<td>1.23 ± 0.53 (8)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>20.6 ± 1.8</td>
<td>10.9 ± 2.2</td>
<td>8.25 ± 0.71</td>
<td>1.43 ± 1.36 (7)</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>19.0 ± 2.6</td>
<td>11.5 ± 1.8</td>
<td>7.32 ± 1.51</td>
<td>0.23 ± 0.53 (2)</td>
</tr>
<tr>
<td>Nicotiana tabacumb</td>
<td>20.2 ± 2.6</td>
<td>4.86 ± 0.57</td>
<td>9.79 ± 1.92</td>
<td>5.58 ± 2.57 (28)</td>
</tr>
</tbody>
</table>

a All plants except tobacco were fed with 20 mM K15NO3 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 K15NO3 for 1 week. 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 KNO3 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. The 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 synthase 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 15N-labeled NO2 for 4 h. The amount of UNNO2 in the transgenic plants with comparison with that of the wild-type (WT) was analyzed.
peroxide anion (\(O_2^-\)) to peroxynitrite (\(\text{ONOO}^-\)), 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 (\(\text{NO}^+\)) 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 \(^1\text{H}, ^{13}\text{C}\) and \(^{15}\text{N}\) NMR spectra (1D/2D NMR) as well as MS fragmentation patterns (Miyawaki et al., 2004), a \(\Delta^2\)-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 \(\Delta^2\)-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-\(\alpha\)\(\beta\)-carotene (Fig. 2B) was found (Hirata et al., unpublished results). Authentic \(\alpha\)\(\beta\)-carotene was reacted with tetranitromethane to obtain 4-nitro-\(\alpha\)\(\beta\)-
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 NO2, which accounted for about 5% of the UNNO2 (Morikawa et al., 2004). Nitrated protein tyrosines were found to form by fumigation with NO2 (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 NO2 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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