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Nitric Oxide and Reactive Nitrogen Oxide Species in Plants

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Introduction

We now know that nitric oxide (·NO) bursts are early and transient characteristics of plant stress under many biotic and abiotic conditions. ·NO originates non-enzymatically from nitrite (NO_2^-), as a by-product from nitrate reductase (NR). It arises enzymatically from nitrite:nitric oxide reductase (NI-NOR), and putative nitric oxide synthase (NOS) activity. NOS substrates (L-arginine, NADPH, and oxygen), and products (·NO and L-citrulline) are ubiquitous in plants, and pivotal components of intermediary N metabolism. ·NO has both beneficial and harmful effects, depending on its concentrations and milieu. At low concentrations, it is a chemical messenger that directly integrates and differentiates time-dependent responses to stress and defence against pathogens. Prime direct targets of ·NO are the haems that shuttle three gases key to plant life, *viz.* ·NO, carbon dioxide, and oxygen. ·NO and thiols are nitrosated to *S*-nitrosothiols, stored, and probably shuttled by transnitrosylation of proteins. At high levels, and in the presence of reactive oxygen species (ROS), ·NO produces

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CaM, calmodulin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CO, carbon monoxide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNA, deoxyribonucleic acid; EPR, electron paramagnetic resonance; ESR, electron spin resonance; FAD, flavin-adenine dinucleotide; FMN, flavin-adenine mononucleotide; cGMP, cyclic guanosine 3',5'- monophosphate; GS, guanylate cyclase; GSNO, *S*-nitrosoglutathione; GTP, guanosine triphosphate; Hb, haemoglobin; HMA, N^G-hydroxymethylarginine; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; NADH, reduced nicotinamide-adenine dinucleotide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; IRP, iron regulatory protein; NI-NOR, nitrite:nitric oxide plasma membrane reductase; L-NMMA, N^{ω}-monomethyl-L-arginine; NO_x, nitrogen oxides; NOS, nitric oxide synthase; PM-NR, plasma membrane nitrate reductase; NR, nitrate reductase; NiR, nitrite reductase; OPDA, 12-oxo-phytodienoic acid; RCC, red catabolite of chlorophyll; RCCR, red decline reductase; RNA, ribonucleic acid; ROS, reactive oxygen species; RNOS, reactive nitrogen oxide species; ·RS, thiyl radical; RSNO, *S*-nitrosothiol (thionitrite); $RSNO_2$, thionitrate; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; UV, ultraviolet.

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reactive nitrogen species (RNS) that create *oxidative* and *nitrosative* stresses. These free radicals have indirect damaging effects that predispose ageing and impact developmental programmes through various forms of ontogenetic death.

To complicate matters, plants, bacteria, and fungi emit ·NO, NO_x, and other volatiles that are signals in the life cycles of organisms. Under these conditions, individual plants not only adapt to their environment, but also determine their environment. This review draws heavily on comparative studies with ·NO sources and targets in animals. We will deal with the properties of ·NO and RNS, endogenous sources of ·NO, the roles of arginine as a substrate and precursor of inhibitors for NOS, citrulline, one-carbon metabolism, and the increasing importance of thiols and *S*-nitrosothiols in ·NO biotransport. We review what little is known about ·NO as a systemic signal in plant development and its significance for agricultural, forestry, and horticultural practices.

Properties of ·NO and RNS

·NO is a lipophilic free radical and a paramagnetic gas that reacts rapidly with molecular oxygen in both the gas and aqueous phase (Stamler *et al.*, 1992). The atoms of ·NO do not have permanent magnetic dipole moments. When placed in a strong magnetic field, they act as a magnet as long as the field is present. ·NO chemically combines with oxygen to form the highly poisonous nitrogen dioxide, dinitrogen tetroxide, or both. With a halogen, it forms a nitrosyl halide, e.g. NOCl. The redox status and biochemical activity of ·NO is altered by adding an electron to form the nitroxyl anion (NO^-). The anion reacts with sulfhydryls and redox metals. Removal of an electron forms the nitrosonium cation (NO^+). The cation causes nitrosation to occur at –S, –N, –O, and –C centres in organic molecules. The main reactions for ·NO (uncharged) are with oxygen, superoxide, and redox metals at iron–sulphur centres in proteins (Buerk, 2001).

Biological ·NO is of ancient origin (Durner *et al.*, 1999). In plants, ·NO is chemically formed in the presence of ascorbate and as a by-product in NR activities under hypoxic conditions. It is enzymatically produced from nitrite by nitrite:nitric oxide reductase (NI-NOR, Stöhr *et al.*, 2001), and from L-arginine and oxygen by putative nitric oxide synthase (NOS) activity (*Figure 12.1*). ·NO is inactivated and removed by oxidation, reaction with superoxide or shuttled via *S*-nitrosothiols (*Figure 12.2*). Most physiological studies have dealt with the auto-oxidations of ·NO that yield biologically active oxidative cogeners (nitrogen dioxide, nitrite, peroxynitrite, and dinitrogen trioxide). The rate of auto-oxidation is dependent only on ·NO and oxygen concentrations, and not on pH, temperature, or hydrophobicity (Davis *et al.*, 2001). By contrast, the reduced cogeners of ·NO, *viz.* nitrosyl hydride (HNO), and its conjugate base, nitroxyl anion (NO^-), are less understood and their biological roles are unclear (Bartberger *et al.*, 2001). HNO, which has both cytotoxic and protective activity, may be formed during the oxidation of N^{ω}-hydroxy-L-arginine (NOS intermediate); in reactions of *S*-nitrosothiols with thiols; and in the direct reduction of ·NO by mitochondrial cytochrome *c*.

·NO is a versatile messenger with high diffusibility, selected reactivity, and regeneration (Lancaster, 1992). ·NO generated under signalling conditions are in the picomolar to nanomolar range. The effects of ·NO are dependent on its source and

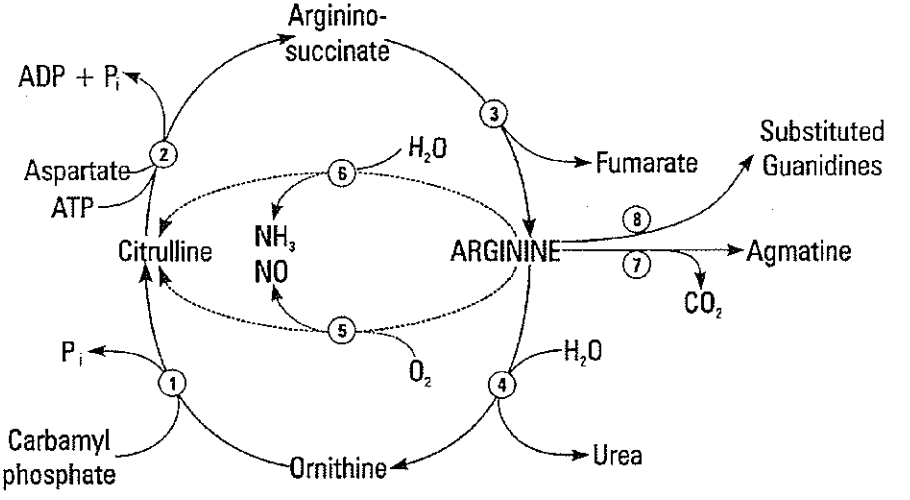

Figure 12.1. The reactions of the Krebs–Henseleit (urea) cycle, and their relations to putative NOS activity, and L-arginine deiminase (dihydrolase, iminohydrolase). Enzymes: 1) ornithine carbamoyl transferase; 2) argininosuccinate synthetase; 3) argininosuccinate lyase; 4) arginase; 5) nitric oxide synthase; 6) arginine deiminase; 7) arginine decarboxylase; 8) numerous enzymes contributing to the formation of substituted guanidino compounds. Reactions 1 to 4 comprise the urea cycle. Reactions 2, 3, and 5 may account for NOS activity in plants (citrulline–NO cycle, or arginine–citrulline cycle). Arginine deiminase was reported in chloroplasts but is mostly found in microorganisms. Reactions 7 and 8 comprise decarboxylation, oxidation, methylation, transamidination, phosphorylation, and keep the guanidino group intact or modify it by methylation, phosphorylation, etc. These reactions remove arginine as a substrate from the urea cycle, and from reactions 5 and 6. Arginine contributes to protein synthesis and turnover (not shown). The production of ammonia, urea, and ·NO are significant in determining N-use efficiency and in mediating stress responses.

concentrations (Davis *et al.*, 2001) (*Figure 12.2*). In aqueous solution, the half-life of ·NO is 0.09 to > 2 seconds, when its decomposition depends on reactions with oxygen. In the aqueous phase, ·NO is auto-oxidized by oxygen to dinitrogen trioxide (N_2O_3). N_2O_3 also arises in the peroxidase catalyzed reactions of nitrite, and from the reactions of ·NO with peroxynitrite (Wink *et al.*, 1999). N_2O_3 contributes to nitrosative damage by the nitrosation of amines (nucleic acid deamination, *N*-nitrosamine formation), and to *S*-nitrosothiol formation (chemical *S*-nitrosylation of haems, metallothioneins), zinc finger degradation, and inhibition of alkyl transferases.

The reaction of ·NO with oxygen within cell membranes is ca. 300 times more rapid than in the surrounding aqueous medium (Davis *et al.*, 2001). In the gas phase and in hydrophobic media, this reaction results in the formation of nitrogen dioxide (·NO_2). ·NO_2 is found mainly in cellular hydrophobic domains (Espey *et al.*, 2001). At ·NO levels found *in vivo*, ·NO_2 slowly dimerizes to dinitrogen tetroxide (N_2O_4), followed by the nitrosation of water, giving nitrite and nitrate. ·NO is quickly converted to ·NO_2 by reaction with peroxy radicals, ozone, and some volatile organic compounds (Menzel and Meacher, 1999).

For plants and animals, the most important physiological factors are the sites and spatial distribution of ·NO formation, fluxes of ·NO, and the duration of ·NO production. At ·NO concentrations below 1 μM, and distant from the site of production, the *direct effects* of ·NO predominate (*Figure 12.2*). Direct ·NO targets are most often

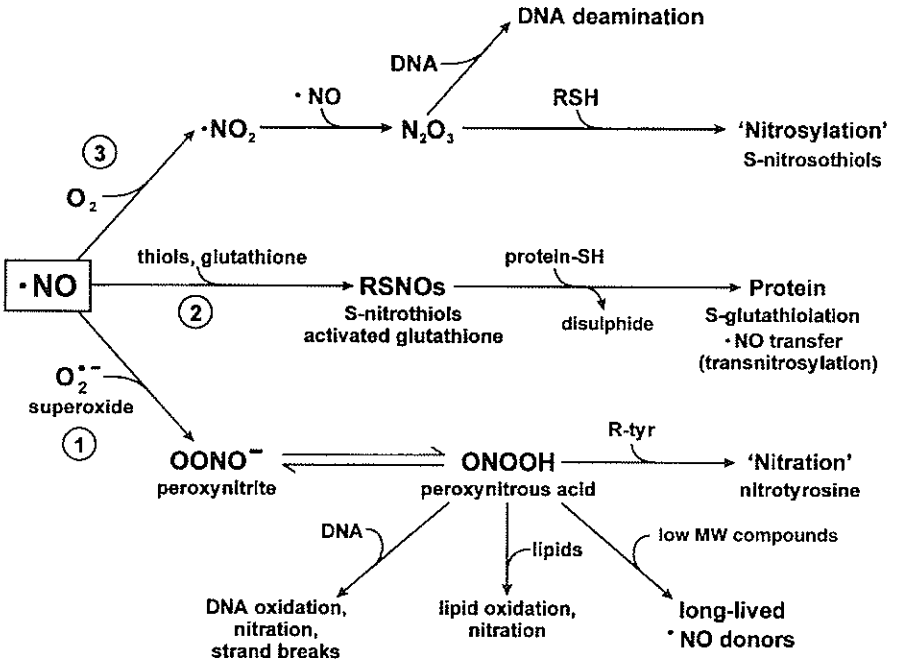

Figure 12.2. Reactions of ·NO and RNS. 1) ·NO reacts with superoxide to form peroxynitrite and peroxynitrous acid leading to indirect cellular damage. The protonation of peroxynitrite is enhanced by acidification resulting in peroxynitrous acid and nitrate. Peroxynitrite may react with carbon dioxide and catalytically decompose via nitrosoperoxycarbonate ($ONOOCO_2^-$) releasing carbon dioxide and nitrate (not shown). Nitrosoperoxycarbonate can nitrate phenols and the tyrosine residues in proteins faster than either peroxynitrite or ONOOH. These reactions represent a sink for ·NO; 2) the bioactive formation of *S*-nitrosothiols (RSNOs) for the storage, transfer, and production of ·NO. Protein functions are regulated by thiols and *S*-glutathiolation to transfer and shuttle ·NO from protein to protein (transnitrosation, transnitrosylation), deliver ·NO to oxygen-poor tissues, and to elicit specific biological responses. ·NO from RSNOs is transferred by sulphur–to–nitrogen and sulphur–to–sulphur transnitrosation, and may involve the formation of carcinogenic N-nitroso derivatives. RNSOs also activate guanylyl (guanylate) cyclase (*Figure 12.3*). *S*-Nitrosoglutathione reductase may control the intracellular levels of GSNO and *S*-nitrosylated proteins independently of oxidative stress; 3) reactions with oxygen producing N_2O_3 lead indirectly to DNA deamination. N_2O_3 also reacts with thiols producing S-nitrosothiols (RSNOs) that contribute to nitrosylation. These reactions strike a balance among the damaging effects of RNS, ROS, and the regenerative repair capacity of plants. The damaging reactions are important factors in ageing. The beneficial reactions contribute to survival, growth, and developmental throughout plant life histories.

metal complexes (e.g. haems, guanylate cyclase, cytochrome P450), non-haem iron proteins (iron–sulphur proteins), zinc and copper proteins, and powerful free radicals (hydroxyl, tyrosyl) that are lipid- and carbon-centred (*Figures 12.2* and *12.3*; Wink *et al.*, 1999). Haems have the highest association rate constant for ·NO in forming a metal nitrosyl complex. The rapid *oxidation* and *addition* reactions of ·NO with haems disarm and preserve ·NO bioreactivity, respectively. Haemoglobins occur in all living organisms (Weber and Vinogradov, 2000). Haems transport, store and facilitate the diffusion of oxygen. They use ·NO to control the oxygen levels; protect against sulphides; sense and scavenge oxygen; and are useful in the detoxification of halogens. Haems help to extend inhospitable environments available to organisms.

