Summary

Recently nitric oxide (NO) has emerged as a key signalling molecule in plants. Here we review the potential sources of endogenous NO, outline the biological processes likely to be mediated by NO, and discuss the downstream signalling processes by which NO exerts its cellular effects. It will be important to develop methods to quantify intracellular NO synthesis and release. Classification of the biosynthetic origins of NO is also required. NO can be synthesised from nitrite via nitrate reductase (NR) and although biochemical and immunological data indicate the presence of enzyme(s) similar to mammalian nitric oxide synthase (NOS), no NOS genes have been identified. NO can induce various processes in plants, including the expression of defence-related genes and programmed cell death (PCD), stomatal closure, seed germination and root development. Intracellular signalling responses to NO involve generation of cGMP, cADPR and elevation of cytosolic calcium, but in many cases, the precise biochemical and cellular nature of these responses has not been detailed. Research priorities here must be the reliable quantification of downstream signalling molecules in NO-responsive cells, and cloning and manipulation of the enzymes responsible for synthesis and degradation of these molecules.

1. Introduction

Nitric oxide, NO, is a small, water and lipid soluble gas that in recent years has emerged as a major signalling molecule of ancient origin and ubiquitious importance (Durner et al., 1999). In 1992 it was named ‘Molecule of the Year’ by Science (Koshland, 1992) and since then there has been a huge number of studies on NO biology. NO emission from plants and its effects on plant growth were described in the early 1970s (Anderson & Mansfield, 1979; Klepper, 1979). However, research on NO and plant signalling was mainly restricted to a few ‘pioneers’ such as Leshem (Leshem & Haramaty, 1996) and Lamattina (Laxalt et al., 1997) until the two landmark publications in 1998 describing NO as a plant defence signal (Delledonne et al., 1998; Durner et al., 1998). Since then, studies on NO and plant biology have increased dramatically, with some of this work being reviewed relatively recently (Durner & Klessig, 1999; Beligni & Lamattina, 2001; Wendehenne et al., 2001; Neill et al., 2002b). In this article, we attempt to describe our current understanding of NO biochemistry in plants, highlight the growing number of biological processes likely to involve NO signalling, and outline the signalling mechanisms by which NO may exert its cellular effects.

2. Why does NO make a good signal?

Nitric oxide (NO) is a gaseous free radical. It contains an unpaired electron in its π orbital, but remains uncharged. However, because of its free radical nature, it can adopt an energetically more favourable electron structure by gaining or losing an electron, so that NO can exist as three interchangeable species: the radical (NO·); the nitrosonium cation (NO+); and the nitrosoyl radical (NO−) (Stamler et al., 1992; Wojtaszek, 2000). NO is sparingly soluble in water (0.047 cm3 cm−3 H2O; 20°C 1 atm), with the solubility increasing in the presence of ferrous salts (Anderson & Mansfield, 1979). Therefore, it is able to move by diffusion in aqueous parts of the cell, such as the cytoplasm, but also move freely through the lipid phase of membranes. Once produced, it can move from one cell to another or within a cell. However, being a reactive free radical, it has a relatively short half-life, in the order of a few seconds. Typically, NO rapidly reacts with O2 to form nitrogen dioxide (NO2), and rapidly degrades to nitrite and nitrate in aqueous solution (Fig. 1). In fact, the formation of nitrates and nitrates is often used diagnostically to measure the previous presence of NO. Thus, the range of its effects is limited to the cell in which it is generated, or to cells in the near neighbourhood.

For a signalling molecule to be effective, it needs to be produced quickly and efficiently on demand, to induce defined effects within the cell, and to be removed rapidly and effectively when no longer required. Alternatively, it is possible that signals function in concert. For example, it could be that continuous NO synthesis is essential for other signalling pathways to operate, such that removal of NO would be inhibitory, even though induction of NO synthesis was not required per se. Another potential scenario might be that NO, constitutively generated, inhibits a particular cellular process. Any stimulus that inhibited NO production would increase the cellular event by virtue of its inhibition of NO synthesis. As detailed below, there are several potential cellular sources of NO in plants, while its chemical structure enables it to move and relay a signal, and be removed efficiently, terminating the message it originally was sent to convey.

NO can also react with other potential signalling molecules, that are likely to be produced temporally and spatially alongside NO. One such chemical is the free radical superoxide anion (O2−) (Pryor & Squadrito, 1995), as depicted in Fig. 1. Superoxide can arise from several sources in plants, including electron leakage from mitochondria and chloroplasts, or from more dedicated sources such as NADPH oxidases (Neill et al., 2002b,c). Superoxide will readily dismute to hydrogen peroxide (H2O2), especially at low pH, or in a reaction catalysed by superoxide dismutase (SOD). In fact, if superoxide is produced, the presence of hydrogen peroxide becomes virtually inevitable. Both superoxide and hydrogen peroxide have been suggested as signalling molecules in plants (Jabs, 1999; Neill et al., 2002b). Consequently, if NO reacts with superoxide or H2O2, this could potentially abrogate superoxide/hydrogen peroxide signalling, as shown in Figs 1 and 2. The product of the reaction between superoxide and the nitric oxide radical, or H2O2 and NO+, is the ion peroxynitrite, itself a reactive and destructive compound. Such interaction between NO and reactive oxygen species (ROS) has been reported particularly during plant-pathogen interactions (Delledonne et al., 2001). NO can also react with proteins, particularly with thiol side groups, or low molecular weight thiols, as discussed in Section 5.5.
3. NO biosynthesis

There are several potential sources of NO in plants (Fig. 2), and it would seem likely that the importance of each of these to the physiological production of NO will depend on the species, the cells/tissues, the conditions under which the plants are grown, and, of course, the signalling pathways active under those specific conditions. There is evidence for NO production in plants from nitric oxide synthase (NOS), nitrate reductase (NR), xanthine oxidoreductase or nonenzymatic sources. Once generated, NO can induce various effects, or react with reactive oxygen species to generate peroxynitrite. It should be noted that NO can exist in three forms, and although it is implied here that only the radical is biologically active, both the NO$^+$ and NO$^{-}$ may have biological effects.

3.1 Nitric oxide synthase (NOS)

A family of enzymes, the nitric oxide synthases (NOS), is instrumental in the generation of NO in animal systems, and this knowledge has prompted a search for homologous mechanisms in plants. In mammals, there are three well-characterised genes encoding NOS. eNOS, or endothelial NOS, is constitutively produced in a wide variety of cells. nNOS, originally characterised from neuronal tissue, is also constitutively produced. On the other hand, iNOS (alternately referred to as mNOS) is an inducible form, expression being increased in the presence of lipopolysaccharide or interferon. nNOS is the largest of the three forms, with a relative molecular mass of approximately 160 kDa. eNOS is approximately 135 kDa, and iNOS approximately 130 kDa (Furchgott, 1995). Mitochondrial NOS (mtNOS) has also been reported (Tatoyan & Ginlivi, 1998).

All NOS isoforms appear to exist in vivo as homodimers, with a topology of each subunit having two distinct domains, with proteolytic cleavage releasing two active enzymes. The oxygenase domain contains the haem and tetrahydrobiopterin binding sites (Fig. 3); so far tetrahydrobiopterin has not been unambiguously identified in plants. The reductase domain contains binding sites for FAD and NADPH, while a calmodulin binding site lies between the oxidase and reductase domains. iNOS is Ca$^{2+}$ independent, and permanently bound to calmodulin, but in the case of nNOS and eNOS the calmodulin is used as a regulatory module and generation of NO is Ca$^{2+}$ dependent.

NOS catalyses the formation of NO from L-arginine, which undergoes a five electron oxidation to L-citrulline. NADPH and molecular oxygen are essential requirements. Arginine is first converted to hydroxyarginine, a nonreleased catalytic intermediate, with the final products being citrulline and nitric oxide (Furchgott, 1995). Activity can thus be assayed using radiolabelled arginine as a substrate, with the formation of radiolabelled citrulline, although it is worth noting that arginine can also be converted to citrulline without the production of NO (Ninnemann & Maier, 1996). It should also be noted that mammalian NOS is capable of producing superoxide ions (Pou et al., 1992), and therefore there is the capacity for the generation of peroxynitrite from this enzyme.

With the well characterised animal NOS as a model, and the availability of anti-NOS antibodies raised against the...
### Table 1: Sources of nitric oxide (NO) in various plants

<table>
<thead>
<tr>
<th>Sources of NO</th>
<th>Evidence</th>
<th>Species (Reference)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide synthase (NOS)</td>
<td>• Activity demonstrated in extracts</td>
<td>Pea (Barroso et al., 1999) Maize (Ribeiro et al., 1999) Tobacco (Durner et al., 1998) Soybean (Delledonne et al., 1998) Lupinus albus (Cueto et al., 1996) Mucuna hassjoo (Ninneman &amp; Maier, 1996)</td>
<td>• ABA-induced NO synthesis and stomatal closure inhibited by NOS inhibitors in pea • Mechanical stress in Arabidopsis induced NO • Bacteria-induced NO production not inhibited by NOS inhibitors • No NOS-like sequences have been identified • Homology to mammalian NOS might be limited • Little known about cellular location of NOS in plants • False positives reported, casting doubt on immunological data • Little evidence correlating NOS activity with NO synthesis in the same species</td>
</tr>
<tr>
<td></td>
<td>• NOS activity inhibited by inhibitors of mammalian NOS</td>
<td>Tobacco (Durner et al., 1998) Soybean (Delledonne et al., 1998) Pea (Barroso et al., 1999) Lupinus albus (Cueto et al., 1996)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• NO released reduced by NOS inhibitors</td>
<td>Soybean (Delledonne et al., 1998) Tobbaco (Foissner et al., 2000) Pea (Neill et al., 2002a) Arabidopsis (Garcés et al., 2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Anti-NOS antibodies used to identify plant proteins</td>
<td>Pea (Sen &amp; Cheema, 1995; Barroso et al., 1999) Wheat (Kuo et al., 1995; Sen &amp; Cheema, 1995) Maize (Ribeiro et al., 1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Anti-NOS antibodies inhibit activity</td>
<td>Pea (Barroso et al., 1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• NO release not inhibited by NOS inhibitors</td>
<td>Arabidopsis (Clarke et al., 2000) Sunflower (Rockel et al., 2002) Chlamydomonas reinhardtii (Sakihama et al., 2002) Scenedesmus obliquus (Mallick et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>Nitrate reductase (NR)</td>
<td>• In vitro production of NO by NR • In vivo production of NO by NR • NR-deficient mutants have reduced NO generation compared to wild type plants</td>
<td>(Yamasaki &amp; Sakihama, 2000) Maize (Rockel et al., 2002) Spinach (Rockel et al., 2002) Arabidopsis (Desikan et al., 2002) Soybean (Dean &amp; Harper, 1986) Arabidopsis (Desikan et al., 2002) Chlamydomonas reinhardtii (Sakihama et al., 2002)</td>
<td>• NR-mediated NO synthesis required for ABA-induced stomatal closure in Arabidopsis • Important to determine nitrite and nitrate concentrations in cells • Sub-cellular localisation of NR needs to be determined • Need to measure NR activities altering following treatments</td>
</tr>
<tr>
<td>Xanthine oxidoreductase (XOR)</td>
<td>• NO shown to be produced by mammalian enzyme under low oxygen tensions in vitro</td>
<td>Reviewed by Harrison, 2002</td>
<td>• No reports of XOR generation of NO in plants, although many tissues may be anaerobic to allow such activity</td>
</tr>
<tr>
<td>Nitrite: NO-reductase</td>
<td>• Nitrite-reducing activity resulted in NO production</td>
<td>Tobacco (Stöhr et al., 2001)</td>
<td>• A potentially important enzyme which needs further characterisation</td>
</tr>
<tr>
<td>Nonenzymatic sources</td>
<td>• Nitrogen dioxide to NO catalysed by carotenoids • Nitrite to NO conversion • NO(_2) converted to NO</td>
<td>(Cooney et al., 1994) Barley aleurone apoplast (Bethke, P and Jones, R, pers. comm.) Rush, Lawn Grass, Ginkgo leaves (Nishimura et al., 1986)</td>
<td>• Requires low pH and a reductant such as ascorbate</td>
</tr>
</tbody>
</table>
mammalian enzymes, several groups have provided evidence for the existence of NOS in plants. Kuo et al. (1995) used a mouse anti-brain NOS anti-body to show the presence of NOS in wheat germ. At the same time, others reported the presence of immunoreactivity to anti-NOS antibodies in plant tissues. Using a rabbit anti-nNOS antibody, pea embryonic axes were shown to contain a 105-kDa protein whilst analysis of wheat germ revealed two bands: 58 kDa and 90 kDa (Sen & Cheema, 1995). However, despite the now fairly prevalent immunological data for the presence of NOS in plants (discussed further below), caution should be exercised, as false positives can and do occur (Lo et al., 2000).

