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Hydrogen peroxide and nitric oxide as signalling molecules in plants

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Abstract

It is now clear that hydrogen peroxide (H_2O_2) and nitric oxide (NO) function as signalling molecules in plants. A wide range of abiotic and biotic stresses results in H_2O_2 generation, from a variety of sources. H_2O_2 is removed from cells via a number of antioxidant mechanisms, both enzymatic and non-enzymatic. Both biotic and abiotic stresses can induce NO synthesis, but the biosynthetic origins of NO in plants have not yet been resolved. Cellular responses to H_2O_2 and NO are complex, with considerable cross-talk between responses to several stimuli. In this review the potential roles of H_2O_2 and NO during various stresses and the signalling pathways they activate are discussed. Key signalling components that might provide targets for enhancing crop production are also identified.

Key words: Abiotic and biotic stress, hydrogen peroxide, nitric oxide, signalling molecule.

Introduction

There is now compelling evidence that hydrogen peroxide (H_2O_2) and nitric oxide (NO) function as signalling molecules in plants (Foyer *et al*., 1997; Neill *et al*., 1999; Bolwell, 1999; Durner and Klessig, 1999; Dat *et al*., 2000). H_2O_2 is a form of reactive oxygen species (ROS) generated as a result of oxidative stress. Oxidative stress arises from an imbalance in the generation and metabolism of ROS, with more ROS (such as H_2O_2) being produced than are metabolized. H_2O_2 is generated via superoxide, presumably in a non-controlled manner, during electron transport processes such as photosynthesis and mitochondrial respiration. H_2O_2 generation via electron transport is increased in response to environmental stresses such as excess excitation (light) energy, drought and cold (Bartosz, 1997; Dat *et al*., 2000). H_2O_2 and other reactive forms of oxygen derived from it can react with various cellular targets. Well established deleterious effects of ROS include damage to DNA and proteins, and lipid peroxidation (Halliwell and Gutteridge, 1989). However, plants possess a battery of antioxidant mechanisms, both enzymatic and non-enzymatic, by which ROS are removed from the cell (Noctor and Foyer, 1998). Thus, a critical balance between the production and metabolism of ROS determines the fate of the cell.

H_2O_2 generation is also induced in plants following exposure to a wide variety of abiotic and biotic stimuli (Fig. 1). These include extremes of temperatures, UV irradiation, excess excitation energy, ozone exposure, phytohormones such as ABA, dehydration, wounding, and elicitor and pathogen challenge (Prasad *et al*., 1994; Lamb and Dixon, 1997; Karpinski *et al*., 1999; Orozco-Cardenas and Ryan, 1999; Guan *et al*., 2000; Langebartels *et al*., 2000; Pei *et al*., 2000; A-H-Mackerness *et al*., 2001). The enzymatic sources of H_2O_2 generated in response to specific stimuli have not been resolved, and there is likely to be more than one. Potential sources include NADPH oxidase, cell wall peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidases (Fig. 1; Bolwell and Wojtaszek, 1997; Bolwell *et al*., 2002). Whatever the source of ROS, it is now apparent that H_2O_2 acts as a signal to induce

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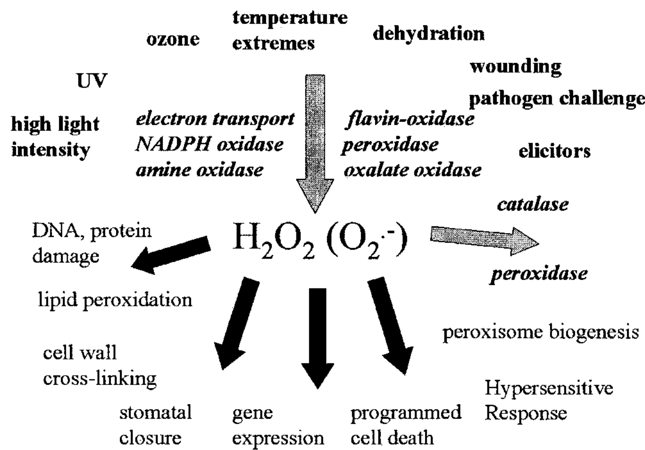

Fig. 1. H_2O_2 signalling in plants. Grey arrows represent potential synthesis and degradation, black arrows represent potential cellular effects of H_2O_2.

a range of molecular, biochemical and physiological responses within cells and plants. Some of these will be discussed in this review; it is probable that additional H_2O_2 responses will be characterized in the future.