At the site where high and sustained amounts of ·NO are produced, the *indirect*

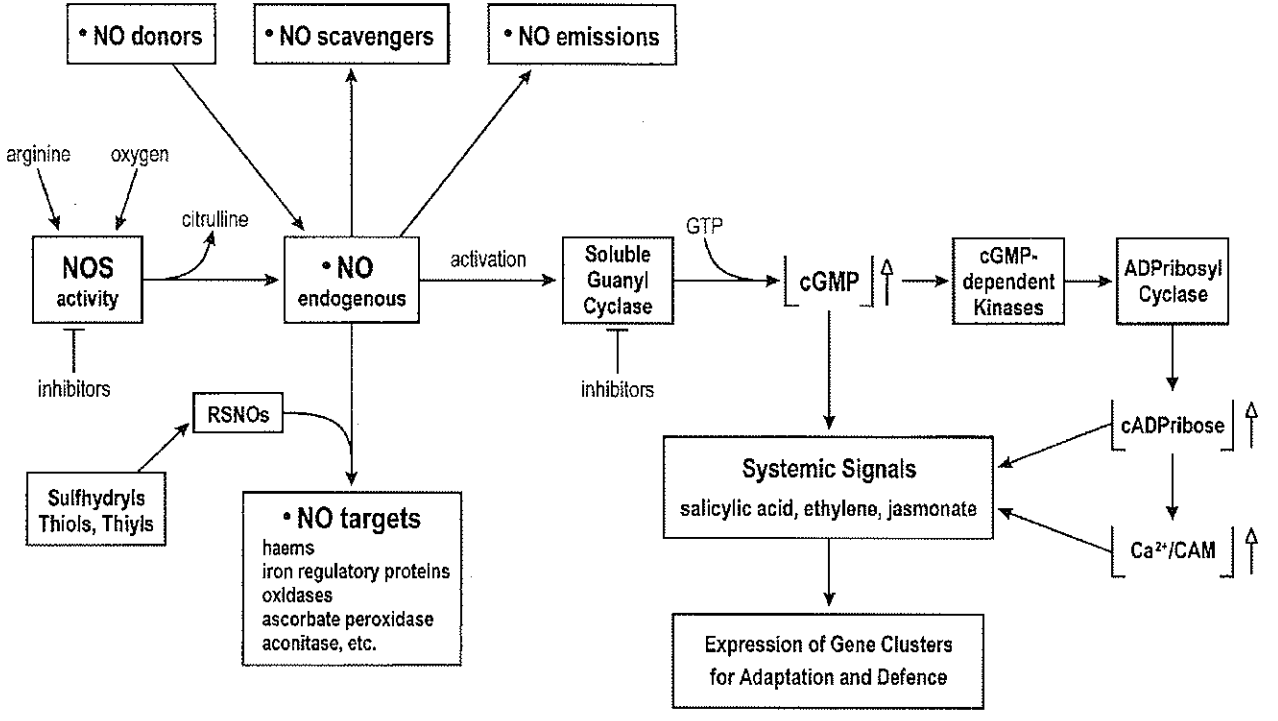

Figure 12.3. ·NO signals use membrane-bound receptors to discriminate among a multitude of stimuli. The sensitivity, speed, and reliability of the response to stress are facilitated by RSNOs and transnitrosylation. The reactions elicited by cellular ·NO are dependent and independent of the activation of soluble guanylyl cyclase (sGC) and/or cyclic guanosine 3'5'-monophosphate (cGMP). The activation of sGC drives inter- and intracellular signal transduction cascades in systemic responses to mechanosensory transduction, pathogens, and environmental stresses. Activation mechanisms comprise kinases, ADP ribosyl cyclase, changes in cellular calcium levels, and other protective responses. The control of up-regulated reactions (upward arrows) are important in post-harvest biology. Hypersensitive responses to invading organisms are modulated by ethylene, jasmonate, salicylic acid, and by changes in intracellular free calcium. These reactions activate defence genes and may contribute to apoptosis. When local calcium stores are depleted, calcium cannot deliver sustained calcium signals. This model implies that calcium spark frequency can be altered by inhibition of NR, NiR, NI-NOR, and NOS activities. ·NO reactions, independent of cGMP, target the haems, ion-channels, proteins and enzymes that alert the plant to real or impending injury.

effects prevail (*Figure 12.2*). They arise from interactions of NO with oxygen and superoxide, giving reactive nitrogen oxide species (RNS) and ROS, causing nitrosative and oxidative stress (Gutteridge and Halliwell, 1999). These stressful chemical reactions comprise *nitrosation* when nitrosonium cation (NO^+) is added to an amine, thiol, or hydroxy aromatic group; *oxidation* when electrons are removed from a substrate; and *nitration,* when NO_2^+ (nitryl, nitronium) is added to a molecule. NO directly nitrates tyrosyl radicals in ribonucleotide reductase and photosystem II (Davis *et al.*, 2001). At physiological NO concentrations, N_2O_3 forms inside hydrophobic cores and causes protein nitration. Dinitrosyl adducts of aconitase formed by NO disrupt the citric acid cycle (Wink *et al.*, 1999). Aconitases are a family of dehydratases that catalyze the reversible isomerization of citrate and isocitrate via *cis*-aconitate and are important targets for NO in plants (Wendehenne *et al.*, 2001).

NO production and emission can be bioimaged in real-time with fine temporal and spatial resolution with diaminofluoresceins (Kojima *et al.*, 1998) in plant cells (Foissner *et al.*, 2000; Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b). The bioimaging of NO depends on the *permeability* of the probe, chemical transformation of diaminofluoresceins by endogenous esterases, and reactions of aromatic vicinal diamines with NO *in the presence of oxygen*. The diacetyl derivative of diaminofluorescein (DAF-2A) permeates cells quickly but NO detection is limited by low photostability, overlap with autofluorescence, and dependency on pHs greater than six. It does not react with NO itself but with NO^+ equivalents, such as nitric anhydride (N_2O_3), which are formed by autoxidation of NO. Glutathione inhibits the nitrosation of intracellular DAF (Espey *et al.*, 2001) and reacts with NO to produce *S*-nitrosothiols (*Figure 12.2*). The NO released from *S*-nitrosothiols accumulates in membranes where it reacts with oxygen to produce N_2O_3 (Ramachandran *et al.*, 2001). Intracellular thiols are then nitrosated by N_2O_3 at the membrane–cytosol interface. New fluorescence indicators for NO, *viz.* diaminorhodamines, feature high photostability, long-wavelength excitation, and no pH dependency over a wide pH range (Kojima *et al.*, 2000).

NO can be measured in single cells by a porphyrinic-based microsensor (Malinski and Taha, 1992), and followed by a NO-specific probe (Pfeiffer *et al.*, 1998; *cf.* Buerk, 2001). Non-invasive photoacoustic spectroscopy has been used to determine the relative endogenous levels of NO in climacteric and nonclimacteric fruits (Leshem and Pinchasov, 2000). NO bioactivity is measured by electron paramagnetic resonance (EPR) spectrometry (e.g. Szalai and Brudvig, 1996). EPR is also known as electron spin resonance (ESR) or electron magnetic resonance (EMR). EPR is the process of resonant absorption of microwave radiation by paramagnetic ions or molecules, with at least one unpaired electron spin in the presence of a static magnetic field. Flash photolysis electron spin resonance is the only technique that allows the observation and unambiguous identification of free radicals in solution on a sub microsecond scale (Woodward *et al.*, 2000). The reactions of radical pairs are susceptible to the influence of external magnetic fields and offer insight into the effects of overhead power cables, electrical equipment, and electrical fields on plant biochemistry.

Sodium nitroprusside, *N,N'*-dimethyl-*N,N'*-dinitroso-*p*-phenylenediamine, and *S*-nitrosoglutathione are membrane permeable NO donors. Photolabile, caged nitric oxide compounds (*N,N'*-dimethyl-*N,N'*dinitroso-*p*-phenylenediamines), when

irradiated with a xenon lamp or a laser flashlight, deliver ·NO into cells in microseconds without immediate cytotoxic effects (Yoshida *et al.*, 2000). Thiols do not interfere with the release of ·NO from these caged compounds. Diffusion and reaction patterns reflect the chemical, biochemical, and fractal properties of cells. ·NO traps, *viz.* 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), are used to control ·NO levels in cells. Carboxy-PTIO (·NO scavenger), nitrate-free media, and selenomethionine also reduce or eliminate endogenous levels of ·NO. N^G-Monomethyl-L-arginine (L-NMMA), and other guanidino and thiourea derivatives are inhibitors of NOS (*Figure 12.1*). In plants, D-NMMA does not block putative NOS activity, and is a useful negative control for L-NMMA (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b; Garcês *et al.*, 2001). Aqueous nitrite ions can be determined by selective reduction and gas phase ·NO chemiluminescence (Dunham *et al.*, 1995).

Protection against oxidative and nitrosative damage

Oxygen is a universal electron acceptor in aerobic organisms. Oxygen sensing is necessary for adaptation to variable habitats and physiological situations. Current models of oxygen sensing are based either on a haem protein that reversibly binds oxygen, or on the production of reactive oxygen species (ROS) by NADPH oxidases. The production of ROS is an unavoidable consequence of aerobic metabolism. ROS alter the redox status of signalling molecules and the functions of their effectors. ROS are derived from the reduction of molecular oxygen producing superoxide, hydrogen peroxide, and hydroxyl radicals. Carbon-centred radicals react with molecular oxygen to give peroxyl, alkoxyl radicals, and hydroperoxides (Huie and Neta, 1999).

The most prolific source for reactive oxygen species (ROS) is the leakage of electrons from the mitochondrial electron-transport chain (Møller, 2001). Photosynthetic electron transport produces oxygen, and ROS (Bolwell, 1999). Oxidative stress comprises hydroxylation, DNA fragmentation, lipid peroxidation, and haem protein oxidation. In cells, ·NO limits the formation of powerful ROS, e.g. metallo-oxo oxidants and hydroxyl radicals produced by the reaction between ferrous iron and hydrogen (or alkyl) peroxide (Fenton reaction). ·NO binds tightly to ferrous iron forming methaemoglobin. It inhibits haem functions in photosynthetic electron flow, nitrate reduction, photorespiration, and other energetic aspects of plant metabolism. In the presence of hydrogen peroxide, ferrous nitrosyl complexes yield ferric iron and nitrite without the generation of strong oxidants (Wink *et al.*, 1999). In general, nitroxides protect against the toxicity from superoxide, hydrogen peroxide, organic hydroperoxides, mutagenic ROS, ionizing radiation, and redox-cycling chemotherapeutic drugs.

Superoxide is found in all organisms in aerobic environments. It reacts with ·NO to form the potentially damaging peroxynitrite and peroxynitrous acid (Arteel *et al.*, 1999) (*Figure 12.2*). Peroxynitrite forms at near diffusion-limited rates, and rapidly decomposes upon protonation by isomerization to nitrate while generating the highly reactive hydroxyl (·OH) and nitrogen dioxide ($\cdot NO_2$). It is a powerful oxidizing agent that initiates lipid peroxidation, oxidizes sulfhydryls, and nitrates the aromatic residues of proteins. In plant peroxisomes, ROS are controlled by catalase, ascorbate peroxidase, and superoxide dismutase (Corpas *et al.*, 2001). When ·NO and hydrogen

peroxide permeate the peroxisomal membrane, superoxide radicals are produced on the cytosolic side of the membrane. The formation of superoxide and ·NO are thought to inhibit catalase and ascorbate peroxidase, causing an increase in hydrogen peroxide due to enhanced fatty acid β-oxidation. Cell functions are protected by intercepting radicals, repairing oxidative damage, eliminating damaged molecules, and by promoting the death of cells having damaged DNA.

In the 'water–water cycle' of chloroplasts, the photoreduction of oxygen by the thylakoid photosystem I complex generates superoxide as a primary product (Asada, 1999). Superoxide and hydrogen peroxide are rapidly scavenged by ·NO so that ROS do not inactivate this complex. During the photoreduction of oxygen to water, superoxide and hydrogen peroxide are also scavenged by electrons derived from water in photosystem II. ·NO reversibly binds to the tyrosyl radicals in the photosystem II of spinach and quenches an electron paramagnetic resonance (EPR) signal (Szalai and Brudvig, 1996). This photosystem is responsible for the light-driven oxidation of water to oxygen and for the reduction of plastoquinone to plastoquinol. In an oxygen-evolving photosystem II, ·NO binds to the non-haem Fe(II). The elimination of the EPR signal provided evidence for the formation of a ·NO adduct. The nitration of protein tyrosine residues by peroxynitrite prevents phosphorylation, and tyrosine kinase signalling, thereby blocking many cell regulatory proteins (Reiter *et al.*, 2000).