In 1996, Ninnemann and Maier showed, for the first time, the presence of NOS activity in higher plants. They used inhibition by the arginine analogues N\textsuperscript{\textgreek{n}}-nitro-argonine (L-NNA) and N\textsuperscript{\textgreek{omega}}-nitro-1-arginine methyl ester (\textsuperscript{\textgreek{l}}-NAME), and the production of radiolabelled citrulline as evidence. Ribeiro et al. (1999) used a combination of antibody and activity assays to provide evidence for the presence of NOS in maize. Western blot analysis of the soluble fractions from roots and young leaves using anti-mouse iNOS and anti-rabbit nNOS revealed a band at 166 kDa. In addition, the enzyme extracts were capable of converting \textsuperscript{\textgreek{14}}C-arginine to \textsuperscript{\textgreek{14}}C-citrulline. Together, these data suggest the existence of NOS in this plant. Further work using the antibodies revealed that the NOS protein was located in the cytoplasm of the cells, but translocated to the nucleus, with translocation being dependent on the phase of cell growth (Ribeiro et al., 1999).

NOS activity was also reported in peroxisomes from pea leaves. Again using the arginine-citrulline assay, a calcium-dependent activity was found. The presence of NOS was further substantiated by the use of a raft of NOS inhibitors, of which aminoguanidine was found to be the most effective. Interestingly, the NOS activity was also inhibited by the addition of an anti-iNOS antibody (Barroso et al., 1999). Further analysis with antibodies revealed a band on Western blots of approximately 130 kDa, and the immunoreactive protein was localised to peroxisomes and chloroplasts, but not mitochondria. Recent work using antibodies has shown that NOS and catalase co-localise to peroxisomes, the latter enzyme being a characteristic marker for these organelles, and laser confocal immunofluorescence has been used to visualise the location of NOS. The presence of NO in peroxisomes was also further substantiated by fluorometric analysis and electron paramagnetic resonance spectroscopy (EPR) using Fe-MGD (Corpas et al., 2002).

Other workers have reported NOS-like activity in plants in response to various stimuli. Bacterial challenge induced rapid NO generation in soybean suspension cultures (Delledonne et al., 1998), with the effect reduced by the NOS inhibitor L-NNA. Tobacco mosaic virus (TMV) infection of resistant tobacco also resulted in increased NO production that was inhibited by a NOS inhibitor (Durner et al., 1998). NOS activity was detected in roots and nodules of Lupinus albus, and inhibited by the NOS inhibitor L-NMMA (N\textsuperscript{\textgreek{G}}-monomethyl-l-arginine; Cueto et al., 1996). In Arabidopsis, mechanical stress also induced NO production, and inhibition by L-NMMA led Garcès et al. (2001) to conclude that an inducible form of NOS is present. Foissner et al. (2000) showed the release of NO from tobacco using real-time imaging with confocal microscopy and the NO sensitive fluorophore diaminofluorescein diacetate (DAF-2DA; Kojima et al., 1998). This activity was induced by a fungal elicitor from Phytophthora cryptogea, and also inhibited by the NOS inhibitor L-NMMA.

However, despite this growing evidence for the existence of NOS in plants, other groups have reported conflicting data. For example, NOS inhibitors had no effect on NO synthesis in leaf extracts or intact tissues (Rockel et al., 2002), while Clarke et al. (2000) found no effects of NOS inhibitors on the release of NO in Arabidopsis thaliana cells in response to bacterial challenge. NOS inhibitors did not reduce nitrite-dependent NO generation in Chlamydomonas reinhardtii, nor did the addition of \textsuperscript{\textgreek{l}}-arginine, the known substrate for NOS, induce activity (Sakihama et al., 2002). \textsuperscript{\textgreek{l}}-arginine was similarly ineffective as a substrate for NO production in the green alga Scenedesmus obliquus, and two known NOS inhibitors had no effect (Mallick et al., 2000).

There is little evidence for the presence of genes encoding NOS in the Arabidopsis genome, suggesting that in this species at least, the presence of NOS is unlikely. However, it cannot be ruled out. Considering that NOS is a bi-domain enzyme, with a reductase domain very similar to that of P450 reductase (an enzyme to which many of the antibodies used to show the presence of NOS might be binding), it is possible that in plants all that is required for NO generation from such a system would be a dedicated oxidase enzyme, being fed electrons from a common reductase. Such an oxidase could be relatively small, as long as it held a haem in the correct redox mid-point potential. As haem chelation requires only two histidines, such an oxidase could be hard to identify using antimammalian antibodies, or mammalian-like gene sequences.

### 3.2 Nitrate reductase (NR)

It seems clear that even if NOS can be a source of NO in plants, alternate sources must also be present, with NR being seen as the most likely candidate (Yamasaki et al., 1999; Yamasaki & Sakihama, 2000; see Table 1). In fact, over 20 years ago, nitrogen oxides were reported in in vivo assays of soybean leaves (Harper, 1981). Using nitrate reductase-deficient mutants of soybean, Dean & Harper (1986) found that such plants did not evolve NO, unlike wild type plants, indicating NR as a likely enzyme candidate for NO production. These workers isolated and characterised the soybean NR activity, showing that it was NAD(P)H-dependent, had a pH optimum of 6.75, and was cyanide sensitive (Dean & Harper, 1988).
Nitr ate reductase is a key enzyme of nitr ate assimilation in higher plants (Pattanayak & Chatterjee, 1998; Lea, 1999), often catalysing the rate-limiting step. It uses NAD(P)H as an electron source for the conversion of nitr ate to nitrite (Lea, 1999). NR also has the capacity to generate NO, an activity that has been demonstrated in vitro (Yamasaki & Sakihama, 2000) and in vivo (Rockel et al., 2002). The enzyme generates NO from nitrite, again with NAD(P)H as an electron donor (Kaiser et al., 2002). NO is probably generated using MoCo (molybdenum cofactor) as the site of catalysis, as found in another MoCo NO-producing enzyme, xanthine oxidoreductase (Harrison, 2002). However, in vitro, the NO generating capacity of NR could only account for a small part (<1%) of the total NR activity extracted (Rockel et al., 2002). The $K_m$ for nitrite has been found to be approx. 100 µM, a concentration higher than the endogenous nitrite concentration estimated in illuminated spinach leaves (10 µM), and the activity was competitively inhibited by nitrate (Kī – 50 µM). For example, the infusion of nitrate through leaf petioles decreased NO generation. Therefore, the rate of NR-generated NO in vivo will be dependent on the intracellular concentration of both these compounds, as well as the enzymatic turnover rate of the enzyme itself (Rockel et al., 2002). Intracellular nitrite has been estimated to be between 10 µM and 4.8 mM in spinach (Rockel et al., 2002), concentrations that correlate with the findings of others (Siddiqi et al., 1992), while nitrate concentrations have been reported to be in the millimolar range (Miller & Smith, 1996). Using nitrite reductase (NiR) antisense NiR tobacco, Morot-Gaudry et al. (2002) detected elevated endogenous nitrite levels, with a concomitant rise in NO release, indicating a link between nitrite and NO. It should be noted that like other NO-generating enzymes such as NOS, NR can also produce peroxynitrite (Yamasaki & Sakihama, 2000).

In higher plants NR is usually found as homodimer, with subunits of 100–115 kDa, depending on the species studied, although in some species it is tetrameric. The spinach NR has 926 amino acids, while others may be a little smaller: the bean NR being 881 amino acids (Hoff et al., 1996a). Its catalytic action requires electron transfer, a process that involves three prosthetic groups, FAD, haem and MoCo. Kinetic analysis revealed that no single step in the electron transfer was rate-limiting (Skipper et al., 2001). Interestingly, the topology of the protein reveals three structural domains, one for each prosthetic group, separated by hinge regions that are susceptible to proteolytic cleavage (Fig. 3). Towards the N-terminus is the MoCo domain, similar to mammalian sulphite oxidase, with the haem binding region, involving histidine residues and showing similarities to cytochrome $b_5$, lying in a central domain. The FAD binding site is found in the third domain, towards the C-terminal end of the polypeptide, and this domain is similar to cytochrome $b_5$ reductase (Campbell, 1996). Thus, like NOS, NR appears to be constructed of domains which when cleaved from the holoenzyme can have autonomous activity.

Expression of the NR genes is light-dependent, following a diurnal pattern, as well as being nitr ate-inducible (Hoff et al., 1994). Light appears not only to induce the transcription of NR genes, but also influence the production of the protein itself, either through control of translation, or by influencing the stability of the protein once synthesised (Vincentz & Caboche, 1991).

Control of NR activity is via covalent modification, involving phosphorylation and dephosphorylation. NR is rapidly inactivated by phosphorylation following a light to dark transition, the site of phosphorylation being serine-543 (in spinach), an amino acid conserved in NR sequences of higher plants (Rouze & Caboche, 1992; Hoff et al., 1994; Bachmann et al., 1996a). Phosphorylation may be Ca$^{2+}$-dependent (Bachmann et al., 1996a,b; Huber et al., 1996). The NR phosphoprotein is recognised by a NR inhibitory protein (NIP), a member of the 14-3-3 family of controlling polypeptides (Bachmann et al., 1996b; Moorhead et al., 1996; Kaiser & Huber, 2001). Binding of NIP and NR inactivates NR. The rates of degradation of NR are also thought to be dependent on its phosphorylation state and association with the 14-3-3 protein.

