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Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*

Hideo Yamasaki

Laboratory of Cell and Functional Biology, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan (*yamasaki@sci.u-ryukyu.ac.jp*)

Air pollution studies have shown that nitric oxide (NO), a gaseous free radical, is a potent photosynthetic inhibitor that reduces CO_2 uptake activity in leaves. It is now recognized that NO is not only an air pollutant but also an endogenously produced metabolite, which may play a role in regulating plant cell functions. Although many studies have suggested the presence of mammalian-type NO synthase (NOS) in plants, the source of NO is still not clear. There has been a number of studies indicating that plant cells possess a nitrite-dependent NO production pathway which can be distinguished from the NOS-mediated reaction. Nitrate reductase (NR) has been recently found to be capable of producing NO through one-electron reduction of nitrite using NAD(P)H as an electron donor. This review focuses on current understanding of the mechanism for the nitrite-dependent NO production in plants. Impacts of NO produced by NR on photosynthesis are discussed in association with photo-oxidative stress in leaves.

Keywords: active nitrogen; nitric oxide; oxidative stress; photoinhibition

1. INTRODUCTION

Irradiation of high light (or excess photon energy) on plants causes reduction of photosynthetic activity, a phenomenon broadly referred to as photoinhibition. Photoinhibition involves complex biophysical, biochemical and physiological processes, which can be defined by their differing relaxation times ranging over 15 orders of magnitude in size and time (Osmond 1994). Numerous studies indicate that photoinhibition can be distinguished into two qualitatively distinct classes on the basis of their relaxation time, i.e. dynamic photoinhibition and chronic photoinhibition (Castro *et al*. 1995; Osmond 1994; Skillman & Osmond 1998).

Dynamic photoinhibition (also designated as short-term photoinhibition, photoprotection or photon protection) is a reversible process on a time-scale of minutes to hours without significant hysteresis. Dynamic photoinhibition is considered to be the engagement of down-regulation, which dissipates excess photon energy in photosystem II (PS II) antenna as heat (Demming-Adams *et al*. 1996; Gilmore 1997). The photoprotective heat dissipation has been measured as non-photochemical quenching (NPQ) of the PS II chlorophyll *a* fluorescence (Osmond 1994). The NPQ mechanism is known to be controlled by the special xanthophyll cycle pigments (Gilmore 1997). Formation of ΔpH across the thylakoid membranes contributes to the downregulation of PS II through activation of the xanthophyll cycle (Gilmore & Yamamoto 1992; Gilmore & Yamasaki 1998). Chronic photoinhibition (also designated as long-term photoinhibition, stress-induced photoinhibition, photodamage or photon damage) is more slowly reversible on a time-scale of hours to days. In contrast to dynamic photoinhibition that is associated with the primary event of photosynthesis, chronic photoinhibition involves a range of inactivation of photosynthetic machinery, which eventually decreases photon-using capacity (Asada 1999; Osmond 1994).

It is now evident that activated harmful molecular species produced upon illumination participate in the inhibition or impairment of photosynthesis (Asada 1999; Asada *et al*. 1998). Singlet-excited oxygen ($^1O_2$) is generated by the interaction of ground-state oxygen ($^3O_2$ or more simply O_2) with triplet-excited chlorophyll ($^3Chl^*$) produced in the PS II reaction centre (Macpherson *et al*. 1993). The electron transfer to O_2 in photosystem I (PS I) generates superoxide radicals (O_2^-), and the disproportionation of O_2^- catalysed by superoxide dismutase (SOD) results in the production of hydrogen peroxide (H_2O_2). If O_2^- and H_2O_2 are not scavenged rapidly at the site where they are produced, their interaction with transition metal ions may produce hydroxyl radicals (·OH), the most reactive and harmful species of active oxygen. These active oxygen species ($^1O_2$, O_2^-, H_2O_2 and ·OH) can potentially oxidize target molecules in the photosynthetic apparatus, such as the D_1 protein in the PS II reaction centre and some Calvin–Benson cycle enzymes, which eventually leads to chronic photoinhibition (Asada 1999). Therefore, understanding the mechanisms for generation and relaxation of activated molecular species during photosynthesis is of especial importance to reveal how plants cope in environments having excess light energy.

Recent studies have suggested that plant cells, as well as animal cells, produce active molecular species of nitrogen in addition to active oxygen species (Delledonne *et al*. 1998; Durner & Klessig 1999). Nitric oxide (NO) and nitric dioxide (NO_2) have been reported to be produced in leaves of higher plants (Dean & Harper 1986; Harper 1981; Klepper 1990). However, the mechanism of the active nitrogen production and its effects on photosynthesis are largely unknown. This review focuses on the mechanism by which plants produce NO and discusses a possible involvement of active nitrogen species in oxidative damage associated with photoinhibition.

2. NITRIC OXIDE AS A PHOTOSYNTHETIC INHIBITOR

NO is a well-known air pollutant as NOx ($NOx = NO + NO_2$), which is produced by the combustion of fossil fuels or the manufacture of nitrogenous fertilizers through industrial activities. NOx concentrations may range up to 100 ppb in the air over industrialized areas (Wildt *et al*. 1997). Because NO plays a key role in atmospheric chemistry via the photochemical production of ozone (O_3) in the troposphere, the molecule is important for atmospheric radical balance and generation of photooxidants.

NOx is a cytotoxic agent to both plants and animals (Wellburn *et al*. 1980). However, in contrast with other air pollutant gases, such as sulphur dioxide (SO_2) and O_3, phytotoxicity of NOx has been considered to be relatively weak. Fumigation with NOx sometimes does not show deleterious effects but it often stimulates plant growth probably acting as a nitrogen fertilizer (Yoneyama *et al*. 1979). There has therefore been controversy as to whether NOx at atmospheric concentrations is phytotoxic (Wellburn 1990).

Taylor & Eaton (1966) reported that long exposure to a low concentration of NO_2 suppressed the growth of bean and tomato seedlings. Hill & Bennett (1970) found that NO inhibited net photosynthetic activity of intact leaves of oats (*Avena sativa* L. var. Park) and alfalfa (*Medicago sativa* L. var. Ranger) at concentrations below those required to cause visible injury symptoms. They reported that the inhibition of CO_2 uptake activity by NO was rapid and reversible. The activity was fully recovered by stopping fumigation with NO (Hill & Bennett 1970). Saxe (1986*a*) showed that reduction of net photosynthesis occurred at a lower dose of NO than that required to reduce respiration. Saxe also showed that NO uptake had no significant dependence on stomatal opening, suggesting that the main effect of NO is on mesophyll cells rather than guard cells (Saxe 1986*b*). Based on the effective uptake, he estimated the toxicity of NO towards net photosynthesis as 22 times higher than that of NO_2 (Saxe 1986*b*).