Glutathione scavenges the peroxynitrite arising from NOS activity by producing *S*-nitrosothiols (RSNOs, e.g. *S*-nitrosoglutathione) (Schmidt *et al.*, 1996). Under aerobic conditions, thiols and sulfhydryl groups of proteins are oxidized to their corresponding thionitrites. Thionitrites, e.g. *S*-nitrosoglutathione, *S*-nitrosocysteine, are not produced in the absence of oxygen. Thionitrite formation in plants has not been studied. Under anaerobic conditions, the oxidation of protein thiols by ·NO gives a sulphenic acid (RSOH) product and nitrous oxide (N_2O) (DeMaster *et al.*, 1995). The sulphenic acid can react with glutathione. The anaerobic oxidation of glutathione and ·NO produces a disulphide and N_2O.

·NO is often assumed to reach protein targets by simple random diffusion. However, ·NO irreversibly reacts with haems, competing proteins, and thiols that rapidly consume, and shuttle ·NO (*Figure 12.2*). The addition of ·NO to haemoglobin (Hb) chemically *S*-nitrosates a cysteine residue giving *S*-nitrosohaemoglobin, which shuttles ·NO from protein to protein by *transnitrosylation (transnitrosation)* (Gow *et al.*, 1999; Klatt and Lamas, 2000). RSNOs arising from the reactions of ·NO with thiols, do not react with Fe(II)-Hb. Their non-reactivity with haems provide a protected route for the delivery of bioactive ·NO equivalents to sites where oxygen tension is lowest. The predominant RSNOs are mostly *S*-nitrosoproteins, where the ·RS radical is a thiyl group, although simpler RSNOs forms exist, e.g. thionitrite, thionitrate, *S*-nitrosoglutathione (Wang *et al.*, 2000b). In animals, and possibly plants, nitrite and nitrate generated from RSNOs may reduce the levels of cellular ·NO, superoxide, and RNS.

Reactions with carbon dioxide, carbon monoxide, and free radicals

The reaction of CO_2 with peroxynitrite in tissues can outcompete water-soluble antioxidants (ascorbate, glutathione) for the same reaction. High levels of CO_2 but not bicarbonate, react with peroxynitrite to give metastable nitrosating, nitrating, and

oxidizing intermediates (Squadrito and Pryor, 1998; Dweik *et al.*, 2001). In this model, ·NO reacts with superoxide to form the damaging peroxynitrite and has an antioxidant role by consuming ROS. Sacrificial thiol/thiolate scavengers effectively quench the total oxidative yield of peroxynitrite, whereas the catalytic porphyrins redirect this oxidation, and may enhance the total nitration and oxidative yield. *S*-Nitrosothiols (RSNOs) would remove ·NO and provide a safe reservoir of bioactive ·NO, thereby reducing the harmful levels of ·NO and related RNS derivatives. RSNOs are relatively resistant to toxic reactions with oxygen and superoxide.

The biological targets for peroxynitrite depend on the relative concentrations, rates and duration of peroxynitrite formation (*Figure 12.2*). Peroxynitrite is converted under acidic conditions to peroxynitrous acid or to nitrosoperoxycarbonate ($ONOOCO_2^-$) depending on CO_2 levels and pH (Squadrito and Pryor, 1998). Thiols may be nitrosated either via nitrosoperoxycarbonate or during the formation of thiyl (·RS) radicals. Nitrosoperoxycarbonate is also rapidly decomposed to CO_2 and nitrate. Compared to peroxynitrite, nitrosoperoxycarbonate enhances the non-enzymatic nitration of tyrosine residues, phenolic compounds, and hindered phenoxy radicals (Ramezanian *et al.*, 1996).

Carbon monoxide (CO) is assimilated by plants giving products similar to CO_2 fixation (Bidwell, 1974). Nothing is known about the relation of CO assimilation to plant ·NO activity. CO and ·NO generate gaseous haem ligands that are intimately linked to one another. The affinity of ·NO for haemoglobin is ca. 1500 × greater than for CO. In animal cells, the cellular steady-state levels of ·NO were elevated by exposure to CO (Thom *et al.*, 2000). Levels of manganous superoxide dismutase and haem oxygenase were increased to protect against the lethal effects of CO. Elevated levels of antioxidant enzymes and cell death (apoptosis) were inhibited by a NOS inhibitor (*S*-isopropylisothiourea), and a peroxynitrite scavenger (selenomethionine). Results showed that the biochemical effects of CO occurred at environmentally relevant levels, that apoptosis followed high concentrations of CO, and that these actions were mediated by ·NO (Thom *et al.*, 2000).

Endogenous sources of ·NO in plants

·NO is formed in nearly every plant tissue; however, it is still unclear how many endogenous sources of ·NO exist, and if, when, and where they co-exist. Currently, the main recognized sources derive from the activities of NiR, NR, NI-NOR, and from putative NOS activity. In animals, other sources are recognized *viz.* those derived from haems, RSNOs, e.g. *S*-nitrosoglutathione, and anaerobic xanthine oxidase activity that reduces nitrite back to ·NO (Buerk, 2001).

Plant experiments do not always distinguish between ·NO endogenous production and emission to the atmosphere. Some assays are not chemically defined, and may not be carried out under aseptic conditions. Most studies dealt with ·NO production strictly as a gaseous emission based on the availability of nitrate, without considering separate or concurrent putative NOS activity, nor external sources of ·NO generation. Mycoplasma, bacteria and fungi may contaminate cell-free and tissue assays, and compromise studies with whole plants. Denitrifying bacteria have three different types of respiratory nitrite reductases (NiRs) that produce ·NO (Watmough *et al.*, 1999). These are: cytochrome *c* NiR, cytochrome cd_1 NiR, and a NiR with a trimeric

copper. The latter occurs in the mitochondrial respiratory chain of fungi. Oxygen binding proteins in microorganisms convert ·NO aerobically to nitrate, or anaerobically to nitrous oxide (N_2O) (Poole and Hughes, 2000).

NITRITE:NITRIC OXIDE REDUCTASE, AND NITRATE AND NITRITE REDUCTASE ACTIVITIES

A plasma membrane-bound enzyme from tobacco roots catalyzes the gaseous formation of nitric oxide from nitrite (Stöhr *et al.*, 2001). This enzyme, nitrite:nitric oxide reductase (NI-NOR) was not detected in leaf-soluble protein fractions nor in plasma membrane vesicles. Optimal activity was observed at pH 6.0. ·NO formation was also followed indirectly and colorimetrically with litmus paper by measuring the decrease in nitrite. Assays involved the addition of sodium nitrite, reduced cytochrome *c*, and reduction with ascorbate. Nitrite was reduced to ·NO with cytochrome *c* as an electron donor, and at a rate comparable to the nitrate-reducing activity of a root-specific, succinate-dependent nitrate reductase (PM-NR) bound to the plasma membrane. NADH was not required for PM-mediated ·NO production, thereby excluding the participation of NR. In this system, PM-NR only reduced nitrate and produced nitrite as a substrate for NI-NOR. Enzymatic purification and separations resulted in a severe loss of NI-NOR activity. The NI-NOR fraction was insensitive to cyanide and anti-NR IgG, thereby distinguishable from PM-NR. Cyanide specifically binds to the Mo centre of NR, and to the oxidized forms of cytochromes. The apparent molecular masses for NI-NOR and PM-NR were 310 and 200 kDa respectively.

NI-NOR in the plasma membrane was postulated to reduce the apoplastic nitrite produced by PM-NR, and to play a role in nitrate signalling via ·NO formation. A specific activity of ·NO formation at the plasma membrane of 300 nm (mg protein)$^{-1}$ h^{-1} would be sufficient to reduce all nitrate produced by PM-NR at pH 6.0 (Stöhr *et al.*, 2001). Apolar ·NO can further enter the cell and induce secondary reactions in the cytosol (*cf.* Pedroso and Durzan, 2000; Pedroso *et al.*, 2001a,b; Garcês *et al.*, 2001). Earlier, a ·NO-inducible guanylate cyclase and signalling via cGMP was demonstrated in tobacco (Durner *et al.*, 1998).

Plant NR (EC 1.6.61-3) catalyzes the NADPH-dependent reduction of nitrate to nitrite for the acquisition of N and for iron reduction (Campbell, 1999). It also provides reduced N for the synthesis of L-arginine, a substrate for NOS activity. NR is located on the outer membranes of chloroplasts and plastids. The NR monomer has one FAD, haem-iron, and molybdenum-molybdopterin. It contains domains for cytochrome *b*, co-factors, and a binding protein. Plant NR is highly regulated by transcriptional and post-transcriptional mechanisms in response to nitrogen type and supply, light, pH, temperature, carbon dioxide, and oxygen availability. In light, electrons for nitrate assimilation are transported in the thylakoid membranes of chloroplasts. Cytoplasmic NR has been shown to reduce nitrite to ·NO with NADH (Dean and Harper, 1988). Nitrite is translocated in a pH gradient from the cytosol across the chloroplast envelope. Excess nitrate in the apoplast could lead to ·NO production when nitrate uptake is inhibited. It remains unclear how and why NR and NI-NOR produce ·NO under environmental triggers (Kaiser and Huber, 2001).

Plant NiR (EC 1.7.7.1; ferredoxin-nitrite reductase) receives electrons from reduced ferredoxins to produce ammonia, oxidized ferredoxin, and water. A nitrate-inducible

ferredoxin in maize roots serves as an electron carrier from NADPH to NiR (Matsumura *et al.*, 1997). Reduction to ammonia may form dinitrosyl iron complexes with thiol-containing ligands, and contribute to the transport of ·NO (Vanin, 1998). Vanin described the reaction of ·NO with ferredoxin in 1972. The significance of this reaction is still not fully understood. Higher plant NiR is a monomeric chloroplastic protein that is controlled at the transcriptional level by light and the N source (Crete *et al.*, 1997). Spinach leaf NiR contains two arginine residues that electrostatically bind ferredoxin (Dose *et al.*, 1997). The accumulation of nitrite by inhibition of NiR would provide nitrite as a substrate for ·NO production.

Under N-limitation, algal mitochondria support the reduction of nitrate and nitrite (Dennis and Turpin, 1990). A sudden imbalance between nitrate vs. nitrite reduction caused an instantaneous ·NO burst (Mallick *et al.*, 1999). Nitrite was the ultimate substrate for ·NO emission. However, the cellular production of low levels of ·NO, possibly produced by NOS, was not ruled out by the use of NOS inhibitors. The inhibition of photosynthesis by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), ATP synthesis by dicyclohexylcarbodiimide, and the uncoupler (2,4-dinitrophenol) and its analogue, arsenate, showed that the *inhibition of nitrite assimilation through the blockage of NiR was primarily responsible for ·NO emissions*. Nitrite levels in the culture medium were linearly related to the ·NO in the exhaust gas. The inhibition of photosynthesis by DCMU imposed a dark metabolism that depleted ATP, NADPH, and inhibited NR, oxygen production, and ·NO release. When nitrate was the sole N source, a failure of cells grown in a medium substituting tungstate for molybdenum, to produce either ·NO and nitrite in light or during a 'light-off' peak, and the resumption of these activities when molybdenum was added, proved that *a functional NR was responsible for the production of nitrite and ·NO*. The appearance of a ·NO peak immediately after nitrite supplementation in darkness in tungstate-substituted cultures, with or without glucose, ruled out an enzymatic role of NR in ·NO emissions. In mammalian mitochondria under hypoxia, the leakage of electrons and NiR were responsible for the production of ·NO. This indicated the existence of a NOS-independent ·NO source in mammals (Kozlov *et al.*, 1999).

NO production was first assayed *in vitro* with soybean NR and NiR. Acetaldehyde oxime and N_2O were identified as products in dark aerobic NR assays (Mulvaney and Hageman, 1984). Assays for NR may produce hydroxylamine (NH_2OH). Hydroxylamine can be converted to nitrite and water by hydroxylamine oxidase (Murad, 1999). In soybean assays, the production of ·NO and $·NO_2$ from nitrite by NR was probable at a Mo-pterin centre (Dean and Harper, 1986, 1988). In animal cells, the molybdenum-containing xanthine oxidase may generate ·NO via NiR at low oxygen levels that impair NOS (Zhang *et al.*, 1998). The redox formation of ·NO required NADH as an electron donor, and was oxygen independent. The reduction of nitrate occurred at the molybdenum centre of xanthine oxidase while NADH was oxidized at the FAD centre. Heparin binding of xanthine oxidase increased nitrite reduction. The xanthine oxidase-dependent formation of ·NO was considered important for the redistribution of blood flow to ischaemic tissue as a supplement to NOS activity, since both nitrite and NADH were elevated in hypoxic tissue. When xanthine oxidase was used as a source of superoxide, tyrosine nitration was inhibited by urate formation from hypoxanthine oxidation (Reiter *et al.*, 2000).

In mitochondria from isolated soybean cotyledons, oxygen consumption via the

cytochrome pathway was inhibited by ·NO (Millar and Day, 1996). Respiration via the cyanide-insensitive alternative oxidase was not significantly affected. Inhibition of the cytochrome *c* oxidase activity was rapidly reversible upon depletion of added ·NO. The inhibition of cytochrome *c* oxidase pathway was dependent on nitrite concentrations. Alternative pathway respiration was not significantly affected under similar conditions. The alternative oxidase was viewed as a factor in plant ·NO tolerance.

In soybean assays, twenty minutes after the light was turned off, ·NO was released by constitutive NR activity. Millimolar nitrite in the presence of ascorbate and other reductants produced ·NO non-enzymatically (Day *et al.*, 1998). In darkness, NR activity was inhibited and ascorbate as nitrite accumulated in chloroplasts. Putative NOS activity was not known at that time. ·NO production increased when the air in the chamber was replaced with nitrogen gas. The reaction of ·NO with superoxide in mitochondria to give peroxynitrite was known to inhibit cytochrome *c* oxidase and adjust oxygen use. However, oxygen uptake did not change during the ·NO burst. This indicated that soybean mitochondria switched from cytochrome *c* oxidase to an alternative oxidase (AOX)-mediated respiration, and that AOX became a sink for electrons. Electron transport for ·NO production decreased superoxide levels.