Experimentally, the activity of NR has been modulated by the addition of tungst ate. Certainly, pretreatment with tungst ate reduces the subsequent NR activity in cells. Tungst ate serves as a molybdenum analogue, and the reduction in NR activity in plants is caused by the synthesis of an inactive tungstostoprotein (Notton & Hewitt, 1971a). In fact, mRNA levels encoding NR, and levels of NR protein, are increased on tungst ate treatment, although activity is diminished (Deng et al., 1989). No direct inhibition of NR by tungst ate has been reported. However, we have found that abscisic acid (ABA)-induced NO synthesis in Arabidopsis guard cells, and concomitant ABA-induced stomatal closure, are both inhibited by tungst ate (R. Desikan et al. unpublished). Moreover, these ABA effects are not inhibited by the NOS inhibitor L-NAME. These effects are in contrast to those observed for pea, suggesting the data are physiologically relevant. In Arabidopsis, tungst ate did not inhibit stomatal closure induced by NO, H$_2$O$_2$, or darkness, indicating that its effects were specific for NO synthesis. Clearly, it will be important to assess the effects of tungst ate on NR activity, both in vitro and in vivo. NR can also be inhibited by cyanide (Notton & Hewitt, 1971b) or azide (Yamasaki & Sakihama, 2000), but these have limited experimental value as they are known to inhibit many other enzymes, for example cytochrome oxidase.

Undoubtedly, the identification of plants lacking in NR activity, or at least with severely depleted activity, will aid greatly in the identification of the role of NR in plants – for example, the nia1, nia2 Arabidopsis mutant, in which both NR genes are mutated (Wilkinson & Crawford, 1993), as well as those of other species such as soybean (Dean & Harper, 1986). NR-deficient mutants of Arabidopsis were instrumental in our work to investigate the role of NR in ABA-induced NO generation (see section 4 below). Further work will no
doubt show that NR, and its generation of NO, are involved in many more physiological responses in plants.

NR was also found to be the source of NO in the green alga *Chlamydomonas reinhardtii* (Sakihama *et al*., 2002). Using a NO-specific electrode and the fluorescence probe DAF-2DA, nitrite was shown to induce NO generation in the dark, a response that was suppressed in light. Furthermore, in a NR-lacking mutant, cc-2929, the response was annulled, providing good evidence that NR was the source of NO.

As might be expected, NR activity in spinach leaves was reduced by the addition of phosphatase inhibitors (Rockel *et al*., 2002). NR activity was also increased by the addition of uncouplers, while inactive NR was activated by rises in 5′AMP (Kaiser *et al*., 1999). Along with anoxia, both 5′AMP and uncouplers led to a rise in nitrite concentration in the cells, and a rise in NO generation (Rockel *et al*., 2002). Similar observations, showing the modulation of NR activity by 5′AMP, have also been made in cucumber (de la Haba *et al*., 2001).

3.3 Other enzymatic sources of NO

Other enzymes can also generate NO (Table 1). For example, Stöhr *et al*., (2001) demonstrated nitrite-reducing activity in tobacco roots, which resulted in the generation of NO. The plasma membrane-enzyme was designated as nitrite: NO-reductase (Ni-NOR). It was insensitive to cyanide and to anti-NR IgG, and gel-filtration showed that it had an apparent mass of 310 kDa, much larger than the 200 kDa estimated for NR. Such an enzyme could be very important, especially if NO is acting as an intercellular signal, as it is well placed to release NO from the cell. Clearly, there is a need to characterise this enzyme and to determine its species and tissue distribution.

Xanthine oxidoreductase (XOR: otherwise referred to as xanthine oxidase (XO) or xanthine dehydrogenase (XDH)), is also an enzyme recently shown to produce NO (Harrison, 2002). Like NR, this is also a redox enzyme with a molybdenum cofactor. NO-generating activity is increased at low oxygen tensions, in catalysis which, like NR, sees the conversion of inorganic nitrite to NO (Millar *et al*., 1997; Godber *et al*., 2000a). If oxygen is present, superoxide is also produced. Therefore, at near anaerobic oxygen tensions, a competition for electrons is established, with the enzyme producing both superoxide and NO. The superoxide can subsequently react with NO to form peroxynitrite (Godber *et al*., 2000b), and this has been suggested as an antibacterial mechanism in mammals (Hancock *et al*., 2002). Therefore, this enzyme has the capacity to produce two signals – either superoxide (which will dismutate to H₂O₂ and ROS signalling if oxygen tensions are high, or NO signalling if oxygen tensions drop. As some plant tissues such as roots can become temporarily anaerobic, this enzyme offers an exciting signalling scenario, where signals can be modulated by the availability of oxygen. Xanthine oxidase activity has been found in plant peroxisomes (Corpas *et al*., 2001). The role of this enzyme in plant signalling needs to be clearly established, and little work in this area has been carried out to date.

3.4 Nonenzymatic sources of NO

NO can also be generated from nonenzymatic sources under the correct conditions. If the environment is particularly acid or reducing, then chemical reduction of nitrite will yield NO, with the chemistry proceeding through a step involving nitrous acid, while ascorbate can react with nitrous acid to yield dehydroascorbic acid and nitric oxide (Weitzberg & Lundberg, 1998). Such reactions can occur in plant tissues. For example, it has been shown recently that barley aleurone cells can generate a sufficiently acidic apoplastic environment to support nitrite to NO conversion using ascorbate as a reductant (P. Bethke & R. Jones pers. comm.).

Light-mediated conversion of nitrogen dioxide to nitric oxide can be catalysed by carotenoids (Cooney *et al*., 1994), although this requires an acid pH and will only occur in selected compartments in the cells. NO₃⁻ was also reported to be absorbed by rush, lawn grass and ginkgo leaves, and released as NO. The potential reductant was fractionated and identified as a polysaccharide (Nishimura *et al*., 1986).

4. NO biology

4.1 Experimental approaches

Application of NO to plants and induction of subsequent responses does not, of course, by itself prove that endogenous NO mediates the particular developmental or physiological process under study. Rather, as with plant hormones, various criteria should be met in order to provide convincing evidence of a role for endogenous NO. These include induction of the process following NO application; inhibition correlated with inhibition of NO synthesis via chemical or genetic means; inhibition of the response following removal of the NO via NO-scavengers; and correlation of NO synthesis/concentration with the particular biological process. Typically, NO is applied to plants (and most organisms) via an NO-donor – that is, a molecule that will generate NO, sometimes after passage into cells. This approach is technically simple (compared to the application of NO gas which has only rarely been used). A range of concentrations can be used and, as NO release can often be measured, it is possible to investigate NO dose–response responses. Various NO donors have been employed, with the assumption that they release NO. Probably the most commonly used is sodium nitroprusside (SNP), a compound that in fact is likely to generate NO⁺ (Stamler *et al*., 1992). Other NO donors include S-nitrosothioglutathione (GSNO), a compound that does release NO but that may have other effects (Hogg, 2000),
 SNAP (S-nitroso-N-acetylpenicillamine; Durner et al., 1998), RBS (Rousin's Black Salts; Clarke et al., 2000) and NOR-3 ((+/-)-(E)-4-ethyl-2-[(E)-hydroximino]-5-nitro-3-hexanamide; Huang et al., 2002). In order to be confident that the effects of NO donors are truly due to NO release, and not due to the chemicals per se, it is useful to use more than one donor. It is also useful to include suitable controls – for example the residual chemical after cleavage and release of NO, and to show that the effects of the donor can be negated by application of an NO scavenger. The NO scavengers PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) and cPTIO (carboxyPTIO) are commonly used. PTIO is supposedly specific for NO but cPTIO has been reported to inhibit NOS; in fact, any pharmacological approach has to be edged with caution, as most biologically active molecules are likely to have side-effects. Several NOS inhibitors such as L-NAME widely used in mammalian studies have also been used with plants (see section 3 above). As no NOS has yet been cloned from plants, there are clearly no NOS\textsuperscript{−} mutants yet available. However, NR\textsuperscript{−} mutants are available, and have been used to provide evidence both for and against the NO-generating involvement of NR in biological processes (Garcês et al., 2001; Desikan et al., 2002).

4.2 Demonstration and quantification of NO

Ideally, NO should be quantified accurately and reliably at its cellular site of action. This means that any technique should measure NO and not other molecules, and not be prone to interference. In fact, NO quantification is problematic for various reasons. In a similar way to ethylene measurements, NO emission from plants has been estimated by gas chromatography and mass spectrometry (Dean & Harper, 1986; Magalhaes et al., 2000; P. Bethke & R. Jones, pers. comm.), chemiluminescence (Wildt et al., 1997) and using laser photo-acoustic spectroscopy (Leshem & Pinchasov, 2000). This latter technique is very sensitive and specific and is being used to record reliable changes in NO emissions from plants subject to various treatments, e.g. pathogen infections (L. Mur, pers. comm.). It is assumed that rates of NO emission reflect global rates of NO synthesis and reactions although it is difficult to know if they actually reflect NO concentrations in target cells. NO can be assayed in solution using an NO-electrode, that measures the steady-state concentration attained by release from the source of NO, be that an NO-donor or cells releasing NO. NO can also be measured by reaction with molecules such as haemoglobin, that reacts with NO and displays a spectral shift that can be detected and used to quantify the accumulation of NO (Murphy & Noack, 1994). Haemoglobin can also react with other molecules such as ROS that may be present with NO. NO-sensitive fluoros have been developed, such as DAF-2DA (Kojima et al., 1998). DAF-2DA is a cell-permeable molecule that does not fluoresce until it reacts with NO, and can thus be used to monitor relative intracellular NO content using fluorescence and confocal microscopy. Although DAF-2DA does not react with ROS (Foissner et al., 2000) there are reports of nonspecific (i.e. non-NO) fluorescence (Beligni et al., 2002). The non-NO-reactive analogue 4-AF is a useful control (Desikan et al., 2002; Mata & Lamattina, 2002). Reaction with calcium has also been reported to increase the sensitivity of DAF-2DA to NO (Brouillet et al., 2001), an important point as calcium elevations represent a common signalling response in cells. DAF-2DA fluorescence can clearly report changes in NO within plant cells, but it is difficult to equate changes in fluorescence with the actual concentration of NO in solution. In epidermal and guard cells, DAF-2DA fluorescence is commonly at its most intense in chloroplasts (Foissner et al., 2000; Desikan et al., 2002; Nelll et al., 2002a) but this might simply reflect DAF-2DA accumulation in these organelles. EPR spectroscopy can also be used to quantify NO within tissues (Pagnussat et al., 2002). NO will react with ‘spin-trap’ probes to form a stable adduct with characteristic EPR spectral properties that can then be monitored.

Given the technical and conceptual problems, it is not surprising that there are relatively few data available on quantification and detection of NO. Leshem & Haramaty (1996) reported up to 160 nmol/g f. wt of NO from wilted pea leaves. In Arabidopsis, Magalhaes et al. (2000) observed 500 nl/g f. wt h\textsuperscript{−1} of NO being emitted from wild type plants. In Arabidopsis cells, up to 180 nmol/g f. wt h\textsuperscript{−1} of NO release has been detected following pathogen challenge (Clarke et al., 2000), and an endogenous NO content of 55 nmol/g f. wt was measured in auxin-treated cucumber roots (Pagnussat et al., 2002).