Because both NO and NO_2 produce nitrite in an aqueous solution, it has been widely presumed that the toxicity is ascribed to the nitrite toxicity on photosynthesis (Wellburn 1990). Nitrite was reported to acidify the stroma, which may inhibit the enzymatic reactions in the Calvin–Benson cycle (Purczeld *et al*. 1978). Zeevaart (1976) argued that the chloroplastic pH would only change if the number of protons entering the chloroplasts exceeds the amount removed by the nitrite reductase (NiR)-catalysed reduction of nitrite. Although nitrite has no significant inhibitory effect on *in vitro* phosphorylation, a possible site of inhibitory action for nitrite has been demonstrated (Wellburn 1990). Wellburn found that nitrite enhanced the release of bound manganese from thylakoid membranes, a phenomenon having the potential to cause a donor-side photoinhibition (Wellburn 1984). Nitrite does not affect the light-induced formation of ΔpH across the isolated thylakoid membranes at concentrations below 1 mM but it inhibits at lower concentrations when SO_3^- is co-present (Robinson & Wellburn 1983). Robinson & Wellburn (1983) suggested that a free radical mechanism may be involved in the synergistic effects of nitrite and sulphite. Later, Shimazaki and others demonstrated light-dependent NO_2-induced chlorophyll bleaching of kidney bean leaves, which was closely correlated to the accumulation of nitrite (Shimazaki *et al*. 1992). Based on the observation that radical scavengers such as tiron effectively suppressed chlorophyll bleaching, they suggested that nitrite would stimulate formation of the PS-I-dependent production of active oxygen species in the stroma (Shimazaki *et al*. 1992).

Because of many contradictory results, probably due to differences in experimental conditions and in the plant species used (Wellburn 1990), it is difficult to deduce generalized effects of NOx. Nevertheless, it appears reasonable to summarize the inhibitory effects of NOx as follows.

(i) Photosynthesis is more sensitive to NOx than respiration.
(ii) The toxicity of NOx on photosynthesis is ascribed to the nitrite accumulation in leaves.
(iii) The *in vitro* activities of photosynthetic electron transport and photophosphorylation are relatively tolerant to nitrite.
(iv) The decline in net photosynthetic activity caused by NOx could be explained by its inhibitory effect on the Calvin–Benson cycle.
(v) Light has both a protective effect and a stimulative effect on the inhibition.
(vi) Oxidative damage mediated by active oxygen species may be involved in an impairment of photosynthesis and subsequent chlorophyll destruction.
(vii) The molecular mechanism of NOx inhibition of photosynthesis is not clear.

3. NITRIC OXIDE AS AN ENDOGENOUS METABOLITE IN LIVING CELLS

Until recently, research on the effects of NO in plants focused only on the aspect of atmospheric pollution as described in § 2. Since the discovery of enzymatic production of NO in cells and its pivotal roles in signal transduction, the endogenous NO production and its regulation has become a big issue in life sciences (Titheradge 1998). This drastic change in our appreciation of NO can be seen in the awards of 'Molecule of the Year' by *Science* magazine in 1992 and the Nobel Prize for physiology and medicine in 1998. Although there has been a growing body of literature that deals with NO,

most literature is in the fields of mammalian physiology, biochemistry and molecular biology. In contrast to improved knowledge about NO in animal systems, our understanding of NO in plants is still very limited.

In mammalian cells, NO is produced by NO synthases (NOS) that catalyse the production of NO from L-arginine in the presence of NADPH and molecular oxygen. A wide variety of NOS isoforms has been reported in vertebrates as well as invertebrates (Hemmens & Mayer 1998). In 1996, evidence for the presence of NOS activities in plants was reported (Cueto *et al*. 1996; Leshem 1996; Ninnemann & Maier 1996). It has been shown that NO plays important roles in establishing symbiosis (Cueto *et al*. 1996; Mathieu *et al*. 1998), inducing phytoalexin production (Noritake *et al*. 1996) and regulating growth and development (Leshem & Haramaty 1996). Recently, it has been demonstrated that NO and NOS are involved in the activation of the plant defence system against pathogens (Delledonne *et al*. 1998; Durner *et al*. 1998). Inhibition of the conversion of L-arginine to citrulline by mammalian NOS inhibitors has suggested the presence of mammalian-type NOS in plants (Cueto *et al*. 1996; Delledonne *et al*. 1998; Ninnemann & Maier 1996). Although a protein of 166 kDa, which cross-reacted with antibodies raised against mouse macrophage or rabbit brain NOS, was identified in maize cells (Ribeiro *et al*. 1999), no protein or gene homologues to mammalian NOS have been isolated to date (Beligni & Lamattina 1999*a*; Bolwell 1999; Durner & Klessig 1999).

4. NON-ENZYMATIC NITRIC OXIDE PRODUCTION FROM NITRITE, A CHEMICAL PATHWAY

In addition to the NO-producing reaction catalysed by a putative NOS from L-arginine along with O_2 and NADPH, the presence of an alternative pathway for NO production has been known for many years, i.e. an inorganic nitrogen pathway (Evans & McAuliffe 1956; Greenwood & Earnshaw 1997). The slow and spontaneous liberation of NO can be observed with nitrite at neutral pH. Nitrite ion (NO_2^-) exists in equilibrium with its conjugate acid nitrous acid (HNO_2; pK_a 3.1–3.5). Because of this equilibrium, a small amount of HNO_2 could be present even at physiological pH. HNO_2 spontaneously produces dinitrogen trioxide (N_2O_3) as the result of a disproportionation reaction. NO can be generated by a decomposition of N_2O_3 *in vitro* from a nitrite solution through decomposition of nitrous acid as follows (Greenwood & Earnshaw 1997):

$$NO_2^- + H^+ \leftrightarrow HNO_2, \quad (1)$$

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O, \quad (2)$$

$$N_2O_3 \leftrightarrow NO + NO_2, \quad (3)$$

$$2NO + O_2 \leftrightarrow 2NO_2. \quad (4)$$

Because spontaneous NO production from nitrite is slow (Miles *et al*. 1996), NO production through the above pathway may be beyond detectable limits of NO detection systems (Schmidt & Mayer 1998) when a submillimolar nitrite solution is acidified at room temperature.