Two constitutive NRs (c_1NR and c_2NR) were found in soybean leaves (Dean and Harper, 1988). The c_1NR had a K_m for nitrate at 5 mM, and preferred NADPH as an electron donor at a pH optimum of 6.5. The c_2NR preferred NADH and had a K_m of 0.19 at the same pH. Induced NR activity preferred NADH at a lower K_m for nitrate (0.13 mM). ·NO emission was detected only with the c_1NR. This enzyme had a higher affinity for nitrite than for nitrate. Reaction mixtures with corn NR also produce ·NO under hypoxia (Yamasaki *et al.*, 1999; Yamasaki, 2000). This one-electron reduction of nitrite uses NADPH as an electron donor. The apparent ·NO production rate had a K_m for nitrite that was 5-fold higher than for nitrate (Yamasaki and Sakihama, 2000; *cf.* Dean and Harper, 1988). *In vitro* assays showed that corn and soybean NR preferentially emitted ·NO. ·NO production was blocked in a soybean NR mutant (Klepper, 1990). In a double NR mutant (*nia1, nia2*) of *Arabidopsis*, ·NO emission, but not the cellular production of ·NO, was blocked, indicating residual putative NOS activity (Magalhaes *et al.*, 2000; Garcês *et al.*, 2001). Exposure of Scots pine to nitrate and NO_x induced NR activity in needles (Wingsle *et al.*, 1987).

Plants use several pathways for ·NO production from inorganic sources, and have developed adaptive systems for hypoxia. Under ambient conditions, the assimilation of nitrate and ammonia for amino acid biosynthesis requires a supply of keto acids. Keto acid formation depends on an increased carbon flow through respiratory pathways and on whether or not N assimilation is occurring in photosynthesizing or non-photosynthesizing tissues. During nitrate assimilation in light, reducing power may be transported from the mitochondria to the chloroplast for use in nitrite reduction (Dennis and Turpin, 1990). It is not yet clear how this changes during hypoxia, nor if RSNOs and transnitrosylation are involved in nitrate respiration, NI-NOR, and in putative NOS activity. Transnitrosylation implicates γ-glutamyl-transpeptidases that shuttle ·NO-carrying and signalling molecules among thiols, haems, and proteins. Genomic analysis of the responses of *Arabidopsis* to low and high levels of nitrate revealed mRNAs for proteins of unknown functions. Some of these proteins may be related to ·NO biotransport, e.g. non-symbiotic haemoglobin, to

senescence, and to methyltransferase activity, *viz.* one-carbon metabolism (Wang *et al.*, 2000a).

PUTATIVE NITRIC OXIDE SYNTHASE ACTIVITY

This review recognizes that parallels for NOS activity in animal systems are very far from being proven in plants. The rapid developments in both fields may result in models that will no longer be accepted. In plants, the formation of ·NO by a mammalian-type cytosolic NOS activity (putative NOS activity) would compete with the urea cycle, consume oxygen, ATP, and draw soluble N from the free amino acid pool (*Figure 12.1*). ·NO production via NOS activity also appears to be quantitatively linked to N flux through nitrate assimilation.

In mammalian cells, NOSs are ubiquitous, constitutive and inducible redox enzymes (Moncada *et al.*, 1997). Oxygen and L-arginine are NOS substrates giving N^{ω}-hydroxy-L-arginine as an intermediate. The products of NOS are ·NO and L-citrulline. In the presence of superoxide, the ·NO that is eliminated from N^{ω}-hydroxy-L-arginine may not always be accompanied by the formation of L-citrulline. Superoxide anions produced during NOS and cytochrome P450 activity are implicated in the oxidative denitrification of compounds having a C = N(OH), e.g. hydroxyguanidines, amidoximes, ketoximes, and aldoximes (Jousserandot *et al.*, 1998). Nitrosyl hydride (HNO) may also be formed during the oxidation of N^{ω}-hydroxy-L-arginine (Bartberger *et al.*, 2001). NOS requires NADPH, oxygen, and a Fe(III) complex for activity, and tetrahydrobiopterin (H4B), FAD and FMN as co-factors. H4B is not known in plants. In the absence of H4B, citrulline formation was uncoupled relative to NADPH oxidation, and generated nitrate without forming ·NO. The sole role of H4B was to enable NOS to generate ·NO instead of the nitroxyl anion (NO^-). Nitroxyl may serve as an efficient pro-oxidant by oxidizing NADPH unless scavenged by superoxide dismutase (Reif *et al.*, 2001).

NOS is a homodimer that oxygenates arginine at a haem site in the N-terminal oxygenase domain (Hobbs *et al.*, 1999). The oxygenase domain has a cytochrome P450-type haem centre, and a binding site for H4B.The reductase domain has binding sites for NADPH, FAD, and FMN, with significant homology to cytochrome P450. Bridging the reductase and oxygenase domains is a calmodulin (CaM) binding site. The binding site acts as a switch to regulate electron flow between the two regions. Three NOS isoforms are in the cytochrome P450 reductase-like gene family. Two of these are expressed constitutively, and a third is inducible. All constitutive isoforms of NOS have a requirement for calcium and CaM (Guzman and Amoah-Apraku, 1999). Inducible isoforms are calcium independent. Isoenzymes are activated by an increase in intracellular calcium that binds to CaM in NOS. NOS is regulated by cell-type-specific transcription, and stimulated by growth factors, hypoxia, hyperosmotic conditions, oxidative stress, and UV light. NOS may generate both ·NO and superoxide when L-arginine or H4B are low. NOS is inhibited by L-canavanine, guanidines, steroids, peptides, and by kinase activators and inhibitors (Guzman and Amoah-Apraku, 1999).

A base sequence was found in the *Arabidopsis* genome that could code for part of a plant NOS or an enzyme with putative NOS activity plant (The *Arabidopsis* Genome Initiative, 2001). The sequence shares some resemblance to the oxidase

domain of cytochrome P450 reductase in animals. However, no obvious homologues for an *Arabidopsis* NOS gene, nor for mammalian respiratory oxidases, were found for this. Molecular-genetic analysis detected many plant cytochrome P450 monooxygenases with unknown functions. Two partial amino acid sequences for a putative NOS protein in *Arabidopsis thaliana* 'Columbia' were recorded. The chromosome 4 sequence (residues 1–1106) has similarities to *Rattus novegicus* NOS (EC 1.14.13.39) (Bevan *et al.*, 1999). Another sequence in the same plant variety, but on chromosome 3, was reported for residues 1–441 (Choisne *et al.*, 1999).

Pea peroxisomal NOS (130 kD) was localized in leaves by electron microscopy and immunoblots with a polyclonal antibody against the C-terminus of murine inducible NOS (Barroso *et al.*, 1999). Chloroplasts also exhibited NOS activity. The calcium-dependent NOS activity was sensitive to NOS inhibitors. The plant mitochondrial electron transport chain contains two calcium dependent, rotenone-insensitive, non-proton-pumping NADPH dehydrogenases on each side of the inner membrane that may be active when plants are stressed (Møller, 2001). However, NOS was not found in plant mitochondria, although it occurs in mammalian mitochondria (e.g. Sarkela *et al.*, 2001). In tobacco and maize, cross-reactivity with rabbit brain NOS and mouse macrophage NOS was linked to proteins with a M_r of 56 kD and 166 kD respectively (Huang and Knopp, 1998; Ribeiro *et al.*, 1999). NOS activity was also reported in the nucleus (Ribeiro *et al.*, 1999). However, in *Kalanchoë* and *Taxus*, NO production in nuclei was not detected by fluorescence microscopy (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b).

Plant NOS is reported to have NADPH oxidase activity that generates superoxide, i.e. it is a diaphorase. Electrons are transferred from NADPH to small molecules, such as paraquat and quinones (Cueto *et al.*, 1996; Day *et al.*, 1999). Paraquat in illuminated chloroplasts traps all the electrons from photosystem I and generates superoxide and hydrogen peroxide (Asada, 1999). The immunohistochemical detection of NOS in fixed sections requires the estimation of both NADPH-diaphorase and NOS activity (Gonzalez-Hernandez *et al.*, 1996). Reliable immunohistochemical and cytochemical detection of NOS activity for plants remains a significant shortcoming.

Putative NOS activity was evident in a double NR mutant (*nia 1, nia 2*) of *Arabidopsis* when excised leaves were incubated in darkness, and centrifuged in the absence of NOS inhibitors (Garcês *et al.*, 2001). Cellular NO production, visualized with DAF-2 DA, was completely eliminated by L-NMMA, a potent NOS inhibitor. Although a 0.5% residual activity from NR was reported for this mutant (Wilkinson and Crawford, 1993), NO production was not detected in the controls in darkness. This would rule out NR and NI-NOR activities, and indicated that endogenous NO was being produced by putative NOS activity (Garcês *et al.*, 2001). The reports of NOS activity in plants need to be defined in terms of responsible gene(s), gene products, an intermediate (e.g. N^{ω}-hydroxy-L-arginine), the required co-factors, locations, binding site properties, turnover rates of inducible and possible constitutive isoforms, integrated signalling cascades, and related to factors that determine adaptations throughout life histories.

L-Arginine iminohydrolase (deiminase), L-ornithine carbamoyltransferase, and carbamate kinase are reported to dissimilate arginine and citrulline in *Arabidopsis* chloroplasts (Ludwig, 1993). The iminohydrolase produces ammonia and citrulline (*Figure 12.1*). This enzyme has not been widely reported in plants, and is mainly

found in single-cell organisms. Since the deiminase may arise from contaminating mycoplasma (Choi *et al.*, 1998), NOS assays employing the conversion of tritiated L-arginine to L-citrulline should rule out possible artefacts due to the masking reaction of arginine deiminase. Out of seven plant systems reporting NOS activity, five use the radioactive arginine–citrulline assay (Wendehenne *et al.*, 2001), and may have not checked for L-arginine consumption and L-citrulline formation via mycoplasma.

ROLES OF ARGININE, GUANIDINE, AND CITRULLINE METABOLISM

In plants, the metabolic roles of L-arginine, homoarginine, and γ-hydroxyhomoarginine have always been difficult to explain (Mothes, 1958; Reinbothe and Mothes, 1962; Steward and Durzan, 1965; Rosenthal, 1982; Durzan and Steward, 1983). Early research recognized the importance of arginine in the coordination of respiration, transpiration, photosynthesis, and protein metabolism through 'oxygen potential'. This provided the background for oxygen and L-arginine as substrates for NOS activity and for the subsequent reactions of ·NO and RNS. Transformations among arginine, citrulline and ornithine were only partially clarified by the Krebs–Henseleit cycle (also known as the urea or ornithine cycle, *Figure 12.1*, *cf.* Naylor, 1959; Durzan, 1969a, 1983; Roubelakis and Kliewer, 1978).

In the urea cycle, arginase is considered a cytosolic enzyme, while ornithine is transported to the mitochondrial matrix where ornithine transcarbamoylase uses ornithine and carbamoyl phosphate producing L-citrulline. The carbamoyl phosphate synthetase is also a mitochondrial matrix enzyme. Citrulline, returned to the cytoplasm, is converted to argininosuccinate and L-arginine. The turnover of storage proteins, histones, and regulatory enzymes provides arginine and may also regulate the urea cycle or enrich the guanidino pool. This occurs especially when protein synthesis is reduced, and when plants enter dormancy. The Krebs–Henseleit cycle also operates to provide nitrogen (urea) for renewed protein synthesis in plants. Ornithine decarboxylase (ODC) may also be a source of polyamines. In *Arabidopsis*, ODC is absent and polyamine biosynthesis occurs via arginine decarboxylase (Hanfrey *et al.*, 2001).

Plant NOS activity originating from a L-citrulline–·NO cycle has not been ruled out (*Figure 12.1*; Wu and Morris, 1998). The guanidino N of arginine is used for ·NO and citrulline production, and derives from L-aspartate and other amino acids in the free amino acid pool, or directly from protein turnover. NOS activity would short-circuit the urea cycle, consume oxygen, ATP, and draw soluble N from the free amino acid pool.

L-Arginine nitrate combines two potential substrates for ·NO production by NR, NiR, NI-NOR, and NOS. Levels as low as 0.63 mM nitrate induced 'neomorphic' changes (Waris, 1959). This response contributed to the early interest in totipotency and somatic embryogenesis (Krikorian and Simola, 1999). The widespread use of inorganic nitrates, L-arginine, and acid protein hydrolysates in culture media for embryogenic induction should be re-examined for their contributions to ·NO production and its developmental signalling pathways (*Figure 12.3*). L-Arginine supplied to barley roots strongly inhibited the transport of nitrate and caused the appearance of nitrite in the nutrient medium; however, the role of nitrite in ·NO production was, and still is, not well understood (S. Goyal, personal communication). Protein arginine

residues are essential in the nitrate uptake from corn roots (Ni and Beevers, 1990). N^G-Nitro-L-arginine is a selective inhibitor of NOS (Griffith and Kilbourn, 1996). Ammonium nitrate used in commercial fertilizers and in cell and tissue culture media, is a substrate for ·NO production via NR, NiR, NI-NOR activities, and via NOS when arginine N is derived from ammonia.