4.3 NO and plant growth and development

It has been known for some time that plants emit NO under normal growing conditions, and that NO can accumulate in the atmosphere from various sources, including industrial pollution (Wildt et al., 1997 and references therein). Detrimental effects of NO on photosynthesis were reported many years ago and subsequently the effects of NO on plant growth were found to be concentration dependent (Anderson & Mansfield, 1979). High levels (40–80 ppm) inhibited tomato growth, whereas low levels (0–20 ppm) enhanced growth, findings repeated for lettuce (Hufton et al., 1996) and pea (Leshem & Haramaty, 1996). Recent data from Takahashi & Yamasaki (2002) show that NO can reversibly suppress electron transport and ATP synthesis in chloroplasts. As nitrite can be a source of NO, it was suggested that, under conditions where nitrite reduction by nitrite reductase is limited, NR-produced NO could inhibit photosynthesis. In accord with this suggestion, antisense-nitrite reductase tobacco plants have been found to accumulate increased NO and exhibit reduced growth (Morot-Gaudry et al., 2002).
The effects of NO on hypocotyl and internode elongation have also been reported. NO donors inhibited hypocotyl growth and stimulated de-etiolation and an increase in chlorophyll in potato, lettuce and Arabidopsis (Beligni & Lamattina, 2000). NO also increased chlorophyll content in pea leaves, particularly in guard cells (Leshem et al., 1997), and retarded chlorophyll loss in Phytophthora infestans-infected potato leaves (Laxalt et al., 1997). The positive effects of NO on chlorophyll retention may reflect NO effects on iron availability. Graziano et al. (2002) have shown that iron availability is improved in the presence of NO. Iron deficiency results in chlorosis caused by reduced chloroplast development, and NO treatment of wild type maize inhibited such chlorosis. Furthermore, iron deficiency in yellow stripe mutants was reversed by NO application (Graziano et al., 2002).

Some reports indicate that NO may have anti-senescent properties. Leshem & Haramaty (1996) found that application of an NO donor to pea leaves under senescence-promoting conditions decreased generation of ethylene, an endogenous driver of senescence, that was subsequently shown to result from an inhibition of ethylene biosynthesis (Leshem, 2001). On the other hand, Magalhaes et al. (2000) observed that exposure of Arabidopsis plants to NO gas increased ethylene levels, and that inhibition of NO synthesis did not affect ethylene accumulation. A potential role for NO in delaying flower senescence is indicated by the increased longevity of several varieties of cut flowers induced by application of NO donors (Leshem, 2001).

Fruit ripening is another senescence-related process promoted by ethylene that can be retarded by NO. Increased ethylene production during ripening of fruits such as banana and strawberries coincides with reduced NO emission (Leshem & Pinchasov, 2000; Leshem, 2001). In addition, application of NO to vegetables and fruits also delayed senescence and extended their postharvest life.

NO can stimulate seed germination in various plant species. Application of NO donors broke dark-imposed seed dormancy in lettuce, that was reversed in the presence of the NO scavenger PTIO (Beligni & Lamattina, 2000). Such effects of NO might also explain the germination of dormant seeds of California chaparral plants induced by smoke containing nitrogen oxides (Keeley & Fotheringham, 1997). Giba et al. (1998) used various NO donors and the appropriate inactive compounds, to demonstrate that phytochrome-controlled germination of Empress tree seeds was mediated by NO. At low pH (2.5 and 3), nitrite promoted seed germination, and acidic conditions minus nitrite did not (Giba et al., 1998). NO was generated from nitrite under low pH conditions (Giba et al., 1998), and nitrite-induced NO synthesis via a nonenzymatic route can occur in plants (see section 3.4 above). Thus, some early reports on the promotion of seed germination by nitrite might be explained by NO generation. For example, Hendricks & Taylorson (1974) and Cohn et al. (1983) reported that nitrite promoted germination of seeds from various plant species. These effects of nitrite (and thus NO) on seed germination are interesting, and suggest that the level of soil nitrite is one factor determining seed germination.

New roles for NO in plant growth and development are likely. For example, a recent report (Pagnussat et al., 2002) has provided exciting data on NO and root development. NO (applied via the NO donors SNP and SNAP) induced adventitious root development in cucumber. Auxin-induced root growth and formation of lateral roots was also blocked by the NO scavenger cPTIO (Pagnussat et al., 2002). A previous report had suggested a role for NO in root elongation in maize, although in this case the effects of auxin were not reversed by a NO scavenger (Gouvea et al., 1997).

4.4 NO and hormones

It may well be that in some cases, NO mediates the biological effects of primary signalling molecules such as hormones, a situation analogous to that being revealed for H2O2 (Neill et al., 2002b,c). For example, hormones may be transported from one location to another and therein induce NO synthesis, or hormone sensitivity may alter developmentally or in response to an environmental stimulus, such that cells acquire the competence to respond to hormones by way of NO synthesis. In either case, NO synthesis might be restricted to specific target cells. So far, cytokinin has been shown to induce NO synthesis in tobacco, parsley and Arabidopsis cell cultures (Tun et al., 2001). Some cytokinin effects can be mimicked by NO – NO donors induced betalaine accumulation in Amaranthus seedlings, and cytokinin-induced betalaine accumulation was inhibited by a NOS inhibitor (Scherer & Holk, 2000), suggesting that NO might mediate some cytokinin effects. A novel role for cytokinins in the induction of programmed cell death (PCD) has recently been proposed (Carimi et al., 2002). Given that NO induces PCD (see section 4.7 below), it is possible that NO mediates this cytokinin-induced process. Cytokinin-response mutants such as cki1 (Kakimoto, 1996) will be useful tools here.

The recent discovery that NO mediates ABA-induced stomatal closure represents a significant development in NO research. Stomatal movements are effected by osmotic fluxes of water across the tonoplast and plasma membrane, such fluxes being driven by movements of K+ and Cl– ions through specific channels that are activated and deactivated in response to various stimuli such as ABA (Schroeder et al., 2001). Guard cell signalling is highly complex, but most signals elicit changes in cytosolic calcium concentrations, often in an oscillating manner (Schroeder et al., 2001). Calcium increases are induced by signalling molecules such as inositol triphosphatase (IP3) and hexakisphosphate (IP6), sphingosine-1-phosphate (S1P), H2O2 and cyclic adenosine 3′,5′-diphosphoribose (cADPR) (Hetherington, 2001). Other signal transduction mechanisms in guard cells include alterations in pH, cytoskeletal arrangement, gene expression and
membrane trafficking (Hetherington, 2001; Schroeder et al., 2001). Recent work has identified novel facets of ABA signal transduction in guard cells, such as mediation of calcium release by H$_2$O$_2$ and S1P (Hetherington, 2001; Schroeder et al., 2001), the involvement of G-proteins (Wang et al., 2001) and the role of protein phosphorylation and RNA-binding proteins (Li et al., 2002).

In our laboratory, we have shown recently that ABA induces rapid NO synthesis in guard cells (and other epidermal cells) of pea (Neill et al., 2002a). This finding has since been extended to *Vicia faba* (Garcia-Mata & Lamattina, 2002) and *Arabidopsis* (Desikan et al., 2002). ABA did not induce NO synthesis in *Arabidopsis* suspension cultures (Tun et al., 2001), indicating tissue specificity. In pea, ABA-induced NO synthesis in guard cells was required for stomatal closure, as removal of NO with the scavenger PTIO substantially inhibited ABA-induced closure. Pharmacological evidence indicated NOS as a source of NO, as ABA-induced stomatal closure and NO synthesis were both inhibited by L-NAME (Neill et al., 2002a), but not by tungstate, a potential NR inhibitor (R. Desikan et al., unpublished). ABA-induced NO synthesis is also required for ABA-induced stomatal closure in *Arabidopsis* (Desikan et al., 2002; Fig. 4). Here, however, the source of NO appears to be NR, not NOS. Tungstate strongly inhibits ABA-induced NO synthesis and stomatal closure (see section 3.2 above) but L-NAME has no effect (Desikan et al., 2002). Consistent with this finding, nitrite induces NO accumulation in guard cells and stomatal closure, both events being negated by a NO scavenger (Desikan et al., 2002 and Fig. 4). Moreover, guard cells of the NR-deficient *Arabidopsis* nia1, nia2 mutant (Wilkinson & Crawford, 1993) do not synthesise NO nor do they close in response to nitrite or ABA (Desikan et al., 2002). It seems possible, then, that there exist species differences in NO synthesis, at least in terms of guard cell responses to ABA.

NO synthesis has recently been found to be induced by auxin in cucumber roots (Pagnussat et al., 2002). NO was required for root growth and the formation of lateral roots, but the source of NO has not yet been determined.

### 4.5 NO and abiotic stress

It is well known that various abiotic stresses such as drought, low and high temperatures, UV and ozone exposure induce the generation of ROS (Neill et al., 2002b; Vranova et al., 2002). ROS initiate several oxidatively destructive processes, but also trigger various signalling pathways (Neill et al., 2002b; Vranova et al., 2002). Thus, maintenance of appropriate ROS levels might represent a survival response. In fact, NO interacts with ROS in various ways and might serve an antioxidant function during various stresses (Beligni & Lamattina, 1999). Modulation by NO of superoxide formation (Caro & Puntarulo, 1998) and inhibition of lipid peroxidation (Boveris et al., 2000) also illustrate its potential antioxidant
role. On the other hand, excess NO can result in nitrosative stress (see section 5.5 below), so a favourable balance of ROS/NO is important.

Drought stress is a major environmental constraint on crop productivity and performance, and understanding the cellular processes that ameliorate the consequences of drought stress and conserve water are clearly important. ABA is synthesised following turgor loss and stimulates guard cell NO synthesis, but the effects of dehydration on NO generation have not yet been resolved. Leshem & Haramaty (1996) reported that wilting increased NO emission from pea plants. However, working with Arabidopsis, Magalhaes et al. (2000) found the opposite. This might represent species differences, but it will be important to determine the effects of water stresses at a range of reduced water potentials and over varying time periods. Nevertheless, application of NO donors does reduce stomatal apertures and thereby reduce transpiration in several species (Mata & Lamattina, 2001). It is likely that NO does not act alone, but interacts with other signalling molecules such as H$_2$O$_2$, to effect stomatal closure. Preliminary data indicate that both H$_2$O$_2$ and NO are required for full stomatal closure in Arabidopsis (R. Desikan et al., unpublished). There is also some evidence that ROS and NO interact to induce ABA biosynthesis. In response to drought stress, an increase in NOS-like activity was observed in wheat seedlings, and ABA accumulation was inhibited by NOS inhibitors (Zhao et al., 2001). ABA-induced NADPH oxidase activity during drought stress, leading to increased ROS levels, has also been reported for maize (Jiang & Zhang, 2002), indicating a close interplay between ABA, ROS and NO levels. The use of ABA, ROS and NO mutants should help to elucidate these complex interactions.

NO responses to other stresses such as heat and chilling have also been noted. Short-term heat stress caused an increase in NO production in alfalfa (Leshem, 2001). Application of NO mediates chilling resistance in tomato, wheat and corn (Lamattina et al., 2001). It is possible that this effect reflects the antioxidant properties of NO, via suppression of the high levels of ROS that accumulate following exposure to chilling or heat stress (Neill et al., 2002b).

Using NO donors and a NOS inhibitor, Mackerness et al. (2001) have shown that UV-B-induced expression of chalcone synthase (CHS) occurs via a NOS-like enzyme. Although this is the first demonstration of a role for NOS and NO in UV-B responses, it remains to be seen whether UV-B treatment actually causes an increase in NO synthesis, and if NO is also involved in other responses to UV-B radiation. In relation to this, treatment of potato tubers with NO donors before UV irradiation was found to result in 50% more healthy leaves compared to plants not pre-treated with NO (Lamattina et al., 2001).