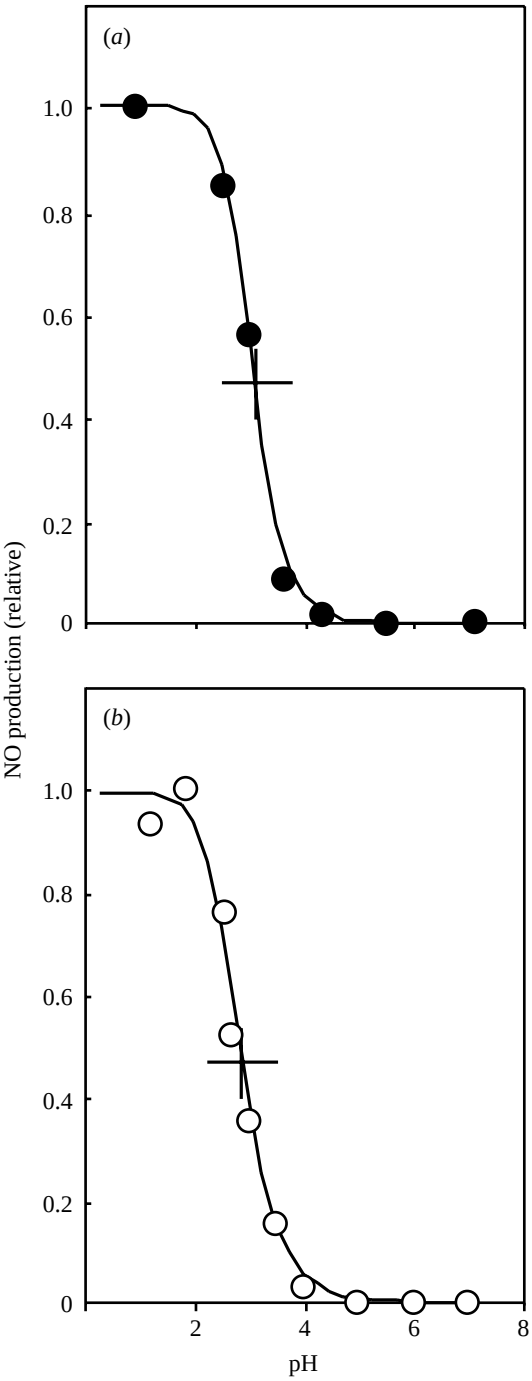

Figure 1. Chemical NO production from nitrite and its pH dependence. The reaction medium contained 20 mM KH_2PO_4, 1 µM $NaNO_2$ and (*a*) 0.1 M KI or (*b*) 2 mM sodium ascorbate. The pH of the medium was adjusted with phosphoric acid.

However, a large amount of NO will be produced from the above pathway in the presence of mild reductant. On a laboratory scale, NO has been synthesized quantitatively by the reduction of acidified nitrite solution with iodide or ferrocyanide or by the disproportionation of nitrous acid (HNO_2) in the presence of sulphuric acid. The chemical generation of NO is almost stoichiometric, thereby being widely used as a convenient method for calibration of NO concentrations (Schmidt & Mayer 1998):

$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + 2K_2SO_4 + 2H_2O + I_2. \quad (5)$$

Ascorbate (AsA), a physiologically important reductant in plants (Asada 1997; Yamasaki *et al*. 1995), has also been reported to induce chemical NO production from nitrite (Evans & McAuliffe 1956; Weitzberg & Lundberg 1998). One-electron oxidation of ascorbate produces monodehydroascorbate (MDA), an oxidized form of ascorbate:

$$2HNO_2 + 2AsA \rightarrow 2NO + 2MDA + 2H_2O. \quad (6)$$

Two molecules of MDA spontaneously disproportionate to ascorbate and dehydroascorbate (DHA):

$$2MDA \rightarrow AsA + DHA. \quad (7)$$

As the result of these reactions, two molecules of NO can be produced by ascorbate:

$$2HNO_2 + AsA \rightarrow 2NO + DHA + 2H_2O. \quad (8)$$

The nitrite–ascorbate system is frequently used to produce NO chemically (Beligni & Lamattina 1999*b*; Kröcke & Kolb-Bachofen 1996). Figure 1 shows the effect of pH on NO production via the chemical reduction of nitrite (H. Yamasaki and Y. Sakihama, unpublished data). Rapid NO production is observed when potassium iodide (KI) is added into an acidic medium. A very similar production of NO is also observed when ascorbate is added into a nitrite-containing acidic solution. The extent of NO production initiated by these reducing agents shows a clear pH dependence that nicely fits to the Henderson–Hasselbalch equation when the pK_a of HNO_2 is assumed to be 3.2. A complete parallelism of the results obtained with KI and ascorbate clearly indicates that the production of NO through the chemical reduction of nitrite requires the protonated form of nitrite (HNO_2), but not the ionic form (NO_2^-) as the NO donor.

Plant tissues contain a high amount of ascorbate, which plays an essential role in detoxifying active oxygen species via the ascorbate–glutathione cycle (Asada 1997; Foyer & Halliwell 1976; Yamasaki *et al*. 1999*b*). In chloroplasts, ascorbate is present at *ca*. 10 mM concentrations (Asada 1997) and nitrite is translocated into the organelles to reduce to ammonia (Crawford 1995). Thus, one can consider that translocated nitrite might be converted to NO via the chemical pathway using ascorbate in the chloroplasts prior to the reduction to ammonia by NiR. However, this is unlikely in physiological conditions. Translocation of nitrite into the chloroplasts requires the activity of photosynthetic electron transport (Shingles *et al*. 1996). Consequently, ΔpH across thylakoid membranes is formed by the electron transport (Yamasaki *et al*. 1991), thereby increasing pH of stroma to an alkaline range (*ca*. pH 8). Furthermore, the nitrite concentration in the chloroplasts has been reported to be at submillimolar levels (Wellburn 1984). As presented in figure 1, therefore, a physiological concentration of NO is not expected to be produced by the chemical pathway at alkaline pH, and even a very high concentration of nitrite (e.g. above 10 mM) will produce only picomols of NO under this condition. Thus, the chemical NO production via ascorbate–nitrite should be virtually negligible in the chloroplasts under favourable conditions. Perhaps, chemical NO production via the nitrite pathway could be pronounced only in acidic compartments or tissues (Lundberg *et al*. 1997; Weitzberg & Lundberg 1998) and degraded cells (Klepper 1990).