GUANIDINO COMPOUNDS

The urea cycle did not explain the natural occurrence of guanidines as products of [^{14}C]L-arginine, nor their role as inhibitors of putative NOS activity (Durzan, 1969a,b; Bidwell and Durzan, 1975; Ventimiglia and Durzan, 1986). During the spring break of white spruce buds, arginase served as a source for ornithine and urea. Glutamine then became the dominant compound in the soluble N pool for the elaboration of amino acids in renewed protein synthesis (Durzan, 1968, 1969a, 1973). Spruce and pine saplings in sand culture accumulated guanidines when ammonium was the sole source of N. However, when nitrate was the sole source of N, the guanidines were difficult to detect (Durzan and Steward, 1967). With the onset of winter dormancy, arginine was diverted as a substrate to increase guanidino compounds. The inhibition of urease activity is another function of guanidines (Mildner and Mihanovic, 1974). In white spruce and jack pine forests, the seasonal competition for light determines the levels of arginine and the composition of the free amino acid pool (Durzan, 1971). Shade to 13% natural light increased arginine and guanidine levels in both species.

Naturally occurring N^{ω}-monomethyl-L-arginine (L-NMMA, *cf.* Durzan and Steward, 1983), and other substituted guanidines, inhibit cellular ·NO production and emissions (Hobbs *et al.*, 1999; Magalhaes *et al.*, 2000; Pedroso *et al.*, 2000a,b). Guanidino compounds are enzymatically produced by oxidative deamination, decarboxylation, transamidination, methylation, *N*-phosphorylation, etc. (Van Thoai, 1965; Durzan and Steward, 1983; De Deyn *et al.*, 1992). Guanidino compounds may generate reactive oxygen species (Mori *et al.*, 1989, 1996). Homologous series of guanidines inhibit plant growth. This effect is reversed by spermine (Srivastava and Smith, 1982). Most plant polyamines are formed from ornithine and agmatine. Arginase is inoperative during soybean development (Goldraij and Polacco, 1999) so that arginine could serve as a substrate for NOS or for the formation of guanidino compounds. The activation of arginase would downregulate ·NO production and prevent NO-mediated nitrosative damage and apoptosis (Gotoh and Mori, 1999).

L-Arginine reacts with oxygen in another enzymatic reaction, *viz.* arginine 2-monooxygenase to form α-keto-δ-guanidinobutyric acid that decarboxylates to γ-guanidinobutyrate. Both guanidines are produced in conifers (Durzan, 1966, 1969a). The monooxygenase is a flavoprotein that acts on canavanine and homoarginine (Olomucki *et al.*, 1968). In *Vicia faba*, γ-guanidinobutyraldehyde originates from agmatine by a γ-guanidinobutyraldehyde dehydrogenase (Masuda and Suzuki, 1984). L-Arginine, fed to jack pine cell suspensions in light and in the presence of nitrate, produced guanidino compounds that contributed 0.6% to the total gain in N, and 0.1% to the autocatalytic gain in dry weight (Durzan and Chalupa, 1976a,b). Guanidino levels increased as the relative growth rate for biomass weight and volume decreased. When cultures reached their final size, γ-guanidinobutryic acid dominated the guani-

dine fraction. Exogenously applied γ-guanidinobutryic acid decreased biomass recovery. Other, yet unidentified guanidines correlated positively with relative growth rates.

ONE-CARBON METABOLISM

One-carbon metabolism comprises: 1) folate-mediated reactions (serine–glycine interconversions); 2) folate-independent reaction (methanol, formaldehyde, and formate metabolism); 3) an activated methyl cycle for methionine synthesis; and 4) the related *S*-methylmethionine cycle for the long-distance transport of methyl groups and reduced sulphur (Hanson and Roje, 2001).

In maize seedlings, formaldehyde (HCHO) is formed by photosynthesis after 10 sec of $^{14}CO_2$ fixation (Trezl *et al.*, 1998). Plants have a NAD-linked formaldehyde dehydrogenase that acts on the glutathione adduct forming *S*-formylglutathione (Hanson and Roje, 2001). The formaldehyde dehydrogenase and *S*-formylglutathione reductase constitute a folate-dependent path to oxidize one-carbon units from HCHO to formate (HCOOH). In red beet, cauliflower, kohlrabi, and grapes, arginine will trap and carry HCHO as N^G-trihydroxymethyl-L-arginine (Trezl *et al.*, 1998). Trapping removes arginine as a substrate for NOS activity, and possibly modifies photosynthesis and the folate cycle. N^G-Hydroxymethylarginine (HMA) is a source for hydroxymethyl groups that are transferred to tetrahydrofolic acid giving N^5,N^{10}-methylene-tetrahydrofolate, a co-enzyme of thymidylate synthase. Aminoguanidine reacts with HCHO to eliminate the C–1 fragment of HMA but not of N^5,N^{10}-methylene-tetrahydrofolate (Hullan *et al.*, 1998). HMA was considered responsible for the endogenous levels of HCHO and for the supply of C–1 fragments to the folate cycle. In soybean root nodules, the reaction of peroxide with oxyleghaemoglobin giving formaldehyde was inhibited by thiourea, but not by L-arginine (Puppo and Halliwell, 1989). Leghaemoglobin-NO complexes are found in intact soybean nodules (Mathieu *et al.*, 1998).

Dimethylarginine dimethylaminohydrolase (EC 3.5.3.18) hydrolyzes asymmetrically methylated arginine residues to citrulline and methylamines (Tran *et al.*, 2000). This enzyme regulates cellular methylarginine levels, which inhibit NOS activity. Protein-arginine *N*-methyltransferases are responsible for the post-translational methylation and demethylation of proteins. Evidence for their turnover to yield naturally occurring methylated arginines and guanidines in plants is lacking. The methyl group of L-NMMA is likely derived from L-methionine, a substrate for ethylene, which is another gaseous signal molecule. Methyltransfer reactions produce betaines that protect against a wide range of environmental stresses. D-NMMA does not block NOS activity (Pedroso *et al.*, 2000b). In *Taxus brevifolia* and *Kalanchoë daigremontiana*, L-NMMA, but not D-NMMA, reduced NO production, DNA fragmentation, and cell death (Magalhaes *et al.*, 1999; Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b).

Anaerobic conditions and iron deficiency in barley roots induce formate dehydrogenase. This enzyme produces CO_2 from formaldehyde and a cytochrome acceptor (Suzuki *et al.*, 1998). It may also produce nitrosoperoxycarbonate that nitrates protein tyrosine residues (*Figure 12.2*). A glutathione-dependent formaldehyde dehydrogenase, which is highly specific for *S*-nitrosoglutathione (GSNO), controls the

intracellular levels of both GSNO and *S*-nitrosylated proteins. Yeast and mice mutants lacking GSNO reductase abolished GSNO consumption, increased GSNO and protein SNO levels, and increased nitrosative challenge. Resistance to oxidative stress was not impaired. Liu *et al.* (2001) concluded that GSNO reductase was critical for homeostasis.

CITRULLINE

In plants, citrulline is formed in the urea cycle and as a product of putative NOS activity (*Figure 12.1*). Naturally occurring ureido products in plants display a great structural diversity, e.g. α-amino-β-ureidopropionic acid, canavanine, desaminocanavanine, the guanidinoureido and amidino moieties of gongrine and gigartinine (*cf.* Durzan and Steward, 1983; Steward and Durzan, 1965). β-Ureidoproprionic acid and ureidoglycolate are intermediates in pyrimidine and purine degradation (Pitel and Durzan, 1975; Muñoz *et al.*, 2001). The latter may include the activities of xanthine oxidase, which in animals represents a source of ·NO.

Wild watermelon survives severe stress and accumulates citrulline to ca. 50% of the total free amino acids (Kawasaki *et al.*, 2000). Citrulline accumulation may implicate the citrulline–NO cycle, with ·NO being the stress-mediating factor (*Figure 12.1*). Water status was also maintained by an increase in enzymes that released free amino acids from peptide bonds, as indicated by cDNA analyses. Water stress is known to increase ·NO emissions (Haramaty and Leshem, 1997; Magalhaes *et al.*, 2000). Stress induced stomatal closing and increased the relative water content of three plant species (Mata and Lamattina, 2001). The use of ·NO scavengers restored transpiration, stomatal apertures, and ion leakage to levels of untreated leaves. The connection of ·NO and citrulline to NOS activity was not evaluated.

Cryptomeria japonica shoots store citrulline rather than arginine (Mori, 1976). Citrulline is commonly found in the vascular systems of several plants. Citrulline, thiols, and RSNOs may become useful markers for ·NO shuttling in drought stress. L-Thiocitrulline inhibits NOS by binding to its haem group and reducing its redox capacity (Guzman and Amoah-Apraku, 1999). Other ureido derivatives are also potent inhibitors of NOS, but their utility is limited by poor uptake (Guzman and Amoah-Apraku, 1999).

ROLES OF THIOLS AND *S*-NITROSOTHIOLS

Thiols, thioredoxins, and glutathione protect against oxidative and nitrosative damage (*Figure 12.2*). Plants have three types of thioredoxins. Two thioredoxins in chloroplasts are linked to ferredoxin and enable enzymes to sense light via a change in their disulphide/thiol status. A third type is linked to NADPH in the cytosol, endoplasmic reticulum, and mitochondria (Ruelland and Miginiac-Maslow, 1999). In maize roots, nitrate induces a ferredoxin that is distinct from constitutive ferredoxins and may transfer electrons from NADPH to NiR and other ferredoxin-dependent enzymes (Matsumura *et al.*, 1997). Pyridine nucleotide disulphide reductase (NADPH) supplies reducing equivalents for redox systems. Reversible thiol oxidoreduction shuttles electrons for DNA synthesis, antioxidant defence, redox regulation of signal transduction, transcription, cell growth and apoptosis. In wheat, the third type of

ferredoxin enhances the mobilization of carbon and nitrogen in germination, and increases the sensitivity to proteases and heat.

Glutathione and ascorbate levels generated by the ascorbate/glutathione cycle and glutathione peroxidases remove ROS in plant organelles (Møller, 2001). Feeding *Nicotiana plumbaginifolia* cells with ^{15}N ammonium sulphate and potassium nitrate resulted in the labelling of arginine, agmatine (*Figure 12.1*), and glutathione (Mesnard *et al.*, 2000). Adaptive stress responses involving peroxynitrite are characterized by increases in glutathione. In transgenic tobacco seedlings, the overexpression of glutathione *S*-transferase and glutathione peroxidase reduced oxidative damage by increasing the scavenging of peroxide (Roxas *et al.*, 2000). ·NO altered the ascorbate–glutathione cycle in peroxisomes of senescing leaves (Del Rio *et al.*, 1998; Corpas *et al.*, 2001). Here, xanthine oxidoreductase and peroxisomal NOS were active and subjected to regulation by ·NO at the Fe–S moiety. This reaction was used to generate superoxide in a fluorescence spectrophotometer assay for an elicitor-induced ·NO burst in tobacco (Foissner *et al.*, 2000). Xanthine oxidoreductase reduces nitrite to ·NO in mammalian cells (Godber *et al.*, 2000).

S-Nitrosothiols (RSNOs) are metabolites of ·NO in intra- and extracellular spaces (Hogg, 2000). The nitrosation of thiols is sufficiently reversible in aqueous buffer to allow low thiol levels at equilibrium to reduce copper and release ·NO and a disulphide from RSNOs. Research with animal cells shows that RSNO bioactivity is involved in: 1) ·NO release for protection against oxidative stress; 2) transnitrosylation or the transfer of the nitroso functional groups from a RSNO to a thiol to prolong the half-life of ·NO and store ·NO. This makes them new factors in membrane transport, in the long-distance transport of glutathione, and possibly in phloem/xylem unloading and water flow. Transnitrosylation occurs chemically or enzymatically, e.g. protein disulphide isomerase. ·NO from extracellular *S*-nitrosothiols is transferred by protein disulphide isomerases on the cell surface into the cytosol (Ramachandran *et al.*, 2001). An oxygen-dependent equilibrium between *S*-nitrosohaemoglobin and iron nitrosylhaemoglobin conserves the ·NO that would otherwise be released in an untimely or deleterious manner (McMahon *et al.*, 2000); 3) *S*-thiolation by a nucleophilic attack on the sulphur of RSNO by a thiolate anion giving a disulphide and a nitroxyl anion. Protein *S*-thiolation is an intracellular response to oxidative stress. *S*-Thiolation is generally a more stable modification than *S*-nitrosation; 4) modulation of intra- and intercellular signal transduction, gene transcription, and apoptosis (Marshall *et al.*, 2000). RSNOs stimulate guanylyl cyclase. They are substrates and inhibitors for enzymes that use glutathione. RSNOs inactivate aconitase and convert it to an iron regulatory protein. Unfortunately, very little is known how oxidation or nitrosylation of transcription factors regulate plant cell activities, or how transcriptional-activating pathways are controlled at other redox-sensitive levels.

In the presence of superoxide dismutase (SOD) and NOS, reduced glutathione reacts with ·NO to produce *S*-nitrosoglutathione (GSNO) (Schmidt *et al.*, 1996). With GSNOs, thioredoxin systems are capable of enabling redox signals and catalytic activity. Thioredoxin cleaves GSNO to liberate glutathione and ·NO (Nikitovic and Holmgren, 1996). This is coupled to the oxidation of NADPH. GSNO also inhibits the thioredoxin system. Genes that regulate glutathione metabolism in *Arabidopsis* coordinately respond to heavy metals and jasmonic acid. Jasmonate and phospholipase D are also involved in wounding reactions in *Arabidopsis* (Wang *et al.*, 2000a).

Wounding an *Arabidopsis* NR mutant led to ·NO burst only in the wounded region (Garcês *et al.*, 2001). This response was blocked by NOS inhibitors and absent when ·NO was scavenged by carboxy-PTIO.