Another atmospheric pollutant that might interact with NO is ozone. Ozone treatment of Arabidopsis plants induced NOS activity that preceded accumulation of salicylic acid (SA) and cell death (Rao & Davis, 2001). In tobacco, NO was found to induce SA synthesis (Durner et al., 1998). Moreover, NO treatment has been shown to increase the levels of ozone-induced ethylene production and leaf injury (Rao & Davis, 2002).

Wounding is a common consequence of pathogen challenge of plants, during which the generation and increased accumulation of NO and H$_2$O$_2$ are frequently observed (Delledonne et al., 1998). Orozco-Cardenas & Ryan (2002) demonstrated that although wounding per se does not induce the generation of NO, treatment with NO donors inhibited H$_2$O$_2$ generation following wounding, as well as the expression of specific wound-induced genes. This suggests that NO produced during pathogenesis might inhibit H$_2$O$_2$ synthesis and the activation of specific wound-induced signalling pathways.

4.6 NO and biotic interactions

The publication of two papers in 1998 describing the role of NO in plant defence signalling (Delledonne et al., 1998; Durner et al., 1998) led to a big increase in NO research. Both these papers demonstrated a key signalling role for NO during the induction of the Hypersensitive Response (HR). HR is a defence process activated in plants in response to pathogen attack. Associated with the HR is an oxidative burst, in which there is greatly increased ROS generation, PCD, and the activation of signalling pathways driving the expression of various defence-related genes. HR results in localised plant cell death, which in turn limits nutrient availability and thus growth and spread of the invading pathogen. Treatment of soybean cultures with avirulent (HR-noninducing) but not virulent (HR-noninducing) Pseudomonas syringae pv glycinea, induced rapid NO synthesis with kinetics similar to H$_2$O$_2$ generation (Delledonne et al., 1998). Moreover, NO donors induced cell death (but only during rapid mechanical agitation of the cells, indicating an interaction between NO and mechanically induced H$_2$O$_2$) as well as expression of phenylalanine ammonia-lyase (PAL) and CHS genes. Furthermore, bacterially induced cell death and PAL gene expression were blocked by NOS inhibitors, and constitutive NOS activity was identified in cytosolic fractions of soybean. Induction of HR in Arabidopsis leaves by P. syringae pv maculicola was also reduced by NOS inhibitors. Together, these data indicate that pathogen-induced NO produced via NOS, interacts with H$_2$O$_2$ to mediate the HR.

Durner et al. (1998) also provided compelling evidence for the role of NO during plant defence responses. Infection of tobacco plants with HR-inducing varieties of tobacco mosaic virus (TMV) induced NOS activity that was inhibited by NOS inhibitors. NO also induced the synthesis of SA and expression of the defence-related gene PR-1. SA is a defence signalling molecule involved in the development of systemic acquired resistance (SAR; Draper, 1997). Thus, the data of
Durner et al. (1998) suggested a role for NO in SAR. Given that SA treatment leads to enhanced NO production (Klepper, 1991) a complex signalling relationship between \( H_2O_2 \) and NO and SA during HR and SAR is likely (Van Camp et al., 1998; Song & Goodman, 2001).

Accumulation of phytoalexins is another phenomenon associated with the HR during pathogen challenge. Treatment of potato tubers with a NO donor stimulated an increase in the accumulation of the phytoalexin rishitin (Noritake et al., 1996). This NO-mediated effect was inhibited by treatment with the NO scavenger PTIO. However, elicitor-induced rishitin accumulation was not affected by PTIO, suggesting that perhaps in this case, endogenous NO is not involved in the defence response. More recently though, a role for endogenous NO in phytoalexin biosynthesis was described. NO treatment of soybean cotyledons triggered the biosynthesis of phytoalexins (Modolo et al., 2002). Furthermore, elicitor-induced phytoalexin formation was inhibited by NO inhibitors, implying NO as a source of NO in this pathway. NO regulation of phenylpropanoid metabolism was reported by Enkhardt & Pommer (2000). In maize shoots, NO appeared to bind to the haem group of cytochrome P450, and thus affect the activity of cinnamic acid hydroxylase, a cytochrome P450-dependent monoxygenase involved in phenylpropanoid biosynthesis.

A role for NO during symbiosis has been suggested in bacteria–legume interactions. NO was detected in soybean nodules using EPR spectroscopy (Mathieu et al., 1998), and, more recently, using confocal microscopy in alfalfa nodules (Herouart et al., 2002). NOS immunoreactivity has been demonstrated in Lupinus nodules (Cueto et al., 1996). However, a clear function for NO during symbiosis has not yet been established. There have been some reports that NO acts as a negative regulator of nitrogen fixation due to its interaction with leghaemoglobin (Herouart et al., 2002). Other data indicate that modulation of NO levels results in alteration of nodule numbers (Herouart et al., 2002).

4.7 NO and PCD

PCD is a genetically determined, metabolically directed cellular process resulting in cell suicide – cells die because of activation of intrinsic signalling and execution processes, rather than by necrosis induced by damage of various sorts. PCD during the HR has been well studied. In our laboratory, research has focussed on establishing a role for NO during pathogen-induced PCD in Arabidopsis cell cultures. Challenging Arabidopsis cells with avirulent but not virulent P. syringae pv maculicola induced NO synthesis, correlated with the generation of \( H_2O_2 \) (Clarke et al., 2000). NO-induced cell death also possessed the characteristics of PCD, such as chromatin condensation, requirement for gene expression, and activation of a caspase-like cascade. Although there is no molecular evidence for caspases in plants, a similar role may be played by metacaspases (The Arabidopsis Genome Initiative, 2000). However, recent work has shown that overexpression of a cysteine protease in Arabidopsis cells resulted in an inhibition of pathogen- and NO-induced cell death (M. Delledonne, pers. comm.), suggesting that differences in NO-mediated signalling pathways leading to PCD are likely to occur in different systems.

Delledonne et al. (2001) have shown that the interaction between NO and ROS can determine whether or not PCD is the outcome. NO by itself does not induce PCD in soybean cell cultures, but it may be that the NO: superoxide ratio determines PCD. If superoxide levels are greater than those of NO, then NO reacts with superoxide to form peroxynitrite, which does not result in PCD. However, if more NO than superoxide occurs, then NO reacts with \( H_2O_2 \) (arising from dismutation of superoxide) to induce cell death. A correlation between \( H_2O_2 \) and antioxidant levels has also been demonstrated recently by de Pinto et al. (2002). In tobacco BY-2 cells, neither NO nor \( H_2O_2 \) alone at low concentrations had any effect on PCD or on the activity of PAL. However, treatment with both \( H_2O_2 \) and NO together induced a substantial increase in cell death with characteristics of PCD, as well as PAL activity. Moreover, this treatment also caused an increase in the activities of enzymes reducing ascorbate and glutathione (De Pinto et al., 2002), implying that both \( H_2O_2 \) and NO regulate cellular antioxidant levels to effect PCD, at least in some systems.

Beligni et al. (2002) have provided data indicating an antioxidant role for NO acting during developmental PCD induced by hormones. In barley aleurone layers, GA-induced PCD was delayed in the presence of NO, which correlated with a delayed loss of activity of the antioxidants catalase and superoxide dismutase. However, NO did not inhibit GA-induced alpha-amylase expression and activity, suggesting that NO does not have a general effect on cellular metabolism, but acts as a specific endogenous modulator of PCD (Beligni et al., 2002).

PCD occurring as a result of mechanical stress may also involve NO. In Kalanchoe daigremontiana, centrifugation of leaves and callus induced NO generation and subsequent DNA fragmentation and cell death (Pedroso et al., 2000). Decreased NO synthesis and PCD in the presence of a NOS inhibitor suggested the involvement of a NOS-like enzyme in this species. Further work by Garcés et al. (2001) showed that mechanical stress of Arabidopsis tissues also induced NO generation via a NOS-like enzyme. Thus in Arabidopsis at least, NO can be generated via different mechanisms under different situations, as is becoming evident for \( H_2O_2 \) (Neill et al., 2002c).

PCD is correlated with altered mitochondrial function and a role for NO is emerging here. In mammalian cells, short-term exposure to NO inhibits mitochondrial respiration via reversible inhibition of complex IV. Prolonged NO exposure results in a gradual and persistent inhibition of complex I,
concomitant with a reduction in intracellular glutathione concentrations, ultimately leading to cell death (Clementi et al., 1998). In plant cells, NO inhibition of ATP synthesis in mitochondria via inhibition of cytochrome oxidase activity has been demonstrated by Yamasaki et al. (2001). Altered mitochondrial activity stimulates PCD in plant cells. NO-induced PCD in Arabidopsis cells occurred via inhibition of respiration and the release of mitochondrial cytochrome c (Zottini et al., 2002). Saviani et al. (2002) showed that treatment of Citrus cultures with NO induced cell death bearing the characteristic hallmarks of PCD. Cyclosporin A, an inhibitor of mitochondrial permeability transition pore (PTP) formation, inhibited NO-induced PCD as well as mitochondrial membrane potential (Saviani et al., 2002). Thus, PTP formation is one of the molecular targets of NO to activate the PCD process in plants.

Therefore, it seems that NO-induced altered mitochondrial function has a harmful effect on the plant cell. However, mitochondrial respiratory shut-down can be compensated by an alternative pathway, using alternative oxidase (AOX), found in plants, fungi and protozoa. Millar & Day (1997) observed that although NO inhibited mitochondrial respiration, electron flow via AOX was not affected. Yamasaki et al. (2001) have since shown that AOX functions to avoid excessive ROS generation, unlike the situation in mammalian cells, where inhibition of cytochrome oxidase leads to increased ROS generation. More recently, Huang et al. (2002) found that exposure to NO induced the expression of various genes, one of which included AOX. Inhibition of the AOX pathway increased NO sensitivity and cell death, suggesting a ‘shunting’ of the respiratory pathway via AOX. Increased AOX activity has also been observed during pathogen challenge of Arabidopsis and tobacco (Simons et al., 1999).

5. NO signal transduction

Although there are an ever-increasing number of NO responses in plants, we still know relatively little of the signal transduction processes by which NO interaction with cells results in altered cellular activities. It seems unlikely that specific receptors exist for NO, as NO is such a simple, small and diffusible molecule. However, cells undoubtedly do sense NO, as various cellular activities are modulated in its presence. Given the ability of NO to react with a range of target molecules it may be that there are in fact several cellular ‘NO sensors’. It is important to reiterate that NO can exist in several different redox forms, that each might activate specific cellular events, and that various NO donors can generate different NO species. Consequently, it would be useful to determine which NO species are present, and when. The ratio of NO, NO+ and NO− and derivatives thereof may well change depending on other prevailing conditions, so techniques to visualise the spectrum of NO species present will be very helpful. Clearly, different cells are likely to respond in specific ways to NO, reflecting their own complement of active proteins, including signalling proteins. However, it may be that cellular specificity in NO responses is also a consequence of variation in NO species (Murgia et al., 2002). Moreover, even though NO is diffusible, it might also be that cells differ, spatially and temporally, in their ability to react with NO – such that in some cases NO effects may be confined to specific cells or even specific microdomains within cells, an accepted concept for calcium signalling and one suggested recently for hydrogen peroxide and cyclic nucleotides (Neill et al., 2002c; Trewavas et al., 2002). The mobile nature of NO, and its chemical reactivity with various cellular targets, mean that downstream effects of NO may be directly induced by interactions of NO with, for example, ion channel proteins or proteins that regulate gene expression, or indirectly, that is, following interaction of NO with signalling proteins such as protein kinases, ion channels or second messenger-generating enzymes.