5. PRODUCTION OF NITRIC OXIDE BY NITRATE REDUCTASE

Nitrate reductase (NR) is the only plant enzyme conclusively shown to have NO-producing ability. The first clue was provided by Harper in 1981 who demonstrated the effects of gas purging on the *in vivo* NR assay (Harper 1981). This assay is a technique to determine the nitrate-reducing activity of tissues (Nicholas *et al*. 1976). Harper found that the level of NOx ($NO + NO_2$) evolved from soybean leaves during the *in vivo* NR assays with anaerobic gas purging (N_2 or argon) was greater than that with aerobic gas purging (air or O_2). A strong correlation between NOx evolution and NO_2^- accumulation was observed. Since no NOx evolution occurred in boiled (heat-denatured) leaf disks, Harper presumed an enzymatic NOx evolution pathway from NO_2^- in leaves (Harper 1981). Using a chlorate screening procedure, Harper and others isolated soybean mutants that lacked the constitutive NR activity in leaves but retained inducible NR activity in response to NO_3 (Nelson *et al*. 1983; Ryan *et al*. 1983). They demonstrated that a soybean mutant lacking constitutive NR activity (designated *nr1*) did not evolve NOx during *in vivo* NR assays (Nelson *et al*. 1983), the first evidence that NOx evolution was associated with the activity of constitutive NR in the leaves. It was later determined that there were two forms of constitutive NR in wild-type soybean leaves, which were designated c_1 NR and c_2 NR (Streit *et al*. 1985). Although there was controversy over the NOx evolution pathway from NO_3 via NO (Mulvaney & Hageman 1984), evidence that NO and N_2O were produced from NO_3 during nitrate reduction was provided by gas chromatography, mass spectrometry and ^{15}N tracer techniques (Dean & Harper 1986). Because the c_1 NAD(P)H:NR (EC 1.6.6.2) is unique to the Phaseoleae tribe of the family Leguminosae (Dean & Harper 1986), the evolution of NOx through the NR-dependent pathway was thought to be limited to legume species among higher plants (Dean & Harper 1988).

In 1997, Wildt and others reported NO emissions from several plant species other than Leguminosae, including sunflower, sugar cane, corn, rape, spruce, spinach and tobacco (Wildt *et al*. 1997). This observation raised a possibility that NO-producing ability may not be a specific phenomenon observed only in legume plants but could be a more general one among higher plants (Yamasaki *et al*. 1999*a*). If NO is produced by a similar mechanism to soybean leaves, other NRs should also produce NO from nitrite. Figure 2 shows *in vitro* NO production by maize NR (EC 1.6.6.1) using a Clark-type NO sensor (Yamasaki *et al*. 1999*a*). As described above, no significant NO production is observed at pH 7.0 when nitrite and NADH are added into the reaction mixture. Addition of NR into the reaction mixture causes a rapid increase in NO production. Because NO can be rapidly degraded through many routes, the lifetime of NO is considered to be several seconds in ambient air. Thus,

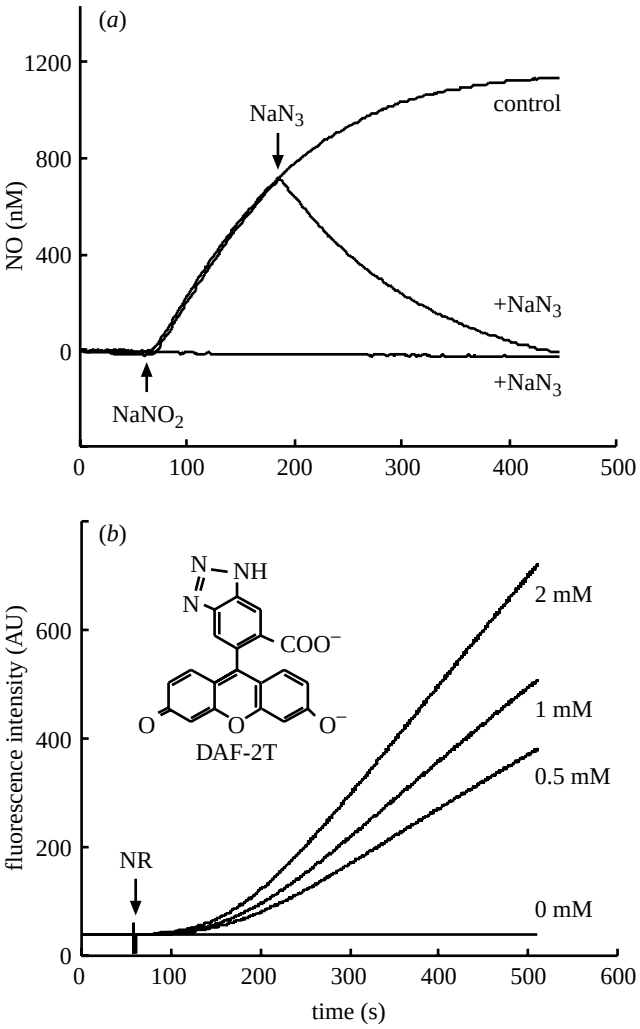

Figure 2. The nitrite-dependent NO production by maize NADH:NR (EC 1.6.6.1). (*a*) Time-courses for NO production measured with an NO electrode. The reaction mixture included 1 mM sodium nitrite, 100 µM NADH and 15 mU ml^{-1} of *maize* NR (Molecular Biologische Technologie, Germany). Sodium azide (1 mM) was added either before (lower trace) or during the reaction (middle trace). The reaction was initiated by adding sodium nitrite. (*b*) Time-courses for NO production measured by the fluorescence indicator DAF-2. The experimental conditions were similar to those in (*a*) except the presence of 10 µM DAF-2. Numbers beside each trace represent the concentration of added nitrite (mM). Redrawn from Yamasaki & Sakihama (2000).

there is a clear steady-state level for NO production even in the absence of any biomolecules. Haemoglobin is a strong quencher of NO, and thus effectively removes NO from a solution (Yamasaki *et al*. 1999*a*). The NO production from NR can be inhibited by sodium azide (NaN_3), a known inhibitor of NR (Yamasaki & Sakihama 2000). Fluorimetric detection of NO with diaminofluorescein-2 (DAF-2) (Nakatsubo *et al*. 1998) also shows NO production from nitrite by NR (Yamasaki & Sakihama 2000). Figure 3 shows a similar nitrite-dependent NO production with NAD(P)H:NR (EC 1.6.6.2) of the fungus *Aspergillus* (H. Yamasaki and Y. Sakihama, unpublished data). Furthermore, NR of *E. coli* was reported to produce NO in the presence of nitrite (Ji & Hollocher 1988). It is

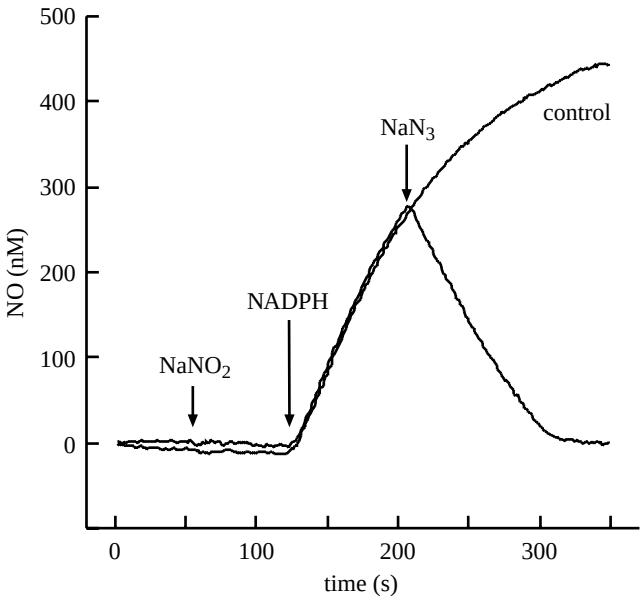

Figure 3. NO production from nitrite by fungus NAD(P)H:NR (EC 1.6.6.2). The experimental conditions were similar to those in figure 2 except for NADPH and *Aspergillus* NR in place of NADH and *maize* NR, respectively.

clear that the NO-producing activity of NR is a more general feature than was thought before.