Arabidopsis trichome cells are detoxification sinks that synthesize glutathione (Gutiérrez-Alcalá *et al.*, 2001). The reaction of peroxynitrite with glutathione or cysteine in the presence of ascorbate eliminates hydrogen peroxide, produces thionitrate ($RSNO_2$), and then thionitrite (RSNO in low yield) (Singh *et al.*, 1996). The reaction of $RSNO_2$ with glutathione yields glutathione disulphide (GSSG) and nitrite. At low pH, the nitrite may release ·NO. Glutathione and GSNO in phosphate buffer yield oxidized glutathione, nitrite, nitrous oxide, and ammonia as end products. From bacteria to humans, nitrosative stress is avoided by a glutathione-dependent formaldehyde dehydrogenase (Liu *et al.*, 2001). This enzyme is responsible for the conversion of GSNO with NAD to GSSG and ammonia.

Most RSNOs are photosensitive and unstable at room temperature. They decompose to their corresponding disulphides and ·NO (Wang *et al.*, 2000b). In anaerobic environments, thiyl radicals (·RS) are formed that react directly with RSNOs to yield a disulphide and ·NO. Metal ions, superoxide, seleno compounds, and glutathione peroxidase catalyse RSNO decomposition. Thionitrites are spontaneous ·NO donors in neutral aqueous solutions and reversibly inhibit ribonucleotide reductase, the rate-limiting step in DNA synthesis (Roy *et al.*, 1995).

S-GLUTATHIOLATION, *S*-NITROSATION, *S*-THIOLATION AND TRANSNITROSYLATION

Protein *S*-glutathiolation is the reversible covalent addition of glutathione to cysteine residues on target proteins (Klatt and Lamas, 2000). The reaction results in a mixed disulphide between a protein thiol and glutathione. This changes the intracellular redox state, and is a potential ROS/RNS sensing mechanism that transduces oxidative and nitrosative stimuli into functional responses at various levels in adaptive pathways. *S*-Glutathiolation occurs in carbonic anhydrase, Cu and Zn superoxide dismutase, glycerol phosphate dehydrogenase, glutathione *S*-transferase, ubiquitinating enzymes, caspases, and many other proteins (Klatt and Lamas, 2000). Enzymes are reversibly inhibited by *S*-nitrothiols (Konarev *et al.*, 2000). Loss of activity is associated with the depletion of 5,5'-dithiobis(2-nitrobenzoic acid)-accessible thiol groups, and not due to ·NO release from RSNO. Full enzyme activity and thiol content are restored by incubation of the *S*-nitrothiol-modified protein with glutathione. *S*-Thiolation and *S*-nitrosation are both relevant reactions for *S*-nitrothiols. The relative importance of these reactions depends on the environment of the protein thiol and on the chemical nature of the *S*-nitrothiol.

The protein–protein transfer of ·NO groups (transnitrosation, transnitrosylation) is a chemical post-translational modification for targetting ·NO in signal transduction (Pawłoski *et al.*, 2001). This precise ·NO delivery challenged the notion of simple ·NO diffusion. ·NO, captured by iron, can be shuttled intramolecularly to a thiol group giving RSNOs that produce *S*-nitrosohaems. In red blood cells, this is followed by the transfer of ·NO to other thiol-containing molecules at the membrane-cytosol interface for the delivery of NO to oxygen-poor cells. In plant cells, transnitrosation may transmit ·NO to distant targets for the same reason or to promote correlative events. Transnitrosation requires γ-glutamyltranspeptidase activity.

In addition to the transfer mechanisms for NO, mobile proteins may determine the fates of cells. In plants, cell fate is determined by location and they become differentiated accordingly. In *Arabidopsis short-root* (*shr*) mutants, the SHR protein is necessary for cell division and endodermis specification (Nakajima *et al.*, 2001). SHR moves from the stele to a single layer of adjacent cells, where it enters the nucleus, and autocatalytically reinforces signalling and alters cell fates, the multiplication of cell layers, and possibly radial root patterning. The movement and translocation of the SHR was postulated as likely being through plasmodesmata. Nakajima *et al.* suggest that the role of this protein in positional signalling and cell-fate determination may have been important in the evolution of land plants to acquire efficient vascular function via radially organized tissues. The connection to the ancient origin of NO was not made (Durner *et al.*, 1999).

Clues as to the diversity and uniqueness of transnitrosation in plants may be offered by the study of naturally occurring sulfhydryl derivatives, e.g. thiothreonine, *S*-(carboxymethyl)cysteine, *N*-methylmethionine, *S*-allylmercapto-L-cysteine, L-homomethionine, and several γ-glutamyl peptides, e.g. homoglutathione (γ-glutamyl cysteinyl-β-alanine), and *N,N'*-bis-(γ-glu)-L-cystine; γ-glu-*S*-(propenyl-1-yl)-L-cysteine (Steward and Durzan, 1965; Durzan and Steward, 1983). The γ-glutamyl peptides are especially abundant in germinating seeds. Strong carcinogens (*N*-nitroso derivatives) are formed by sulphur-to-nitrogen and sulphur-to-sulphur transnitrosation (Al-Mustafa *et al.*, 2001).

RSNOs alter the oxidative folding of proteins by shuffling thiol groups and disulphide bonds (Welker *et al.*, 2001). Irreparable oxidative changes may accumulate unfolded or misfolded proteins and evoke an 'unfolded protein response' (Chapman *et al.*, 1998). This response triggers an intracellular signalling pathway that increases the transcription of heat shock protein in the endoplasmic reticulum (ER), and allows the cell to make more chaperones, i.e. proteins that help other proteins avoid misfolding pathways that produce inactive or aggregated states. Other proteins mediate the up-regulation of genes that encode ER-resident proteins and disulphide-bond forming enzymes. So far, the unfolded protein response has not been studied in plants nor related to the activities of NO.

Nitric oxide and systemic signals in plant development and pathology

The first and crucial step in molecular sensing is the transduction of diverse stimuli into a cellular response. Transduction mechanisms are finely tuned to discriminate among different stimuli with speed and sensitivity. The properties of NO are well suited for sensory systems that use facilitated transfer, membrane-bound receptors, and ion channels. NO-dependent signalling and transduction patterns visualized by fluorescent probes and measured by microsensors that electrochemically oxidize NO provide models for the biotransport and fractal distribution patterns of NO and RNS (*cf.* Vicsek, 1989; Rothschild, 1998; Buerk, 2001).

NO signal transduction pathways comprise guanylyl cyclase (GC) and enzymatic reactions that are *dependent* or *independent* of cyclic guanosine 3',5'-monophosphate (cGMP) (*Figure 12.3*; Davis *et al.*, 2001; Wendehenne *et al.*, 2001). NO increases the activity of soluble guanyl cyclases (sGCs) by interacting with its haem moiety (Murad, 1999). sGC converts GTP to cGMP. In spruce needles, NO stimulates cGMP

production (Pfeiffer *et al.*, 1994). The binding of NO to sGC activates a rise in cGMP. A NO signal is transmitted by sGC to downstream elements of the signalling cascade, *viz.* the cGMP-dependent protein kinases, cGMP-gated cation channels, and cGMP-regulated phosphodiesterases. In tomato, NO stimulates the synthesis of cyclic ADP ribose (cADPR), which mobilizes calcium, calmodulin, and abscisic acid signalling pathways (Leckie *et al.*, 1998; Durner and Klessig, 1999). This integrates with other systemic signals and leads to the expression of gene clusters for adaptation to stress and for defence against invading pathogens. cGMP has repeatedly been proposed in *Arabidopsis* signal transduction, but a protein containing a GC domain has not been identified (The *Arabidopsis* Genome Initiative, 2001). Even so, NO is postulated to signal the defence against plant pathogens by activating GC, NOS, other NO generating systems, and by regulating iron homeostasis (Wendehenne *et al.*, 2001). The roles of calcium and calmodulin, if any, in putative NOS activity in plants remain unclear.

In plants, NO targets various proteins having transition metals, haems, thiols, and tyrosine residues through *cGMP-independent* reactions (*Figure 12.3*; Wendehenne *et al.*, 2001). Calcium-dependent potassium channels, transcription factors, phosphatases, kinases, signalling proteins, iron homeostasis, and cytosolic aconitase are also affected. Enzymes inhibited are lipoxygenase, ribonucleotide reductase, iron-metabolizing proteins, aconitase, proton ATPase, and cytochromes. Factors that determine the balance between cGMP-dependent and independent reactions, and the relative importance of beneficial and toxic effects of NO, relate to the concentrations of NO in cells, redox proteins, and the distribution of enzymes that biotransport NO.

When added to plants, sildenafil (Viagra®) potentiates the activity of NO by selectively inhibiting a cGMP-specific phosphodiesterase that is responsible for the degradation of cGMP. The duration of action of cGMP is controlled by the action and tissue distribution of this enzyme. Sildenafil inhibited ethylene production, doubled the half-life of cut flowers, and kept them 'straight' for a week beyond their natural life span (Leshem *et al.*, 1998; Siegel-Itzkovich, 1999). Leshem (2000) considers NO as a newly defined, gaseous, endogenous hormone. Leshem's book should be consulted for the role of NO in postharvest physiology and for topics not covered here.

In animals, the catabolism of NO in intact mitochondria results in the formation of hydroxylamine, nitrite or nitrous acid, depending on the availability of oxygen, superoxide dismutase, and glutathione (Steffen *et al.*, 2001). In vascular, smooth muscle tissue, the action of NO was cGMP-independent and attributed to the inhibition of ornithine decarboxylase (ODC) (Ignarro *et al.*, 2001). In tobacco, the inhibition of ODC and arginine decarboxylase by suicide inhibitors blocked growth and development and may have involved the inhibitory effects of NO (Burton *et al.*, 1989). At low oxygen concentrations, mitochondrial dihydroorotate dehydrogenase is indirectly and reversibly inhibited by NO (Beuneu *et al.*, 2000). In plants, this enzyme catalyzes the oxidation of dihydroorotate to orotate during pyrimidine biosynthesis (Pitel and Durzan, 1975). Northern-blot analysis showed that wheat leaves under drought stress and treated with the NO donor, sodium nitroprusside, accumulated three late embryogenesis abundant transcripts (Mata and Lamattina, 2001). This was thought to increase tolerance to severe drought in yet unknown ways.

Gene-chip and cDNA technologies reveal multiple signalling systems that 'cross-

talk' throughout plant development. Cross-talk models have yet to connect the sources for ·NO to ·NO sinks, e.g. nitrosation, and to processes that biotransport ·NO. Repeated and cross-talked ·NO signals may be preserved and enhanced by *hysteresis processing* by sGC or other factors that convert ligand-binding free energy into protein conformational free energy. Hysteresis is the effect of prior treatments and environments on the present properties of a sensor or system. Repeated signalling via RSNOs may include mechanisms for the repair or 'subtracting out' system misadjustments. The almond 'bud failure' inherited syndrome is characterized by hysteresis in seasonal nonlinear 'misadjustments' in free arginine, citrulline, ornithine, and proline that contribute to the dysfunctional phenotype (Durzan, 1993; Durzan and Kester, 1997). The roles of seasonal heat stress, ·NO and RNS, in contributing to this syndrome are not yet clear.

The damaging reactions of RNS and ROS contribute to ageing in plants. Genetic lesions and their accompanying syndromes become more evident with increased stress, plant size, and complexity. The beneficial reactions for ·NO transport help to strike a balance between events predisposing ageing and the need for regenerative repair. These include hormone sensitivity and action, and the movement of water, nutrients, and photosynthate over sometimes large distances. Regarding the regenerative potential of meristems, the oldest part of a tree is the youngest part developmentally (Kester, 1982). Pruning, cuttings for rooting, the excision of plant parts for propagation, and mechanical forces elicit ·NO bursts (see below). How the reactions of ·NO contribute with plant hormones to the initiation of new meristems, apart from protection against oxidative and nitrosative reactions, remains unclear. If multiplication rates and whole plant correlations are not re-established, development commonly fails with the 'browning' and death of tissues. The control of ·NO levels by thiols and guanidines may offer useful countermeasures for the browning reactions, and for genomic decay, e.g. DNA fragmentation. This has relevance to gravitational biology and to the support of plant life in extraterrestrial environments.

PLANT RESPONSES TO ·NO AND THE UPTAKE AND EMISSION OF NO_x

In plants, emitted ·NO comprises a 'general adaptation syndrome' (Leshem, 2000). The maturation of non-climacteric strawberries and climacteric avocado fruits decreased their ·NO emissions and enhanced the release of ethylene (Leshem and Pinchasov, 2000). ·NO controlled ethylene production stoichiometrically *in vivo* and *in vitro* (Leshem and Haramaty, 1996; Haramaty and Leshem, 1997). Ethylene emission in senescing pea leaves was significantly decreased by ·NO-releasing compounds. Plants fumigated with ·NO aged more slowly than controls to support a protective role for ·NO (Leshem *et al.*, 1998). Exposure to exogenous ·NO increased the shelf-life of strawberries (Leshem and Pinchasov, 2000). Loss of nitrogen to the soil and atmosphere as gaseous ·NO may be a way to eliminate toxic ·NO levels in tissues (Stöhl *et al.*, 2001).