Although it is not always correct to assume that plant and animal systems are the same, analogies with previously characterised animal signalling pathways can help to direct initial investigations into plant processes (see Wendehenne et al. (2001) for an excellent comparison of NO signalling in mammalian and plant systems). NO signalling in mammalian cells typically involves cGMP (cyclic guanosine monophosphate) as a second messenger, although there are also cGMP-independent pathways (Mayer & Hemmens, 1997; Bogdan, 2001), and there are already data implicating cGMP signalling in plant responses to NO. Core components of most eukaryotic signalling pathways usually include spatially specific elevations in cytosolic Ca2+, either via release from intracellular stores, or via influx from the extracellular milieu. The second major process in cell signalling is reversible protein phosphorylation – signalling events frequently require protein activation by phosphorylation (or, less frequently, dephosphorylation), followed by a subsequent return to a resting state via the actions of constitutive (or sometimes inducible) protein phosphatases. Thus far, there are only limited data regarding these aspects of NO signalling in plants, although it seems likely that they will also represent key components of NO responses.

5.1 cGMP signalling

cGMP is a well-established second messenger molecule, that is, a biologically active intracellular signalling molecule whose concentrations are transiently altered in response to an external stimulus. Typically, cGMP concentrations are increased via enhanced activity of the biosynthetic enzyme guanylyl cyclase (GC), that synthesises cGMP from GTP (guanosine tri-phosphate) (Figs 5 and 6). Concentrations are transiently altered in response to an external stimulus. Typically, cGMP concentrations are increased via enhanced activity of the biosynthetic enzyme guanylyl cyclase (GC), that synthesises cGMP from GTP (guanosine tri-phosphate) (Figs 5 and 6). Concentrations are returned to resting values (and generally kept at low levels) by the constitutive action of phosphodiesterases (PDE) (Fig. 5). In mammalian cells, many of the cellular effects of NO appear
to be mediated by cGMP. NO activates cGMP production via interaction with a soluble (and possibly also membrane-bound) form of GC; as NO is reasonably permeant, it reacts directly with the iron in the haem moiety of GC, inducing a conformational change that results in enzyme activation (Hancock, 1997). Such activation is transient, persisting only for so long as NO is present. Thus the immediate cellular effects of NO are relatively short-lived, as cGMP is rapidly degraded by PDE.

Early reports of the existence of cyclic nucleotides in plant tissues were not generally accepted, in part due to their very low levels and the possibility of experimental artefacts (Newton et al., 1999). However, unambiguous identification by mass spectrometry has demonstrated unequivocally that they do indeed occur in plant tissues, and various experimental approaches have recently generated exciting data showing that the intracellular concentrations of cyclic nucleotides alter in response to various stimuli, indicating that synthetic and degradative enzymes must be present (Newton et al., 1999; Trewavas et al., 2002). cGMP has been identified by mass spectrometry in Zea mays (Janistyn, 1983) and Phaseolus vulgaris (Newton et al., 1984) and quantified in several species by radio-immunoassay (RIA). It is important to emphasise, however, as do Newton et al. (1999), that RIA can generate false positive data. Plant extracts are undoubtedly far more complex than mammalian plasma, and likely to contain compounds that can interfere in some way with the binding of cGMP to an antibody. Thus some purification steps are required before quantitative analysis, and, ideally, RIA data should be confirmed by comparison with data obtained by a rigorous analytical technique that identifies and quantifies cGMP unequivocally. Generally, cGMP is found in plant tissues in the pmol g\(^{-1}\) range, within the range found in mammalian cells, although there is considerable variation in the values reported (see Table 2). This is likely to reflect inherent technical difficulties and there is clearly a need for the development of appropriate technologies. The application of mass spectrometry techniques such as electrospray ionisation, linked to capillary and nanoflow liquid chromatography (LC-ESI-MS) may be particularly useful here. Indeed, Ehsan et al. (1998) have used this technology to quantify cyclic AMP (cAMP), purified via an immunoaffinity technique, in the pmol g\(^{-1}\) range in tobacco cells. cGMP appears similarly amenable to analysis by such a method (S. J. Neill et al., unpublished) and it may be possible to use cGMP labelled with a heavy isotope as an internal standard for isotope dilution studies. It will of course, be essential to determine cGMP concentrations in discrete tissues and cells (for example, stomatal guard cells, or

---

**Fig. 5** Nitric oxide (NO) signal transduction.
those specifically responding to pathogens). Even more challenging, but potentially very revealing, will be the demonstration of cGMP concentrations in specific microlocations, or microdomains, within cells (Trewavas et al., 2002). It seems likely that various components of signalling pathways are not uniformly distributed within cells, and that specific stimuli generate transient events in specific subcellular locations. If cellular responses depend on where within a cell such changes occur, it will obviously be essential to visualise such changes. Honda et al. (2001) have recently described a molecular genetic approach, in which they transformed cells with a construct containing a cGMP-sensitive protein (in this case cGMP-activated protein kinase) linked to variants of green fluorescent protein, to visualise intracellular cGMP via fluorescence microscopy. Using this approach, NO-induced changes in intracellular cGMP were demonstrated in mammalian cells. Interestingly, NO-induced alterations in intracellular cGMP were variable and appeared to reflect different activities of GC and PDE.

Several lines of evidence indicate the essential requirement for cGMP synthesis and action during plant responses to NO. Pfeiffer et al. (1994) used HPLC and RIA to quantify cGMP in spruce needles exposed for 10 min to gaseous NO (60 ppm). Basal levels of c. 0.015 µmol/g increased dramatically to c. 1.9 µmol/g, although these values do seem somewhat out of step with those reported elsewhere (Table 2). Injection of recombinant rat NOS into tobacco leaves, or treatment of tobacco suspension cultures with the NO donor GSNO, induced a rapid and substantial increase in cGMP content, from 5 to 10 to around 70–90 pmol/g within 1–2 h. Moreover, induction of PAL and PR-1 gene expression in tobacco suspension cultures by GSNO was inhibited by the guanylyl cyclase inhibitors LY 83583 and ODQ. Although one should always recognise the caveat that inhibitors are rarely (if ever?) specific (and in fact both LY 83583 and ODQ are clearly not (Hausladen & Stamler, 1998; Feelisch et al., 1999)), treatment with a cell-permeable analogue of cGMP, 8-Br-cGMP, was able to counteract the inhibitory effects of LY 83583, strengthening the case for cGMP involvement. Moreover, treatment with 8-Br-cGMP alone was sufficient to induce the expression of both PR-1 and PAL. In Arabidopsis suspension cultures, NO-induced PCD was inhibited by incubation with ODQ, and relieved by coincubation with 8-Br-cGMP. Treatment with 8-Br-cGMP alone however, did not induce PCD, indicating that cGMP was required, but not sufficient, for NO induction of cell death (Clarke et al., 2000). A similar requirement for cGMP during ABA- and NO-induced stomatal closure has been found for pea (Neill et al., 2002a) and Arabidopsis (R. Desikan et al., unpublished). Again, treatment with 8-Br-cGMP alone was not able to mimic the effects of ABA and NO. Thus, it seems that cGMP is a sufficient intracellular mediator for some signalling pathways, but for others, additional intracellular signals are also required. This is underscored by the apparent contradiction between those data showing that stomatal opening in Col-0 and Arabidopsis induced by auxin, kinetin or a natriuretic peptide require the synthesis and action of cGMP (Cousson & Vavasseur, 1998; Pharmawati et al., 1998a,b) and those demonstrating the same requirement for ABA- and NO-induced stomatal closure in pea and Arabidopsis (Neill et al., 2002a; R. Desikan et al., unpublished). In fact, in our laboratory, 8-Br-cGMP alone induces neither stomatal opening nor closure in Arabidopsis, but does counteract the inhibition of IAA-induced stomatal opening and of ABA or NO-induced closure by the GC inhibitor ODQ. This implies that IAA, ABA and NO activate cGMP synthesis and at least one other

<table>
<thead>
<tr>
<th>Resting levels, Basal concentrations</th>
<th>Stimulus, elevated concentrations</th>
<th>Tissue</th>
<th>Method; comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 pmol/g</td>
<td>GA, 0.28 pmol/g after 2 h; lowered after 4 h</td>
<td>Bean</td>
<td>RIA; identified by mass spectrometry</td>
<td>Newton et al. (1984)</td>
</tr>
<tr>
<td>0.07 pmol/g</td>
<td>Barley aleurone cells</td>
<td>RIA; inhibited by LY 83583</td>
<td>Penson et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>15 pmol/g</td>
<td>NO gas (60 ppm)</td>
<td>Rice shoots</td>
<td>RIA</td>
<td>Reggiani et al. (1997)</td>
</tr>
<tr>
<td>40 pmol/g</td>
<td>Plant natriuretic peptide analogue with PDE inhibitor</td>
<td>Maize root stele</td>
<td>RIA, LY 83583 had no effect</td>
<td>Pharmawati et al. (1998a)</td>
</tr>
<tr>
<td>0.015 µmol/g</td>
<td>Potato</td>
<td>HPLC/RIA, good agreement</td>
<td>Pfeiffer et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>2.8 nmol/g</td>
<td>1.9 µmol/g</td>
<td>Potato</td>
<td>RIA, LY 83583 had no effect</td>
<td>Pfeiffer et al. (1995)</td>
</tr>
<tr>
<td>0.25 pmol/g</td>
<td>Spruce seedlings</td>
<td>RIA</td>
<td>Pharmawati et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>5–10 pmol/g</td>
<td>GSNO, 70 pmol/g after 0.5 h</td>
<td>Tobacco cells</td>
<td>RIA</td>
<td>Durner et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
signalling event that is required for stomatal opening or closure, respectively. In the case of NO responses, the molecular nature of such additional signals also awaits elucidation; calcium is a likely candidate.

The transient increases in cGMP content induced by NO, and inhibition of NO responses by GC inhibitors, mean that the enzymes required to synthesise cGMP are present, and in some cases, rapidly activated, and that the (presumably phosphodiesterase, PDE) enzymes that effect metabolic inactivation, are also present, being either constitutively active or similarly activated. In fact, cGMP-PDE enzyme activity has been found in plant tissues (Newton et al., 1999) and several potential PDE genes exist in the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000), although functional characterisation of a plant cGMP-PDE has not yet been achieved. The reported effects of Viagra™ (Pfizer, UK) in plants suggest that cGMP-PDE activity is present and terminates the effects of NO (Leshem, 2001); Viagra™, sildenafil citrate, the world’s fastest-selling drug, used clinically to treat erectile dysfunction, is a cGMP-PDE inhibitor (Fricker, 2001). Moreover, although cGMP synthesis has been demonstrated in plant extracts (Newton et al., 1999) no obvious plant GC has yet been cloned. A comparison with the situation of adenylyl cyclase (AC) might be useful here. A plant AC gene has recently been cloned from maize pollen (Moutinho et al., 2001). This AC has only limited sequence homology with previously cloned AC enzymes and it is likely that the AC enzyme family is a rather diverse one, such that database searching by simple sequence homology may not be fruitful. In another example of the potential complexity of cyclic nucleotide metabolism, a Dictyostelium gene with homology to mammalian soluble AC was recently characterised that, in fact, encoded a protein possessing GC activity. Although orthologues to this gene are present in mammals, they are not, apparently, in Arabidopsis (Roelofs et al., 2001). A novel gene encoding a protein with GC activity, AtGCl, has recently been characterised by Ludidi & Gehring (2003). It will be interesting to determine the role of this enzyme in cell signalling generally, and NO synthesis in particular.