6. PEROXYNITRITE PRODUCTION BY NITRATE REDUCTASE

Because NO is capable of acting as an intracellular signal messenger, the molecule has recently attracted much interest by researchers who are concerned with plant defence responses against pathogens (Camp *et al*. 1998; Delledonne *et al*. 1998; Durner *et al*. 1998; Durner & Klessig 1999). The regulatory function of NO can be ascribed to the high affinity of NO for Cu and Fe in haem and non-haem proteins (Titheradge 1998). Cytotoxicity of NO itself is rather weak in a comparison with active oxygen species such as O_2^- and ·OH. Even a small amount of NO production has been reported to function as an antioxidant to break chain reactions of lipid peroxidation (Rubbo & Freeman 1996). On the contrary, an excess of NO production has been suggested to lead to an increase of H_2O_2 production in mitochondria via inhibition of cytochrome *c* oxidase in the respiratory chain (Millar & Day 1996, 1997), which is a potential cause of oxidative stress for cells.

NO can be converted to peroxynitrite ($ONOO^-$) in the presence of O_2^- by the following reactions:

$$NO + O_2^- \rightarrow ONOO^-, \quad (9)$$

$$ONOO^- + H^+ \rightarrow ONOOH, \quad (10)$$

$$ONOOH \rightarrow NO_3^- + H^+. \quad (11)$$

The rate constant for the reaction between NO and O_2^- is near diffusion controlled (Koppenol 1998; Squadrito & Pryor 1998). The product peroxynitrite anion ($ONOO^-$) is stable when it is stored at −80 °C at pH 12 in the absence of target molecules. However, $ONOO^-$ at physiological pH

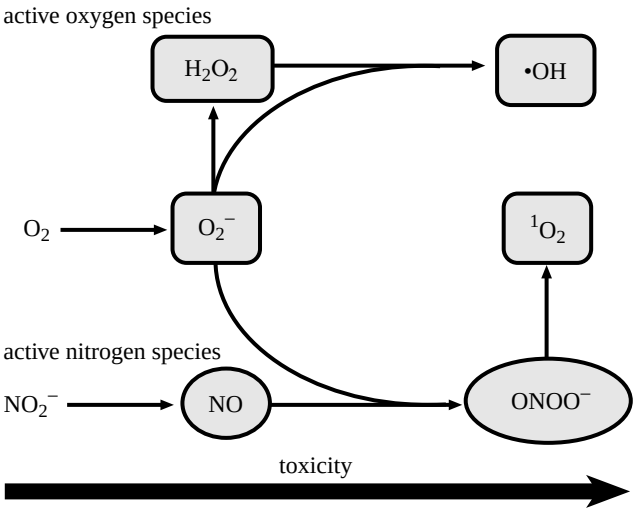

Figure 4. Sequential production of the activated toxic molecules and their interactions. Like hydrogen peroxide (H_2O_2), NO is membrane permeable and easily diffuses in cells. If superoxide radical (O_2^-) reacts with NO, the most toxic compound peroxynitrite ($ONOO^-$) is produced. The rate constant of this reaction is greater than that of the O_2^- disproportionating reaction catalysed by SOD. Singlet oxygen ($^1O_2$) can be produced from $ONOO^-$ in the reaction with H_2O_2 (Di Mascio *et al*. 1996). ·OH, hydroxyl radical.

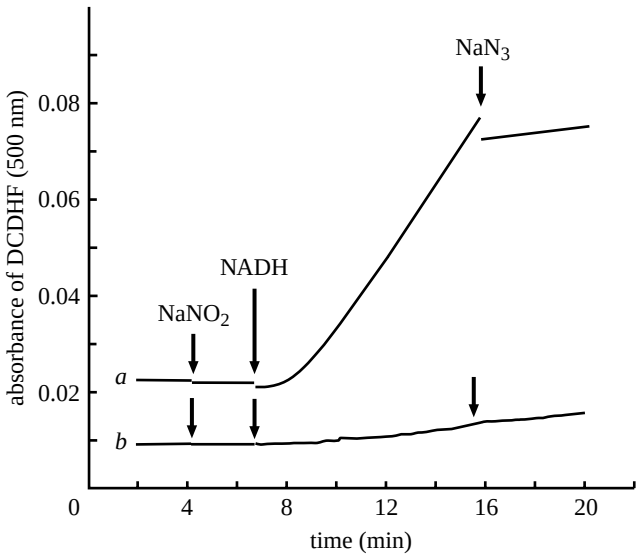

Figure 5. Formation of peroxynitrite ($ONOO^-$) by nitrate reductase. Production of peroxynitrite was monitored by the absorbance increase of DCDHF. The reaction medium included 100 µM DCDHF and 30 mU ml^{-1} maize NR. Nitrite (1 mM), NADH (1 mM) and NaN_3 (1 mM) were added at the arrows as indicated. *a*, Measured under ambient air; *b*, measured under a stream of nitrogen gas to remove oxygen. Redrawn from Yamasaki & Sakihama (2000).

is in rapid equilibrium with its conjugate acid, peroxynitrous acid (ONOOH, pK_a 6.8). ONOOH is a short-lived molecule and it spontaneously decays to nitrate with a half-life of less than 1 s via the formation of an energized intermediate ($ONOOH^*$) (Radi 1996). $ONOO^-$ has been considered to be a major cytotoxic agent of active nitrogen species derived from NO (Arteel *et al*. 1999; Koppenol 1998; Squadrito & Pryor 1998; Wink & Mitchell 1998). Moreover, the reaction between $ONOO^-$ and H_2O_2 may produce $^1O_2$, a highly toxic active oxygen (Di Mascio *et al*. 1996). Interactions between active oxygens and nitrogens are summarized in figure 4.

Barber & Kay (1996) reported that NR could use molecular oxygen as an electron acceptor, producing O_2^- as the product. If NO and O_2^- are both simultaneously produced by NR, nitrite-dependent production of $ONOO^-$ should be detected. Figure 5 shows the *in vitro* formation of $ONOO^-$ by maize NR (Yamasaki & Sakihama 2000). Absorbance of 2′,7′-dichlorodihydrofluorescein (DCDHF), an indicator for $ONOO^-$ (Ischiropoulos *et al*. 1999), is markedly increased by NADH in the presence of NR and NO_2^-. Sodium azide (an NR inhibitor), glutathione (an $ONOO^-$ scavenger) and depletion of molecular oxygen (the source of O_2^-) all completely suppress the formation of $ONOO^-$, indicating that NR is capable of producing three types of toxic molecules (i.e. NO, O_2^-, $ONOO^-$) when nitrite, the normal NR reaction product, is provided as the substrate (Yamasaki & Sakihama 2000).