·NO emissions to the atmosphere are converted to nitrogen dioxide ($\cdot NO_2$) within minutes by reaction with ozone, peroxy radicals, and volatile organic compounds (Menzel and Meacher, 1999; Ryerson *et al.*, 2001). ·NO bursts in guard cells may contribute to ·NO emissions to the atmosphere (Leshem *et al.*, 1997; Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a; Garcês *et al.*, 2001). The diurnal production and

emission of NO has been related to CO_2 flux in photosynthesis, and to water loss through transpiration (Wildt *et al.*, 1997). Plants under drought synthesize sphingosine-1-phosphate. This intermediate is a calcium-mobilizing molecule that links abscisic acid (ABA) to the reduction of guard cell turgor and to the closing of stomata–reducing water loss (Ng *et al.*, 2001). ABA and NO bursts stimulate cADPR production (*Figure 12.3*), alter guard cell oscillations (Allen *et al.*, 2000; Schroeder *et al.*, 2001), and mediate stomatal closing (Mata and Lamattina, 2001). Compared to leaf epidermal and parenchyma cells, guard cells were the first to show NO production and DNA fragmentation (Pedroso and Durzan, 2000).

Changes in net photosynthesis due to light intensity or CO_2 altered NO emissions correspondingly (Wildt *et al.*, 1997). The ratio of the NO emission to CO_2 uptake was similar for many plants. Strong NO emissions occurred at night when nitrate levels in the nutrient solution were increased. NO emissions became comparable to the highest rates found in soils. However, after fertilizer treatment, the release of NO at night was different for different plant species. Plants emitted NO in response to inhibitors of electron transport, after herbicide treatment, or under anaerobic conditions (Klepper, 1979, 1990, 1991). The release of NO at night may relate to the RNS and RSNOs in plants during the day, and to mechanisms that shuttle the NO via RSNOs for the night release of NO. Exogenous NO affects the efficiency of photosynthesis at photosystem II, and contributes to syndromes caused by pollution (Leshem *et al.*, 1997). NO would also become available for competitive and autocatalytic reactions involving NO transport (Nedospasov *et al.*, 2000). The nitrosylation of haems and Fe–S complexes by reassimilated NO would interfere with oxygen consumption and delivery, and possibly reduce NOS activity. An unaddressed question is the possible role of NO on phytochrome chromophores, their thiol linkages, and on the developmental timekeeping in photosynthetic organisms (Johnson, 2001).

Nitrous acid (HONO, HNO_2) is released by wheat leaves during nitrate assimilation (Smart and Bloom, 2001). The wounding mechanical handling of greenhouse plants, the pruning of roots and shoots, and their composting will release not only NO, but also *nitrous oxide* (N_2O) (Hellebrand, 1998). NO from a pyridylphenylurea derivative is a potent inducer of shoots in plant tissue cultures (Kurosaki *et al.*, 2000). Plants may reduce NO to NO_2 (Nishimura *et al.*, 1986). The existence of a NO_2 source in plants remains in debate (Lerdau *et al.*, 2000). Atmospheric NO_2 is recycled back to NO by photolysis (Williams *et al.*, 1992; Lerdau *et al.*, 2000). NO_2 uptake is postulated to have a 'compensation point'. At ambient concentrations below the compensation point, the canopy is a net source of NO_2 to the atmosphere. At concentrations above this point, plants may act as a net sink. NO_2 is emitted (denitrification) from transgenic tobacco expressing antisense NiR mRNA (Goshima *et al.*, 1999).The rapid production of NO in and around guard cells (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a; Garcês *et al.*, 2001) indicates that the formation of some nitrogen oxides (NO_x) may originate as products of emitted NO.

POLLUTION, CLIMATIC STRESS, AND FOREST DECLINE

Living with pollutants in the environment has been critical to successful plants. The major sources of NO_x are fossil fuels, and coal-fired power sites. Uranium-235, thorium-232, along with mercury, arsenic, lead, aluminium, iron, chromium, silicon,

calcium, chlorine and other pollutants, are continually dispersed in millions of tons of coal combustion by-products (Corbett, 1993; Judkins and Fulkerson, 1993). Since aminoethylisothiouronium compounds have radioprotective activity (*cf.* Bidwell and Durzan, 1975), and are inhibitors of NOS activity, they are useful to sort out the impacts of NO as a contributor to NO_x, RNS, ROS, and to radiation damage in conifer decline. This may also provide indicators for monitoring ecosystems that are becoming N-saturated. In tree breeding, it is important to know the prerequisites under which a consistent adaptational superiority and flexibility is guaranteed (Gregorius and Kleinschmit, 2001). Superiority is seen as consistently higher adaptive value of one genotype over another across all environmental conditions. Flexibility relates to the adaptive value of the phenotype.

Oxidative stress, ozone, and nitrogenous atmospheric pollutants (Menzel and Meacher, 1999) reduce growth in greenhouse environments (Hufton *et al.*, 1996), and contribute to the decline of trees in urban (Scott *et al.*, 1998) and forest environments (Skelly and Innes, 1995). In some cities, NO_x pollution significantly reduced tree life from 20 to 8 years (Anon., 1992). Plant contributions to indoor air pollution probably have little impact (Spengler and Sexton, 1983). The NO-dependent inhibition of photosynthesis, transpiration, and respiration is diagnostic for selecting spruce trees against decline, and potted plants against air pollution in nurseries and greenhouse environments (Saxe, 1991). NO emissions by stomata are a result of stress responses, chloroplast ageing, and include the release of volatile organic compounds from isoprenoid pathways (Durzan, 2000b). Isoprene serves as an antioxidant in leaves, considering that it reacts with ozone and hydroxyl radicals (Sharkey and Yeh, 2001). The release of NO, isoprene, and other hydrocarbons allow plants to survive rapid temperature changes.

Reduced N is a main growth-limiting nutrient in boreal forests. Ammonia overload from arginine, and increased content of arginine, ornithine, and urea, were implicated in the 'red' decline of conifers caused by climatic stress (Engvild, 1998). This decline differs from *Waldsterben* caused primarily by air pollution, acid rain, nutrition, growth-altering substances, and many other interacting factors. The 'red' decline of forests involves chlorophyll breakdown. During leaf senescence, a red catabolite of chlorophyll (RCC) is accumulated (Wüthrich *et al.*, 2000). RCC is formed by an oxygenase and a RCC reductase (RCCR). This reaction requires reduced ferredoxin, and is sensitive to oxygen and NO. The accelerated death gene (*ADC*) encodes the RCCR and suppresses the spread of disease symptoms (Mach *et al.*, 2001). Other haems are sites for NO binding with potentially damaging effects. Arginine, NO formation, putative NOS activity, RCC formation, and apoptosis are probable components of the 'red' decline syndrome.

NO, NO_x, CO_2, O_2, and volatile organic compounds are assimilated by tissues to signal, integrate, and differentiate time-dependent strategies for survival and adaptation, especially under constraining environments. High-altitude balsam fir forests show constantly moving, wind-induced, cyclic waves of death, regeneration, and maturation (Sprugel and Borman, 1981). Biomass productivity, species diversity, and nutrient cycling are closely tied to this disturbance, and are an integral part of long-term ecosystem maintenance that may be related to how plants produce NO and RNS. Wind influences every facet of growth and yield, including floristic diversity, dispersal, composition, productivity, and longevity. Many organic volatiles are

significant signals that sensitize the defensive reactions of plants. Wounding and herbivore attacks increase the emission of plant volatiles that attract predators to the damaged parts (e.g. Kessler and Baldwin, 2001; Ryan, 2001).

·NO IN PLANT DISEASE RESISTANCE

·NO-dependent signal transduction alerts plants to real or impending injury and triggers protective responses (*Figure 12.3*; Wendehenne *et al.*, 2001). A related oxidative burst produces superoxide and the accumulation of hydrogen peroxide that are toxic to invading pathogens (Lamb and Dixon, 1997). Hydrogen peroxide cross-links sites at the cell wall and acts as a diffusible signal for genes in surrounding cells. The burst arises from the activation of a membrane-bound NADPH and NADH oxidases at the plasma membrane, apoplastic peroxidases, amine oxidases, and oxalate oxidases (Bolwell, 1999). Hydrogen peroxide signals the transcription of glutathione S-transferase and phenylalanine ammonia lyase. Protection against oxidative damage is mediated by regenerated glutathione, and by glutathione reductase, and mono- and dehydroascorbate reductases.

In soybean cell suspensions, the induction of the hypersensitive response and cell death required a balance between ROS and ·NO production (Delledone *et al.*, 2001). Unlike animal cells, the peroxynitrite formed by the reaction between superoxide and ·NO was not an effective inducer of cell death. Hydrogen peroxide, formed by superoxide dismutase-catalysed dismutation of superoxide, and ·NO, triggered the hypersensitive-related cell death. Fluctuations in oxygen levels were the key indicator for redox stress in unaffected cells. The sensor integrating the coactivation of ·NO and hydrogen peroxide was key in pathogen-induced hypersensitive cell death. Hydrogen peroxide, ·NO, and related RNS are now recognized as redox signals in disease resistance (Delledone *et al.*, 1998; Van Camp *et al.*, 1998) but the significance of RSNOs, e.g. *S*-nitrosoglutathione, remain largely unknown (*Figures 12.2* and *12.3*).

In tobacco, ·NO participates in the redox signalling during defence responses following pathogen attack by producing peroxynitrite (Clark *et al.*, 2000). Catalase and ascorbate peroxidase were inhibited by peroxynitrite. In *Solanum tuberosum* leaves infected with *Phytophthora infestans*, ·NO acted as an antioxidant and strongly counteracted many of the cytotoxic effects due to ROS, such as ion leakage, and cell lesions (Beligni and Lamattina, 1999). It also preserved chloroplast membranes and protected reactions in the chlorophyll metabolic pathway (Lazalt *et al.*, 1997). Infected leaves treated with a ·NO donor showed less DNA fragmentation. In potato tubers, ·NO and its donors induced phytoalexin accumulation. This effect was removed by a ·NO trap (Noritake *et al.*, 1996).

Signal molecules and free radicals initiate cell death to limit the spread of infection. This hypersensitive response is followed by the acquisition of resistance to pathogens at sites distal to the original infection giving 'systemic-acquired resistance'. Disease resistance and stress responses in *Arabidopsis* are regulated by multiple signal transduction pathways involving ·NO, salicylic acid, jasmonic acid, ethylene, and glutathione genes (Xiang and Oliver, 1998; Durner and Klessig, 1999; McDowell and Dangl, 2000).

In tobacco, two mitogen-activated protein (MAP) kinases, designated as salicylic acid (SA)-induced protein kinase (SIPK) and wounding-induced protein kinase

(WIPK) were activated in a disease-specific manner following pathogen infection or elicitor treatment (Kumar and Klessig, 2000). Both ·NO and SA activated SIPK, but not WIPK. Neither jasmonic acid nor ethylene activated SIPK or WIPK. SIPK was thought to function downstream of SA in the ·NO signalling pathway in defence responses (*Figure 12.3*). ·NO and salicylic acid acted synergistically in the expression of the pathogenesis-related 1 gene (*PR-1*) (Klessig *et al.*, 2000). After viral infection, NOS-like activity was found in disease-resistant, but not susceptible, tobacco. *PR-1* expression was activated via a ·NO-dependent, cADPR-independent pathway.

Recombinant mammalian NOS in tobacco plants and cell suspensions triggered the expression of the defence-related genes encoding a pathogenesis-related protein and phenylalanine ammonia lyase (Durner *et al.*, 1998). This indicated that components of animal ·NO signalling were operative in tobacco. Infected plants, susceptible to TMV, did not express increased NOS activity. ·NO-resistance was assigned to cyclic ADP ribose and to the release of calcium, i.e. resistance was independent of cGMP. Hausladen and Stamler (1998) pointed out that the calcium release may be regulated by *S*-nitrosylation/oxidation and that, during a respiratory burst, ·NO reacts with superoxide giving bacteriocidal activity.

Aconitase acts as a ·NO sensor for iron homeostasis in plants (Wendehenne *et al.*, 2001). cGMP production and the Fe–S of cytosolic and mitochondrial aconitase were connected to ·NO activity (Navarre *et al.*, 2000). ·NO changes cytosolic aconitase into an iron-regulatory protein (IRP) that controls iron homeostasis, and binds to mRNAs having an 'iron-responsive element' to further regulate protein metabolism. The conversion into the IRP promotes the loss of the iron–sulphur cluster in aconitase. IRP and aconitase activities are mutually exclusive and regulated in part by ·NO. Mitochondrial aconitase was inhibited by ·NO. The increase in iron was postulated to create a lethal environment that would contribute to the hypersensitive reaction and possibly kill the pathogen.

GERMINATION

Nitrites and nitrates stimulate the germination of many seeds, while ammonia salts are usually ineffective (Giba *et al.*, 1998). Nitrites release ·NO at pH 3 or lower by the dismutation of nitrous acid. In several plants, germination is dependent on nitrite, or on hydroxylamine (Hendricks and Taylorson, 1974). In those experiments, hydroxylamines, nitrites, and other nitrogenous compounds yielded ·NO under strong oxidation and promoted germination. ·NO was produced from nitrite, rather than from nitrate reduction *per se*. Peroxidase was thought not to contribute to the oxidation of these compounds to ·NO. The chelation of hydroxylamine(s) with iron atoms of haems was suggested as a source of ·NO.

The inhibition of catalase was required for the breaking of seed dormancy (Hendricks and Taylorson, 1975). Catalase is a ferric haem enzyme that is critical for the degradation of hydrogen peroxide. ·NO at 1 mM, when fully absorbed, completely inhibited catalase activity, as did substituted nitrogen dioxides. This indicated that the ·NO produced in germination contributed to the ·NO-dependent inhibition of catalase. The binding and inhibition of catalase by ·NO is reversible (Brown, 1995). In soybean embryonic axes, total oxygen consumption was inhibited by ·NO in a concentration-dependent manner (Caro and Puntarulo, 1999). It also decreased superoxide anion

generation by microsomes from the embryonic axes (Caro and Puntarulo, 1998). Oxygen consumption was dependent on cytochrome oxidase activity and more sensitive to ·NO exposure than the alternative oxidase pathway. ·NO exposure increased the production of hydrogen peroxide and germination. Compounds that released ·NO induced the growth of maize root segments (Gouvea *et al.*, 1997).