5.2 cADP-ribose (cADPR) and calcium

Intracellular calcium can be stored in various cellular locations, release from which is predicated by the presence of specific calcium channel proteins that recognise and bind second messengers such as IP3 and cADPR. cADPR (Fig. 6) is a well-characterised calcium-mobilising second messenger in animal cells that activates calcium release from a discrete subset of membrane vesicles. cADPR synthesis in animal cells is commonly activated by NO, such activation being mediated by cGMP, potentially via activation of a cGMP-dependent kinase (Wendehenne et al., 2001). cADPR can also induce Ca2+ release from plant endoplasmic reticulum and vacuoles (Allen et al., 1995; Leckie et al., 1998). In tobacco cells, cADPR was able to mimic NO induction of PR-1 and PAL gene expression, and the cADPR effects were inhibited by Ruthenium Red, an inhibitor of intracellular Ca2+ release (Durner et al., 1998). In addition, 8-Br-cADPR, an antagonist of cADPR, inhibited PR-1 gene expression induced by recombinant NOS (Klessig et al., 2000). NO induces stomatal closure and is an essential intermediate in ABA-induced stomatal closure (Garcia-Mata & Lamattina, 2001; Garcia-Mata & Lamattina, 2002; Neill et al., 2002a). Calcium is a core component of stomatal ABA signalling pathways, with a full response to ABA being calcium-dependent, but a partial response likely to be calcium-independent (Webb et al., 2001). It is not surprising then, that NO-induced stomatal closure requires calcium (Garcia-Mata & Lamattina, 2001). The sources of calcium, however, remain to be determined. Both calcium influx from the cell exterior and calcium release from intracellular stores are required for ABA effects (MacRobbie, 2000). In addition, it appears that intracellular calcium can be released from at least two separate sources – vesicles sensitive to IP3, as well as those sensitive to cADPR. cADPR induces partial stomatal closure in Commelina...
features with cantharidin, a protein phosphatase 2A inhibitor, protein phosphatases. Treatment of soybean suspension cultures with cantharidin inhibited both ABA and NO-induced stomatal closure (Neill et al., 2002a). Zocchi et al. (2001) recently reported that ABA stimulates cADPR synthesis in the sponge Axinella polyoploides, leading to the suggestion that ABA/cADPR signalling may have originated in an evolutionary precursor of Metazoa and Metaphyta. So far, though, cADPR has not yet been identified unequivocally in plant cells nor has its biosynthetic enzyme, cADPR cyclase, been cloned. However, there is good evidence that cADPR mediates ABA-induced gene expression in tomato and that ABA can induce increases in cADPR in tomato and Arabidopsis (Wu et al., 1997; J. Sanchez, pers. comm.). Moreover, in Arabidopsis transformed with an Aplysia cADPR cyclase gene linked to a sterol-inducible promoter, sterol treatment induces partial stomatal closure in the absence of ABA (J. Sanchez & N-H. Chua, pers. comm.). These data would be consistent with ABA inducing NO synthesis in guard cells, leading to subsequent synthesis of cGMP and cADPR.

5.3 NO and cGMP-activated protein kinases/protein phosphatases

A common downstream target of cGMP in mammalian cells is cGMP-activated protein kinase (Wendehenne et al., 2001). Although plants contain an impressive array of protein kinases, thus far a cGMP-activated PK has not yet been identified. However, NO has been shown to activate PKs in Arabidopsis and tobacco (Clarke et al., 2000; Kumar & Klessig, 2000; Fig. 5). It is not known if the effects of NO were direct, or via activation of other signalling molecules that subsequently activate the PKs. In fact, the NO-activated PK in tobacco was identified as SIKP, an SA-activated mitogen activated protein kinase (MAPK), and SA was required for NO activation of SIKP, as it was not activated in the NahG mutant that has a reduced SA content (Kumar & Klessig, 2000). Moreover, at least in tobacco, NO induces increases in endogenous SA (Durner et al., 1998). The NO-activated PK in Arabidopsis has not yet been identified, but did have the characteristics of a MAPK (Clarke et al., 2000). Whether or not activation of these kinases is mediated by cGMP remains to be seen.

NO synthesis and signalling also involves regulation via protein phosphatases. Treatment of soybean suspension cultures with cantharidin, a protein phosphatase 2A inhibitor, led to increased NO synthesis (Delledonne et al., 1998). By contrast, NR-mediated NO synthesis in spinach was shown to be inhibited by cantharidin (Rockey et al., 2002). In Arabidopsis, the ABA-insensitive mutants ab1–1 and ab2–1 in which different PP2C genes are mutated, do not synthesise NO in response to ABA, but their stomata are unable to close in response to treatment with NO (Desikan et al., 2002).

5.4 Cyclic nucleotide gated ion channels (CNGCs)

CNGCs are defined functionally as ligand-gated ion channels that are activated by cyclic nucleotide binding to the channel protein, but there also exist channels that may be gated by other stimuli such as voltage but whose activity is modulated by cyclic nucleotides, as well as those whose activity can be affected by phosphorylation via cyclic nucleotide-dependent protein kinases (Leng et al., 1999). Several cyclic nucleotide-gated ion channels (CNGCs) have now been cloned from plants (Trewavas et al., 2002), including those responsive to cGMP (Gaynard et al., 1996; Schuurink et al., 1998; Kohler et al., 1999; Leng et al., 1999; Arazi et al., 2000). In Arabidopsis, the CNGC gene family contains at least six members, encoding channel proteins with the characteristic six potential membrane-spanning domains and a putative cyclic nucleotide binding domain (Kohler et al., 1999). These proteins also contain a functional calmodulin binding site (Kohler & Neuhaus, 2000), indicating the potential for cross-talk between calcium and cGMP signalling pathways. Mutation of the Arabidopsis AtCNGC2 gene results in the dnd1 (‘defense, no death’) mutant. This mutant displays elevated SA content, salicylic acid-dependent constitutive defence responses and loss of the Hypersensitive Response (HR) (Clough et al., 2000), demonstrating a physiological function for this CNGC. It is interesting to note that, at least in tobacco, NO induces SA biosynthesis and thus responses downstream of SA (Durner et al., 1998; Klessig et al., 2000), suggesting the possibility that one normal consequence of AtCNGC2 channel activity might be to repress SA synthesis. When expressed in yeast or human cells, AtCNGC2 mediates flux of K⁺ and Ca²⁺ ions in the presence of either cAMP or cGMP (Leng et al., 1999), but its channel and activation characteristics in planta are not yet characterised. The expression of AtCNGC2 is developmentally regulated in Arabidopsis, being increased during senescence (Kohler et al., 2001). Determination of the expression profile of all members of the CNGC family activated by cGMP is a clear research priority. For example, some stomatal responses to NO may be effected through cGMP-mediated activation of CNGCs.

5.5 cGMP-independent effects of NO

Although cGMP-independent effects of NO are known, the biochemical mechanisms are not completely understood (Hausladen & Stamler, 1998; Bogdan, 2001; Wendehenne et al., 2001) with novel processes being characterised recently, for example nitrolinolate-mediated NO signalling (Lim et al., 2002). The chemistry of NO means that transition metal- (e.g. iron, copper, zinc) and thiol-containing proteins are major cellular targets (Beligni & Lamattina, 2001; Bogdan, 2001; Wendehenne et al., 2001). Similar to its interaction with GC, NO can complex with iron in other haem and iron-containing proteins. Thus, NO inhibits...
tobacco aconitase, an iron-sulphur-containing enzyme that catalyses the isomerisation of citrate to isocitrate (Navare et al., 2000). In addition to the subsequent effects on metabolism (aconitase is a component of the Krebs cycle), it is possible that NO converts the cytosolic form of aconitase into IRP1 (iron-regulatory protein), a protein that is involved in cellular iron homeostasis. In animal cells IRP1 inhibits the translation of mRNA encoding the iron-binding protein ferritin by binding to iron-responsive elements in the 5′ untranslated region (Wendehenne et al., 2001; Murgja et al., 2002). This would lead to a reduction in cellular ferritin content, resulting in an increase in free iron that may subsequently lead to generation of ROS such as the hydroxyl radical via the Fenton reaction, with consequent cell death (Wendehenne et al., 2001). However, mechanisms of iron homeostasis may differ between plants and animals, with ferritin content being regulated transcriptionally in plants. Murgja et al. (2002) found that NO induced the accumulation of both ferritin mRNA and protein in Arabidopsis, acting through the IDRS (iron-dependent regulatory sequence) present in the ferritin promoter. NO also inhibits the haem-containing enzymes catalase and peroxidases, with potential knock-on effects on ROS levels and xylem development (Ferrer & Barcelo, 1999; Clark et al., 2000; Barcelo et al., 2002).

It is well-known that NO can interact (probably via NO+) with reactive amino acids such as cysteine and tyrosine in proteins and with thiol groups present in other molecules such as the ubiquitous redox-sensitive and potentially regulatory tri-peptide glutathione (Jia et al., 1996; Wendehenne et al., 2001). Thiol modification by ROS such as hydrogen peroxide is already recognised as a potential signalling mechanism in plants (Neill et al., 2002b). Because thiol residues and disulphide bridges can be important for tertiary protein structure, reversible modification of thiol groups and disulphides may have important consequences for protein activity, analogous to protein modifications via reversible phosphorylation. Protein thiol residues have recently been described as potential ‘nanotransducers’ (Schafer & Buettner, 2001); the existence of several forms, generated by reaction with ROS and reactive nitrogen species (RNS) that might compete for the same thiol group, allowing ‘graded’ activity responses as opposed to ‘on or off’ switching effected by phosphorylation (Cooper et al., 2002). S-nitrosylation of proteins and glutathione has been demonstrated in vitro, formation of S-nitrosothiol adducts shown to alter protein activity and some endogenous S-nitrosylated proteins and S-nitrosothiols have been demonstrated (e.g. haemoglobin, Jia et al. (1996); and GSNO, Hogg, (2000)). However, to date no endogenous S-nitrosylated proteins have been characterised in plants, although the recent development of a sensitive proteomic approach to identify endogenous S-nitrosylated proteins may well be useful here (Jaffrey et al., 2001). In this study, various S-nitrosylated proteins were detected and shown to be absent in knock-out mice in which neuronal NOS was deleted, indicating a physiological role for S-nitrosylation. As with high molecular weight S-nitrosothiols, GSNO has not yet been identified in plants; analysis by LC-MS may be possible (Kluge et al., 1997). GSNO is often used as an NO donor, but may induce biological responses via mechanisms not involving NO release, such as trans-nitrosation of thiol groups present in proteins, thereby modifying their activity (Hogg, 2000).