7. NITRITE-DEPENDENT NITRIC OXIDE PRODUCTION *IN VIVO*

Several lines of evidence from both *in vitro* and *in vivo* assays have clearly indicated that NR is responsible for NO production in leaves. The next question is when NR produces NO in plant cells. Obviously, accumulation of nitrite in the cells is the key event to reveal the condition where the NR-dependent NO production occurs.

Under favourable conditions, nitrite produced by the normal NR reaction is translocated into chloroplasts. NiR (EC 1.6.6.4) located in the stroma, converts the translocated nitrite to ammonium ion (NH_4^+) using reduced ferredoxin as an electron donor. NH_4^+ is then assimilated into amino acids via the glutamine-synthase–glutamine: 2-oxoglutarate aminotransferase glutamine synthetase–glutamate synthase (GS–COGAT) pathway (Miflin & Lea 1980). The translocation of nitrite into the chloroplasts requires ΔpH across the chloroplast envelope (Shingles *et al*. 1996). Reduction of translocated nitrite catalysed by NiR needs reducing equivalents produced by the photosynthetic electron transport chain (Crawford 1995). Therefore, the activities of nitrogen assimilation and carbon assimilation are clearly interdependent (Foyer *et al*. 1995). Because of the co-regulated mechanism between nitrogen and carbon metabolism (Foyer *et al*. 1995), it is usually difficult to detect nitrite in healthy plant tissues in contrast with nitrate (Kawamura *et al*. 1996). However, nitrite can be accumulated in cells when photosynthetic electron transport is not operational. Inhibition of photosynthetic electron flow by herbicides such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) results in nitrite accumulation in illuminated leaves (Klepper 1975). DNP (2,4-dinitrophenol), a classical uncoupler, also causes nitrite accumulation in the dark through an indirect inhibition of nitrite reduction in the chloroplasts (Klepper 1976). Accumulation of nitrite has been also reported in an NiR-less transgenic tobacco (Vaucheret *et al*. 1992). These studies indicate that nitrite can be accumulated when photosynthetic activity is absent or inhibited, the condition where NR produces NO. Indeed,

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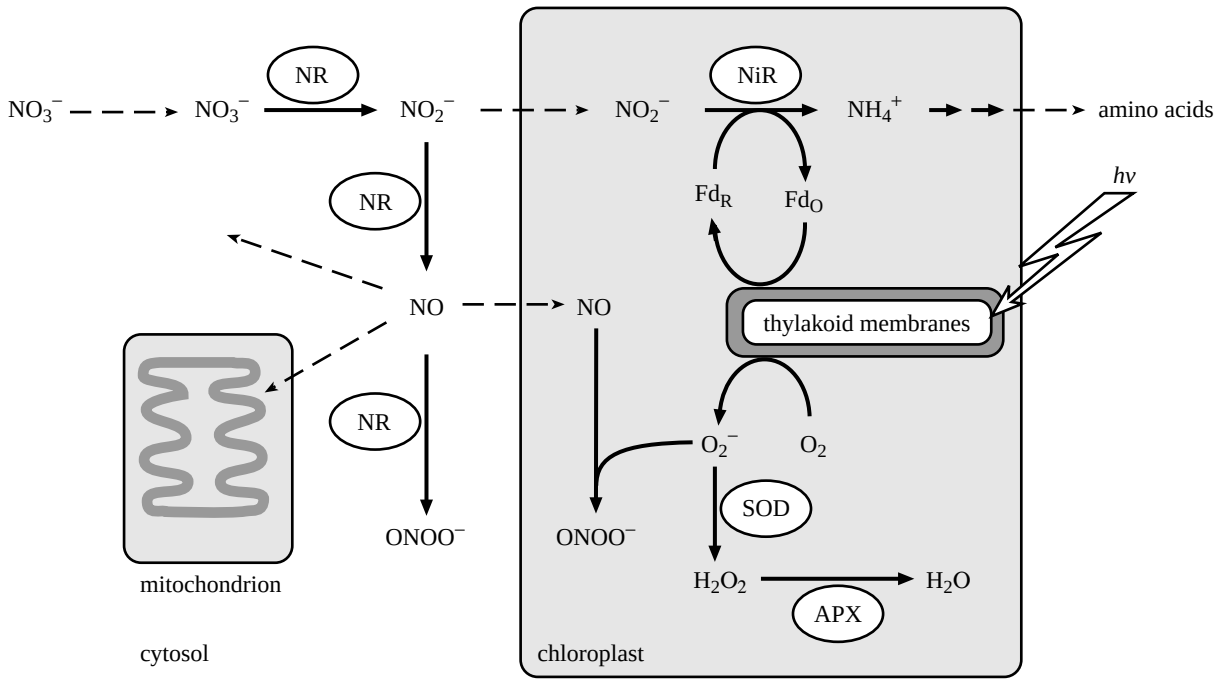

Figure 6. Scheme representing a linkage between photosynthesis and NO production. A decrease in the rate of the reduced form of ferredoxin supplied to NiR causes nitrite accumulation in the cytosol. Accumulated nitrite can be converted to NO by cytosolic NR. Because of the lipophilic nature, NO rapidly diffuses into chloroplasts as well as mitochondria. The NO that penetrates into the chloroplasts may react with the photoproduced O_2^- to form $ONOO^-$. $ONOO^-$ is also generated by NR. NR, nitrate reductase; NiR, nitrite reductase; Fd_R, reduced form of ferredoxin; Fd_O, oxidized form of ferredoxin; SOD, superoxide dismutase; APX, ascorbate peroxidase.

the addition of a photosynthetic electron transport inhibitor, such as DCMU, induces NO production from leaves (Klepper 1979).

Figure 6 illustrates a scheme representing the nitrite-dependent NR-catalysed NO production pathway in leaves. When photosynthetic electron transport systems supply enough of the reduced form of ferredoxin for the NiR reaction, nitrite is efficiently assimilated into glutamine via the GS–GOGAT pathway. Thus, no accumulation of nitrite occurs in the cells. However, a decline of reduced ferredoxin causes a decrease in the conversion rate of nitrite, resulting in an accumulation of nitrite in the cytosol. Under this condition, NR would convert nitrite to NO. Because NO is membrane permeable, any NO produced in the cytosol could easily diffuse into the stroma. If the photosynthetic electron transport system produces O_2^- at PS I under illumination (Asada 1999), $ONOO^-$ may be produced in the reaction between NO and O_2^- (reaction (9)).