Jack pine and other conifers commonly release arginine-rich seeds during forest fires. Seed composition reflects climate at the seed source and preconditions germination (Durzan and Chalupa, 1968; Chalupa and Durzan, 1972). ·NO released by fires stimulates germination (Hayhurst and McLean, 1974; Wright and Bailey, 1982; Keeley and Fotheringham, 1997). During germination, nitrate, and possibly ·NO, may function as a co-factor for phytochrome action by creating active and spare phytochrome receptors (Hilhorst, 1990). In light, ·NO counteracts ROS damage, stimulates de-etiolation, and inhibits hypocotyl elongation (Beligni and Lamattina, 1999, 2000). The exposure of *Arabidopsis* to ultraviolet-B radiation increased NOS activity (Mackerness *et al.*, 2001). Some plants show no seed dormancy and germinate viviparously or shortly after release (Farnsworth, 2001). Recalcitrant and viviparous species cannot tolerate the maturation drying that is prerequisite to dormancy. Differences in the production and function of plant hormones were implicated in the occurrence of recalcitrance and vivipary. From the foregoing, the ability to produce ·NO and its relation to the generation of RNS, ROS, and apoptosis during germination may be worth examining. The role of arginine (*Figure 12.1*) as a component of seed reserve protein, and as a ·NO donor, has not yet been studied.

MECHANICAL FORCES, GRAVITY, AND WOUNDING

·NO-dependent mechanotransduction converts a mechanical stimulus into a biochemical response. Mechanical forces increase peroxidase activity, ethylene and auxin levels, leading to the formation of 'reaction wood' or 'compression wood' (Savidge, 1988).The major oxidase in lignifying compression wood is a laccase-type polyphenol oxidase (McDougall, 2000). Three copper sites of laccases are targets for ·NO (Martin *et al.*, 1981). Another pivotal intermediate in plant mechanotransduction is 12-oxo-phytodienoic acid (OPDA) (Stelmach *et al.*, 1998). OPDA is an intermediate in plant jasmonate biosynthesis. OPDA derives from linoleic acid and yields 3-hydroperoxylinoleic acid, a precursor for traumatic acid. The latter was observed in the traumatotropic curvature reactions during plant wounding. Lipoxygenase, which catalyzes the peroxidation of polyunsaturated acid, acts on 3-hydroperoxylinoleic acid to programme organelle degradation (Van Leyen *et al.*, 1998). Lipoxygenase has a non-haem iron that reacts with ·NO (Leshem *et al.*, 1997). In animals, it catalytically consumes ·NO, and impairs guanylyl cyclase activation (O'Donnell *et al.*, 1999).

In *Kalanchoë* and *Arabidopsis*, centrifugation, clinorotation, and wounding elicit early cellular ·NO bursts (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b; Garcês *et al.*, 2001). NOS inhibitors prevented these ·NO bursts, and even the subsequent DNA fragmentation in chloroplasts and nuclei. This significantly reduced ·NO-dependent apoptosis. The addition of ·NO donors promoted DNA fragmentation (Pedroso and Durzan, 2000). Prior models for genomic decay arising from mechanical and gravitational forces in space vehicles have not recognized the importance of ·NO

production (Krikorian, 1996; Matsuoka *et al.*, 2001). Long-lived and stress-tolerant plants, capable of producing guanidines and RSNOs that regulate ·NO and protect against mechanical forces, wounding, and vibrations, could be of use in long-term studies in space biology (Durzan, 2000a).

We do not yet know how ·NO affects touch (*TCH*) gene expression when plants are subjected to mechanical forces. The *TCH* family comprises *TCH1* encoding calmodulin (CaM); *TCH2* and *TCH3* encoding CaM-related proteins; and *TCH4* that encodes a xyloglucan endotransglycosylase (Xu *et al.*, 1995; Johnson *et al.*, 1998). ·NO bursts from NR, NI-NOR, and NOS activities are early and rapid signals in response to gravitational and mechanical forces (thigmotropism, centrifugation). Together with the expression of the TCH proteins, they may shape functional phenotypes through metabolic changes and apoptosis. A role for ethylene in the *TCH* response was proposed, but in *Arabidopsis* ethylene-insensitive mutants (*etr1-3, ein2-1*), the ETR1 and EIN 2 protein functions were not evident in responses to mechanical stimulation. ·NO bursts occur prior to ethylene release (Leshem and Haramaty, 1996; Magalhaes *et al.*, 2000), and may transmit signals via kinases to triggering the adaptive responses.

Paclitaxel (Taxol®) is an anti-microtubule agent for the effective treatment of human cancers. Its complex taxane ring structure is produced in amyloplasts and chloroplasts of gravisensing *Taxus* cells (Durzan *et al.*, 1998; Durzan, 1999, 2000a). Side chain modifications are completed in part by cytochrome P450 in cytoplasm. Inhibitors of NR and NOS blocked both ·NO bursts and paclitaxel formation (Dong, Pedroso and Durzan, unpublished). Methyljasmonate (*Figure 12.3*), when added to mechanically agitated cultures of *Taxus* sp., significantly increased the recovery of paclitaxel (Yukimune *et al.*, 1996). Connections among mechanical actions, ·NO bursts, isoprenoid metabolism, and paclitaxel formation may be useful in process control models for metabolic engineering (Durzan, 2000a).

·NO IN APOPTOSIS, DEVELOPMENT, AND REPRODUCTION

Apoptosis, a form of developmentally programmed cell death (ontogenetic cell death) is initiated by stress-induced ·NO bursts. This eliminates transitory organs, tissues, cells, and reshapes plants showing adaptive plasticity. Abscission, an example of apoptosis, adapts the cellular make-up of plants throughout the seasons and their life histories (Addicott, 1982; Havel and Durzan, 1996a,b, 1999). Regular and predictable allometric changes and fractal shapes become evident in the proportions of plant organs, modular construction (Niklas and Enquist, 2001), nutrient transport, cell-cycle progression, and homeostasis (Fussenegger and Bailey, 1988).

In plants, ·NO-dependent apoptosis occurs in embryogenesis, organ development, tissue homeostasis, differentiation, and during the rejuvenation of cells. Models for apoptosis in plant development, as distinct from cell death in defence and pathology, were provided for apomixis (Mogie, 1992), asexual leaf-plantlet formation in *Kalanchoë* (Pedroso and Durzan, 2000), xylogenesis (Groover *et al.*, 1997; Havel *et al.*, 1997), and embryogenesis (McCabe *et al.*, 1997; McCabe and Leaver, 2000). The death of reproductive cells before fertilization eliminates mutations in organelle and nuclear DNA, and regulates offspring numbers when resources are scarce (Krakauer and Mira, 1999). In gymnosperms, the latter is seen in 'brood reduction' (Haig, 1992). In conifer oocytes, the metabolites resulting from ·NO bursts are salvaged during

apoptosis. Some appear to have 'hormonal' effects that synchronize free-nuclear and cell divisions, and contribute to latent diploid parthenogenesis (Durzan *et al.*, 1994; Havel and Durzan, 1996a,b, 1999; Durzan, 1999).

Markers for apoptosis in plant development comprise many variations for the loss of nuclear permeability, chromatin condensation, DNA degradation, release of nucleoli, and nuclear fragmentation. The enucleation and differentiation of Norway spruce early embryonal cells during suspensor formation involves protein ubiquitination, proteasome complexes, and proteases of different types (Durzan, 1996; Havel and Durzan, 1996a). Ubiquitination determines the rate of regulatory protein turnover. ·NO levels were highest in the arginine-rich surface cells of early embryos, and absent in the enucleated suspensors (Durzan and Pedroso, unpublished). Near tree lines in the Arctic, conifer proembryos have the capacity for monozygotic cleavage polyembryony. This results in the production of multiple competitive embryos that are often abortive (Ching and Simak, 1971; Simak, 1973). Cells that are responsible for the cleavage furrow in spruce polyembryogenesis display high levels of ·NO and initiate apoptosis (unpublished data). The role of ·NO production in establishing the cleavage appears analogous to the role of apoptosis in the positional control and patterning of hand digits in mammalian embryos (Merino *et al.*, 1999). In animal apoptosis, poly (ADP-ribose) polymerase (synthase) (PARP) is cleaved by caspases, a family of cysteine proteases, and inactivated. In necrosis, PARP is not cleaved. It is activated and consumes NAD, and subsequently ADP, to impair cellular energy (Szabó and Snyder, 2000).

The overproduction of ·NO causes significant metabolic dysregulation that leads to the onset of diseases, apoptosis, necrosis, and even to death of the whole organism. Gilchrist (1998) and Dickman *et al.* (2001) have identified reactions in plant apoptosis that are common to cell death in mammals. The connections to ·NO are only recent (Pedroso *et al.*, 2000a,b). A common view has been that ·NO-induced cell death is caused mainly by oxidative stress (Eu *et al.*, 2000). This hypothesis predicts that interactions between RNS and ROS-produced oxidants initiate the death programmes. Evidence is emerging that cell death can be independent of ROS and is induced by *nitrosative challenge* (Eu *et al.*, 2000). Nitrosative challenge was characterized by the accrual of nitrosated proteins without a major alteration in the cellular redox state. Interactions between ROS and RNS altered the pathways that yielded nitrite and nitrate without impacting the level of *S*-nitrosation or extent of cell death.

The inhibition of apoptosis by low levels of ·NO is linked to the regulation of respiration (Beltrán *et al.*, 2000). In plants, this may include nitrate respiration. In animals, ·NO inhibits complex IV in the mitochondrial respiratory chain, and maintains cytochrome *c* oxidase in competition with oxygen. The loss of mitochondrial membrane potential was prevented by ·NO (Strasser *et al.*, 2000). The membrane potential collapses only after the accumulation of oxidative damage and/or the exhaustion of gycolytic ATP, followed by cell death. In animals, ·NO may promote cell survival by nitrosating several proteins in proapoptotic pathways (Davis *et al.*, 2001). Caspase activity was reversibly inhibited by protein *S*-nitrosylation/denitrosylation at the active-site cysteine (Mannick *et al.*, 1999). NOS activity inhibited caspase activity independently of cGMP. The distinction between apoptosis in development, and oxidative and nitrosative death in defence is seen in the essential role of a mitochondrial apoptosis-inducing factor (AIF). AIF genetically uncouples

caspase-activated death pathways (Joza *et al.*, 2001). In mouse embryogenesis, AIF promotes the first morphogenic wave of cell death. Unlike AIF, the caspases execute the mitochondrial cytochrome *c*-dependent death programme. The release of mitochondrial cytochrome *c* leads to the cytosolic assembly of the apoptosome and induces mammalian apoptosis. These mechanisms provide new hypotheses for the role of NO and its connections to cell death during morphogenesis.

In *Arabidopsis*, similarities with animal proteins that regulate apoptosis were not detected, although 36 cysteine proteases were found (The *Arabidopsis* Genome Initiative, 2001). In the presence of NO, these are liable for the nitrosation of tyrosine residues. Caspase-like proteases have been related to plant cell death (Solomon *et al.*, 1999; Lam and Del Pozo, 2000). Caspase activation during menadione-induced apoptosis was reported in tobacco protoplasts (Sun *et al.*, 1999). In an ozone-sensitive *Arabidopsis* mutant, ethylene and jasmonate were shown to regulate superoxide-dependent cell death in opposite ways (Overmeyer *et al.*, 2000). In *Arabidopsis* suspension cultures, NO-induced cell death by avirulent bacteria was mediated in part by cGMP, and blocked by a specific inhibitor of guanylate cyclase (Clarke *et al.*, 2000). A MAPK with unknown consequences was also activated by NO.

NO is unlikely to damage DNA directly (Halliwell, 1999). Nitrous acid produces deamination (*Figure 12.2*). Peroxynitrite leads to both deamination and nitration. Responses to DNA damage may include DNA repair at cell cycle checkpoints. In eukaryotic cells, checkpoints halt divisional cycles until conditions are suitable for the cell to proceed to the next stage, e.g. progression through the divisional cycle, or cell death (Havel and Durzan, 1996b). In several plants, including an *Arabidopsis* NR double mutant, DNA fragmentation was coupled to the induction of putative NOS activity, and to NO-dependent apoptosis (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000a,b; Garcês *et al.*, 2001). In *Kalanchoë daigremontiana*, NO bursts led first to chloroplast DNA and then to nuclear DNA fragmentation (Pedroso and Durzan, 2000). 3-Nitrotyrosine was recovered from acid hydrolysates of proteins from *Taxus* cells grown in darkness and predisposed to apoptosis by a prior burst of NO (Pedroso *et al.*, 2000a,b). NO production and cell death were prevented by NOS inhibitors, and by the NO scavenger, carboxy-PTIO.

Conclusions

Our understanding of NO in plant life histories comes when systematic genomic information, proteomics, gene knock-outs, mutations, and transgenic plants may be employed to explain how NO metabolism, signalling, and transduction contribute to resting metabolic rates, irritability, and to oxidative and nitrosative stresses during prolonged genomic × environmental interactions, and throughout development. The reactions of NO with haems and thiols extend the opportunities for life in constraining environments, and impact managed horticultural practices. The exact understanding of how and why NO affects plants is highly dependent on finding the responsible genes, clarification of the endogenous sources of NO, the chemistry of free radicals and their targetted biological functions. Volatile compounds, such as NO_x, ethylene, and isoprene, also contribute gaseous signals to plants under environmental and abiotic challenges, and integrate with the biological effects of NO. The current sweeping views provide a wealth of new concepts, many without rigorous justification,

and are bound to meet with criticism. Experiments will have to determine how many truly new concepts survive.

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