5.6 NO metabolism

The biological effects of RNS such as NO and GSNO make it likely that endogenous mechanisms exist to remove them. Stamler’s group recently characterised an enzyme activity from Escherichia coli that degrades GSNO (Liu et al., 2001). The enzyme was identified by mass spectrometry as glutathione-dependent formaldehyde dehydrogenase (GS-FDH). In fact, the GSNO reductase (GSNOR) activity of this protein was much greater than its GS-FDH activity, and also identified in yeast and mammalian cells. Knock-out mice and yeast, in which the GSNOR gene was deleted, had much reduced GSNO-catabolising capacity, and accumulated protein S-nitrosothiols and some GSNO. In addition, the GSNOR-deficient yeast was much more susceptible to nitrosative stress. The endogenous amounts of GSNO were very low in wild type mouse macrophage cells but GSNO readily accumulated in the culture medium of interferon-treated cells (interferon activates NOS) supplied with glutathione. Thus the role of GSNOR may be to maintain low endogenous levels of GSNO and thereby prevent excessive formation of S-nitrosylated proteins to damaging levels, presumably by trans-nitrosation reactions with GSNO. GS-FDH genes have been cloned from various plant species (Sakamoto et al., 2002) and now the Arabidopsis GS-FDH protein has, like its microbial and mammalian counterparts, been shown actually to possess GSNOR activity (Sakamoto et al., 2002). Moreover, the Arabidopsis gene was able to complement the yeast GSNOR knock-out mutant. Expression of the Arabidopsis GSNOR is constitutive and relatively high, in keeping with a potential role in maintaining low endogenous levels of GSNO. No doubt subsequent studies using a transgenic approach to manipulate the level of GSNOR activity will reveal much more about its function.

Other NO metabolising mechanisms have been characterised that may have relevance for plant–pathogen interactions. NO generation by mammalian cells represents a potent defence mechanism against bacterial pathogens, as NO is toxic to bacteria at high concentrations (possibly via conversion to peroxynitrite). Several groups have shown that bacterial flavohaemoglobin (HMP) serves a protective role in bacteria such as Salmonella typhimurium and Escherichia coli against nitrosative stress that might be imposed within the host environment. These bacterial HMP proteins possess NO-dioxygenase activity, converting NO to nitrite and...
nitrates (Crawford & Goldberg, 1998; Gardner et al., 1998; Hausladen et al., 1998). HMP gene expression is inducible by NO, via a mechanism that in E. coli involves NO interaction with the iron-sulphur clusters present in the global transcription factor FNR. In the absence of NO, FNR binds to the promoter regions of hmp, repressing gene expression. Following complex formation with NO, FNR is inactivated, and thus HMP gene expression de-repressed (Ramos et al., 2002). In the phytopathogen Erwinia chrysanthemi, flavohaemoglobin HmpX was described as a virulence determinant (Favey et al., 1995). It may be that HmpX is required for virulence because it mediates bacterial detoxification of host-generated NO. Differing sensitivities to NO have also been reported for other phytopathogenic bacteria (Alamillo & Garcia-Olmedo, 2001). It will be interesting to see if other phytopathogens require NO-metabolising enzymes for virulence; if so, development of inhibitors of bacterial flavohaemoglobins may represent a novel approach to plant protection.

5.7 NO transport

Although NO is a reasonably diffusible gas, it is possible that NO precursors or NO-adducts could act as ‘stores’ of NO, or transport forms operating over short or long distances, analogous to flooding-induced transport of the ethylene precursor ACC from roots to shoots in flooded plants (Jackson, 2002). For example, in animals the presence of S-nitrosohaemoglobin in circulating blood led to the suggestion that it might serve as a source of circulatory NO (Jia et al., 1996). This is now considered unlikely, but instead, evidence has been presented that nitrite is present in circulating blood and can serve as a delivery source of NO (Gladwin et al., 2000). As nitrite can clearly serve as a precursor to NO in plant cells, including guard cells that are continually exposed to the xylem stream (Desikan et al., 2002), it is a possibility that nitrite can serve as a mobile source of NO. There is also the possibility that GSNO, in either phloem or xylem, is another mobile source of NO in plants. There is then the intriguing possibility that these NO-precursors may be delivered from flooded roots to shoots, generating NO in the shoots and inducing the stomatal closure that is observed but not attributable to ABA (Jackson, 2002). Plants also contain haemoglobin genes (Hunt et al., 2001), so it is possible that plant haemoglobins can form reversible NO complexes.

5.8 NO interactions with other signalling pathways

A reductionist approach that evaluates the biological roles of NO and the underlying signalling mechanisms in isolation is inevitably a deficient one. Clearly NO can interact with other signals either directly, for example, with superoxide to form peroxynitrite (see section 2 above) or with other signalling pathways to direct cellular activities. A comprehensive, holistic consideration of the interactions and signalling cross-talk involving NO is outside the scope of this review. However, it is pertinent to make a few observations. In particular, much experimental data indicate that generation of ROS such as hydrogen peroxide (potentially arising from superoxide generated via NADPH oxidase; Neill et al., 2002b) appears to be a common companion to NO generation. Thus, both ROS and NO are generated in response to pathogen challenge (Durner & Klessig, 1999) as well as in stomatal guard cells in response to ABA (Neill et al., 2002a). H2O2 is made in response to many biotic and abiotic stimuli (Neill et al., 2002b) and it may well be that so too is NO, and that subsequent responses depend, in part at least, upon interactions between H2O2 and NO, as outlined in the previous section. This is well illustrated by the data of Delledonne et al. (2001) described in section 4.7 above. It is also possible that NO can inhibit superoxide generation (Caro & Puntarulo, 1998). It is also worth pointing out that both H2O2 and NO activate potential MAPKs and changes in intracellular calcium, further points of cross-talk for interaction of NO with H2O2 and other stimuli. Interactions between ABA and NO during stomatal closure in V. faba were highlighted by Garcia-Mata & Lamattina (2002). Clearly NO can, and does interact with many signals, but a complete ‘NO signalling map’ will take some time to complete.

5.9 NO and gene expression

It is apparent from the previous sections that a growing number of physiological and developmental responses to NO have been demonstrated in plants. Thus there must inevitably be changes in the spectrum of gene expression following exposure to NO. In fact, NO induction of gene expression was first shown during plant–pathogen interactions. In tobacco, infection of resistant plants with TMV induced increased NOS activity (Durner et al., 1998) and injection of recombinant NOS or exposure to the NO donors GSNO or SNAP induced the expression of PAL and PR-1 genes (Durner et al., 1998). PR-1 gene expression was also induced by TMV infection, an effect suppressed by coinjection with the NOS inhibitor L-NAME, providing evidence for the endogenous mediation of TMV-induced gene expression by NO (Klessig et al., 2000). Delledonne et al. (1998) showed that SNP induced the expression of PAL and CHS in soybean suspension cultures. Subsequent to this, there have been only limited reports on NO-induced gene expression. A.-H.-MacKerness et al. (2001) showed that NO scavenging with PTIO or inhibition of NOS by L-NAME inhibited UV-B induction of CHS gene expression in Arabidopsis plants. Direct exposure to NO, via treatment with either GSNO or SNAP also induced CHS gene expression, suggesting that endogenous NO mediates UV-B induction of CHS gene expression. Murga et al. (2002) have shown that NO induces ferritin mRNA accumulation in Arabidopsis plants and suspension cultures. Moreover, NO is required for iron
induction of ferritin transcript accumulation, as removal of NO with cPTIO is inhibitory. A recent microarray study identified a large number of genes that were induced by NO (applied via the NO donor NOR-3) in Arabidopsis suspension cultures (Huang et al., 2002). In this work, particular attention was paid to the AOX1a gene, encoding one member of the small enzyme family of alternative oxidases (see section 4.7 above). AOX1a gene expression was induced within 2 h by treatment with NOR-3, such induction being suppressed strongly by removal of NO with cPTIO, confirming that the effects of NOR-3 were indeed due to NO generation. Furthermore, AOX1a expression was induced within 1 h following exposure of plants to gaseous NO. Other genes transiently induced by NO in suspension cultures included several pathogenesis-related proteins and antioxidant genes including peroxidases and glutathione-S-transferases, as well as some encoding likely signalling proteins (Huang et al., 2002).

What are the potential mechanisms by which NO induces gene expression? The induction of many genes (c. 5% of those on the array) by NO (Huang et al., 2002) suggests a common mechanism for at least some of these genes. Thus far, though, no bioinformatic data are available with respect to the presence of common potential regulatory cis elements in the promoter regions of the genes analysed by Huang et al. (2002). On the other hand, Murgia et al. (2002) showed that NO induction of ferritin gene expression was mediated via the IRDS sequence in the ferritin promoter.

Given that NO can change protein conformation in several ways, including S–nitrosylation, interaction with iron in haem groups and reactions with iron and other transition metals such as zinc, commonly found complexed with histidine and cysteine residues in zinc finger motifs often present in transcription factors, it is possible that NO alters transcriptional profiles directly, by diffusing into the nucleus and activating or inactivating transcription factors (Fig. 7). On the other hand, as NO also activates signalling processes including SA production, cGMP synthesis, calcium fluxes, and reversible protein phosphorylation, it is also likely that NO effects on transcription are mediated via modulation of transcription factor activity in signalling cascades requiring such processes (Fig. 7). For example, NO induction of PR-1 gene expression in tobacco required SA synthesis and action, whereas induction of PAL gene expression did not (Durner et al., 1998). Furthermore, although PR-1 gene expression was induced by cGMP and cADPR, NO induction of PR-1 expression was not completely suppressed by an antagonist of cADPR, indicating that PR-1 expression is mediated by both cGMP-dependent and independent pathways (Klessig et al., 2000). In Arabidopsis suspension cultures, AOX1a gene expression was induced by NO much earlier than was SA biosynthesis, and was still induced in various mutants with impaired SA signalling or reduced SA content (Huang et al., 2002).

**Conclusion**

Recent years have seen a huge increase in research activities aimed at elucidating the biological roles of NO in plants and the underlying signalling mechanisms. There can be no doubt that NO is an endogenous metabolite, but its biosynthetic origins still remain to be completely characterised. Certainly NO can be synthesised from nitrite via nitrate reductase, but the presence of an enzyme similar to mammalian NOS has not yet been confirmed. Other sources of NO, both enzymatic and nonenzymatic, are also possible. Identification and subsequent manipulation of NO biosynthetic enzymes and the encoding genes is a research priority. Application of NO via NO donors indicates that NO can induce various processes in plants, such as PCD, stomatal closure, and root growth, although it should be borne in mind that different NO donors may release different molecular species of NO that could have distinct effects. Nevertheless, pharmacological manipulation using NO scavengers and inhibitors of NO synthesis does indicate very strongly that endogenous NO mediates various responses to developmental and external stimuli. There is now a requirement for methods to assay NO synthesis and quantify its release from cells. Intracellular signalling responses to NO involve generation of cGMP and cADPR and elevation of cytosolic calcium, but in many cases the precise biochemical and cell biological nature of these responses is yet to be detailed. There is a clear need for the development of techniques to identify, visualise and quantify cGMP and cADPR in plant cells, and to clone the genes required for their synthesis and degradation, such that transgenic approaches can be used for functional analyses. Similarly, PKs and PPs, transcription factors, ion channels and other signalling proteins activated or repressed by NO await identification and characterisation. Despite the already
exponential growth in plant NO research, there is a need for much more – given the essential roles of NO in plant growth and development, such research efforts will surely be justified.

Acknowledgements
We are grateful to all those colleagues who provided reprints, preprints and unpublished data for this review.

References


Skinner L, Campbell WH, Mertens J, Lowe D. 2001. Pre-steady-state...