8. TARGET MOLECULES OF ACTIVE NITROGEN SPECIES

Like active oxygen species such as O_2^- and H_2O_2, active nitrogen species, including NO and $ONOO^-$, induce oxidative damage of cells. It has been convincingly shown that active nitrogen species are potent oxidants capable of oxidizing amino acids, lipids, DNA and other biomolecules (Arteel *et al*. 1999; Grace *et al*. 1998; Rubbo & Freeman 1996).

The interaction of NO or $ONOO^-$ with thiol groups in proteins (RSH) or in glutathione (GSH) causes formation of thiyl radical (RS·), though the NO and $ONOO^-$ derived reaction intermediates are different. The thiyl radical reacts with reduced form of thiol (RSH) to produce a disulphide bond (RSSR). During this reaction, O_2^- is generated in ambient air (figure 7). It is important to note that chloroplast stroma contains many thiols that are functionally important for photosynthesis. It has been shown that H_2O_2 inactivates $NADP^+$ glyceraldehyde-2-phosphate dehydrogenase, fructose-1,6-bisphosphatase and ribulose-5-phosphate kinase in the chloroplasts (Asada 1999). These H_2O_2-sensitive stromal enzymes contain catalytically functional thiol groups and they are inactivated by formation of disulphide (Asada 1999). The inhibition of the Calvin–Benson cycle enzymes by H_2O_2 decreases the photon-using capacity and simulates the further photoproduction of O_2^- (Asada 1999). Similar to H_2O_2, NO is membrane-permeable and highly diffusive (Lancaster 1996). Thus, NO produced in the cytosol rapidly diffuses into the chloroplast stroma. Like the case of H_2O_2-induced inhibition, therefore, NO could inactivate the thiol-containing enzymes in the Calvin–Benson cycle. In addition to these enzymes, ascorbate peroxidase (APX), an H_2O_2 scavenging enzyme, may be inactivated by NO via oxidation of functional thiols. Moreover, an exhaustion of the antioxidant GSH in the stroma by $ONOO^-$ enhances oxidative damage by lowering the active oxygen scavenging activity of the ascorbate–glutathione cycle. Overall, NO and $ONOO^-$ are both theoretically capable of inactivating the carbon fixation metabolism in a way similar to H_2O_2, which eventually leads to photoinhibition. The inhibition of CO_2 uptake activity by NOx can be accounted for by inactivation of these enzymes. However, no direct evidence is available to show the

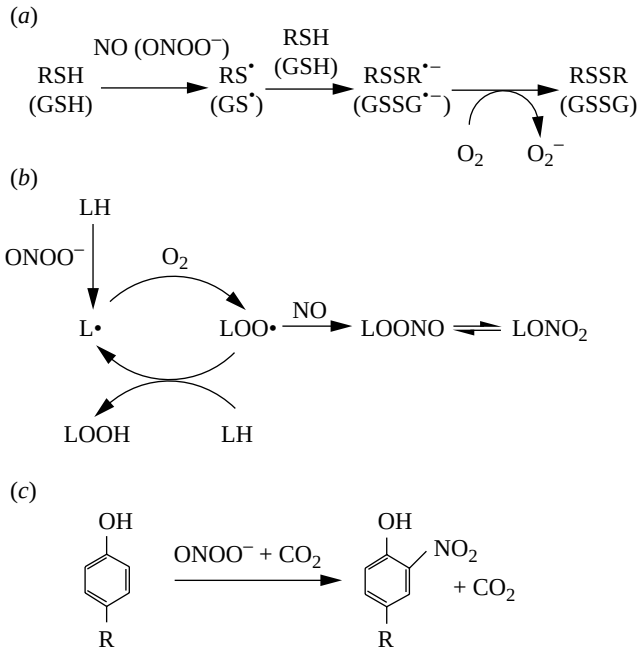

Figure 7. Biological target molecules of NO and $ONOO^-$. (*a*) Thiol groups are oxidized by either NO or $ONOO^-$ and a disulphide bond is formed as the result. During this process, O_2 is reduced to generate superoxide radical (O_2^-). (*b*) $ONOO^-$ can initiate lipid peroxidation. (*c*) In the presence of O_2 and NO, nitrogen-containing products can be produced (Rubbo & Freeman 1996). LH, polyunsaturated fatty acid; L·, carbon-centred radical; LOO·, peroxyl radical; LOOH, hydroperoxide. $ONOO^-$ nitrates phenols including tyrosine and phytophenolic compounds. CO_2 acts as a catalyst for this reaction.

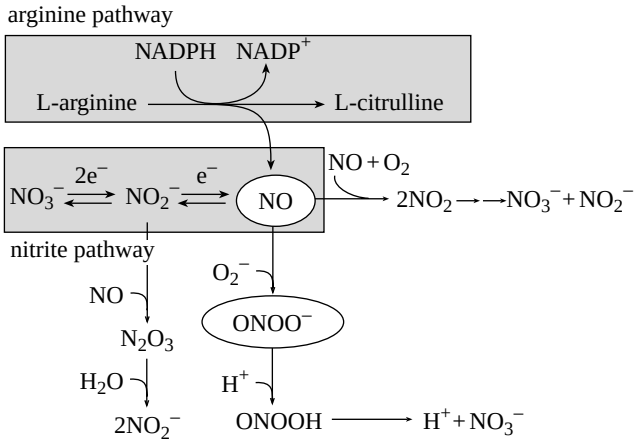

Figure 8. Two pathways for NO production in plants. The arginine pathway includes NO production from L-arginine with NADPH, which is catalysed by a putative nitric oxide synthase (NOS) in plant cells. The other alternative pathway for NO production is through one-electron reduction of nitrite, which occurs by both enzymatic and non-enzymatic routes. Active nitrogen species, including NO and $ONOO^-$, are eventually degraded to nitrate, nitrite or both.

NO-dependent inactivation of the enzymes. It is apparent that further confirmation is necessary to clarify the inhibitory effects.

Nitration, nitrosylation and nitrosation reactions are unique to active nitrogen without an equivalent in the active oxygen-mediated reactions (Arteel *et al*. 1999). Tyrosine residues of protein are nitrated by $ONOO^-$ to produce nitrotyrosine (Arteel *et al*. 1999). Interestingly, of the two substrates for carbon fixation in plants, CO_2, but not HCO_3, stimulates the nitrating reaction by acting as a catalyst (Arteel *et al*. 1999; Squadrito & Pryor 1998). This presents a dilemma for photosynthesis. Although high CO_2 concentration is considered to be favourable for the operation of the Calvin–Benson cycle, there may be an accompanying risk of $ONOO^-$ modification of proteins. Protein nitration would interfere with enzyme functions or metabolism. In animal systems, the formation of nitrotyrosine has been reported to disturb signal transduction systems by inhibiting the phosphorylation–dephosphorylation process of proteins (Kong *et al*. 1996; Li *et al*. 1998).

9. PERSPECTIVES

Figure 8 summarizes the two pathways for NO production in leaves. One is the arginine pathway that produces NO by a putative NOS enzyme with L-arginine, NADPH and O_2. The other is the nitrite pathway that produces NO through one-electron reduction of nitrite. NR catalyses this reduction with NAD(P)H. Although there are so many chemical degradation pathways for NO (Greenwood & Earnshaw 1997), the most biologically important product is $ONOO^-$, which is the reaction product of NO with O_2^-. Whatever the degradation pathways, NO will finally be degraded to nitrite or nitrate.

Nitrate is an important source of nitrogen for photosynthesis and most of the assimilated nitrogen is allocated to photosynthetic components such as chlorophylls, rubisco of stroma and the D_1 protein of PS II (Kolber *et al*. 1988; Seemann *et al*. 1987). Thus, it has been widely appreciated that a high nitrogen supply improves the acclimation capacity of photosynthesis against strong light (Castro *et al*. 1995; Evans 1989; Ferrar & Osmond 1986; Hikosaka & Terashima 1995; Ramalho *et al*. 1997; Skillman & Osmond 1998). The finding of NR-dependent NO production offers the new prospect that the nitrate assimilation pathway may be an alternative source of oxidative stress in plant cells. This is analogous to the situation in carbon assimilation in which O_2^- is photoproduced at PS I (Asada 1999). A rapid turnover of the D_1 protein of PS II is involved in photoprotection (Ohad *et al*. 1994) and D_1 degradation functions as 'an emergency sacrifice' to avoid further production of active oxygens (Asada 1999). NR seems to play a role similar to the D_1 protein. Recent studies have indicated that NR is highly regulated by complex transcriptional and post-translational mechanisms (Campbell 1999; Huber *et al*. 1996; Kaiser *et al*. 1999). The NR enzyme is degraded after several hours in the dark (Kelker & Filner 1971). There are inhibitor proteins called 14-3-3 proteins that inactivate NR in response to the Ca^{2+}-dependent signal transduction system (Athwal *et al*. 1998; Moorhead *et al*. 1996). However, the physiological role of this rapid response to inactivate NR is not fully understood (Huber *et al*. 1996; Stitt 1999). It appears too complex for regulating the efficient assimilation of nitrogen. One possible explanation for this is that the strict regulation may be

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also required to suppress nitrite accumulation in the tissues (Kaiser & Huber 1994), a potential cause of the oxidative damage mediated by active nitrogen species (Yamasaki & Sakihama 2000).

Because the production of NO through the NR-dependent nitrite pathway probably requires low or no activity of photosynthetic electron transport, oxidative damage induced by active nitrogen species could be not directly concerned with the high-light-induced photoinhibition. An increase in the rate of nitrite reduction by photosynthetic electron transport under strong light may function as a photoprotecting mechanism to avoid photoinhibition. NO-dependent photoinhibition would be pronounced in high nitrate and low light intensity. The NOx pollution studies imply that photoinhibition induced by NO is strongly influenced by CO_2 and temperature (Caporn *et al*. 1994; Saxe 1986*a*; Srivastava *et al*. 1975). Mitochondrial respiration stimulated by high temperatures increases the rate of CO_2 release. The increase in level of the CO_2 concentration in leaves may occur when gas exchanges through stomata are limited by stress such as drought. It is important to remember that CO_2 is a strong effector for the $ONOO^-$-dependent nitration of proteins (Arteel *et al*. 1999; Squadrito & Pryor 1998).

Unlike the *in vitro* photoinhibition observed with isolated thylakoid membranes, PS II or reaction centre, the *in vivo* photoinhibition is a complex phenomenon that may be the result of interactions with many compartments and tissues. Stomata closure often enhances photoinhibition by limiting the CO_2 availability for the Calvin–Benson cycle. The plasma membrane H^+-ATPase of guard cells is involved in the stomatal aperture (Shimazaki *et al*. 1986). Interestingly, the NR enzyme shares the same regulating mechanism with the H^+-ATPase through the 14-3-3 proteins (Kinoshita & Shimazaki 1999; Moorhead *et al*. 1996), implicating a mode of co-regulation for these enzymes in response to environmental conditions including nitrogen supply, light, temperature, CO_2 and O_2 availability.

10. CONCLUDING REMARKS

Even though many aspects of the NO-induced photoinhibition are speculative and based on air pollution studies, an outline for the NO production is now established (figure 6). However, much remains to be revealed to draw further a full picture of the NO-induced photoinhibition. Plant cells include abundant phenolic compounds such as flavonoids. These molecules are able to scavenge active oxygen species through enzymatic (Yamasaki & Grace 1998; Yamasaki *et al*. 1997) and non-enzymatic routes (Yamasaki *et al*. 1996). An indirect mechanism for protecting leaves from photoinhibition has been proposed (Yamasaki 1997). Interestingly, these molecules are also capable of scavenging NO and $ONOO^-$ *in vitro* (Grace *et al*. 1998; Haenen & Bast 1999). Furthermore, zeaxanthin, which is an important carotenoid formed by the xanthophyll cycle to elicit the NPQ mechanism, has been reported to be an efficient scavenger of ONOOH (Scheidegger *et al*. 1998). Thus, the presence of *in vivo* mechanisms for scavenging active nitrogen species will be of especial interest to explore the NO-induced photoinhibition.

It must be concluded that we are at an early stage in understanding the effects of NO on photosynthesis. The fact that a large amount of NO can be produced as a by-product during nitrogen assimilation may imply that the physiological roles of the nitrite-dependent NO production pathway may be different from those of the arginine-dependent one. For better understanding the mechanism of photoinhibition *in vivo*, we should be able to answer the simple question: Does photosynthetic apparatus say 'no' to NO?

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Discussion

P. Mullineaux (*Department of Applied Genetics, John Innes Centre, Norwich, UK*). Did you find S-nitrosoglutathione in any plant species? Or has anyone in the audience?

H. Yamasaki. No, I didn't. To the best of my knowledge, there is no published report available on the detection of S-nitrosoglutathione (GSNO) in plants. GSNO is an S-nitrosothiol compound that can be formed by the NO-dependent S-nitrosation of glutathione (GSH). As well as SANP (S-nitroso-N-acetylpenicillamine), GSNO has been widely used as a convenient NO donor because it spontaneously breaks down to release NO in aqueous solution. The reaction between peroxynitrite and GSH has been shown to produce GSNO or nitroglutathione ($GSNO_2$). In mammalian systems, these reaction products have been considered as the biologically active intermediates that can be produced from peroxynitrite. It is apparent that detection of the reaction products of peroxynitrite will be an important subject to understand the biological effects of active nitrogen species in plants.

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