Given that H_2O_2 is produced in response to such a variety of stimuli, it is likely that H_2O_2 mediates cross-talk between signalling pathways, and is an attractive signalling molecule contributing to the phenomenon of 'cross-tolerance', in which exposure of plants to one stress offers protection towards another (Bowler and Fluhr, 2000). For example, previous exposure to sublethal doses of ozone or UV conferred tolerance to infection by a virulent pathogen (Sharma *et al.*, 1996), and exposure to heat stress induced tolerance towards subsequent pathogen attack (Vallelian-Bindschedler *et al.*, 1998). In addition, exposure to low levels of one stress (for example, cold) can induce tolerance towards subsequent higher levels of exposure to the same stress, a phenomenon termed acclimation tolerance (Prasad *et al.*, 1994). Despite this cross-talk, cellular responses to various stresses do exhibit some degree of specificity as well as commonalities, and H_2O_2 does not induce the full range of responses induced by a 'broader' stress such as cold, UV or pathogen challenge (Langebartels *et al.*, 2000; A-H-Mackerness *et al.*, 2001; Knight and Knight, 2001). Moreover, it may be that cellular responses to H_2O_2 differ according to its site of synthesis or perception, for example, whether the H_2O_2 is synthesized in plastids or at the plasma membrane. However, the fact that oxidative stress is a common facet of many cellular stress responses, means that elucidating those intracellular signalling processes mediating H_2O_2 signalling is of potential significance to any programme aimed at improving crop tolerance of environmental stresses.

Nitric oxide (NO) is a free radical gas with well-characterized signalling roles in mammalian systems, acting as a second messenger during vasorelaxation, neurotransmission, immunity, and cytotoxicity (Furchgott, 1995). It is now clear that NO is also a major signal molecule in plants (Durner and Klessig, 1999), and NO signal transduction is discussed here because it seems likely that NO can be synthesized during stress responses at the same time as H_2O_2, and it may be that cellular effects reflect responses to both H_2O_2 and NO. Two landmark publications in 1998 demonstrated the role of NO during the hypersensitive response (HR) of plants to infection by bacteria and viruses (Delledonne *et al.*, 1998; Durner *et al.*, 1998). NO generated at the same time as H_2O_2 in response to pathogen attack was found to mediate defence responses similar to those seen following H_2O_2 generation.

Responses to H_2O_2

A well-established role for H_2O_2 is as a signal molecule during the HR (Lamb and Dixon, 1997; Grant and Loake, 2000). H_2O_2 generated following pathogen challenge mediates cross-linking of cell wall proteins (Bradley *et al.*, 1992) and plant cell wall-bound phenolics (Grant and Loake, 2000), and, although this is still somewhat controversial, may also have microbicidal function (Peng and Kuc, 1992; Wu *et al.*, 1995). A key facet of the HR is localized programmed cell death (PCD) of host cells at the site of attempted invasion, a response that can be reproduced in suspension cultures following various treatments (McCabe and Leaver, 2000). In some systems, H_2O_2 has been shown to be a diffusible signal mediating localized PCD during HR (Levine *et al.*, 1994, 1996), as well as being involved in a systemic signalling network, giving rise to 'micro-HRs' in systemic leaves of plants infected with avirulent bacteria (Alvarez *et al.*, 1998). However, recent studies using transgenic catalase/peroxidase-deficient tobacco (i.e. in which endogenous H_2O_2 will not be readily catabolized) showed that such plants were hyperresponsive to pathogen challenge, thus providing direct evidence for a role for H_2O_2 in HR cell death (Mittler *et al.*, 1999). Recently, PCD triggered in barley aleurone by the phytohormone gibberellin was also found to be mediated by H_2O_2 (Bethke and Jones, 2001; Fath *et al.*, 2002), implying a role for H_2O_2 in developmental PCD in addition to that induced by pathogen challenge.

In the authors' laboratory the *Arabidopsis thaliana* suspension culture system has been used as a model to elucidate the role of H_2O_2 as a signalling molecule, particularly during plant–pathogen interactions. It has been shown that H_2O_2 is generated following elicitor and pathogen challenge (Desikan *et al.*, 1996; Clarke *et al.*, 2000), and that this H_2O_2 acts as a signal to induce PCD

and defence gene expression (Desikan *et al.*, 1998*a*, 2000). Importantly, it was also found that H_2O_2-induced PCD requires a 'presentation time' of about 1 h (i.e. if H_2O_2 is removed within 1 h after addition, PCD can be inhibited) implying that H_2O_2 induces a signalling cascade leading to PCD (Desikan *et al.*, 1998*a*). It is important to note that the effects of exogenous H_2O_2 depend on the rate at which it is degraded, which presumably determines its concentration at its site of action. Much higher concentrations of H_2O_2 are required to initiate PCD in *Arabidopsis* cells, compared to those required with protoplasts (Fig. 2A, B). The increased sensitivity of protoplasts correlates with their decreased H_2O_2-scavenging capacity. H_2O_2 is destroyed rapidly by cells: when 20 mM H_2O_2 is added to cells, it is degraded very quickly, with H_2O_2 having a half-life of 2–5 min (Fig. 2C). However, when the same dose of H_2O_2 is applied to protoplasts, it persists for much longer, with a half-life of about 1 h (Fig. 2D). The reduced scavenging capacity of protoplasts may be due to loss of cell wall-associated enzymes such as peroxidases.

PCD induced by H_2O_2 during the HR in *Arabidopsis* (Desikan *et al.*, 1998*a*) and soybean (Solomon *et al.*, 1999) requires transcription and translation, and several studies have demonstrated that H_2O_2 modulates gene expression during defence responses. In soybean, H_2O_2 induced the expression of the defence-related genes glutathione *S*-transferase (*GST*) and glutathione peroxidase (*GPX*, Levine *et al.*, 1994). In the authors' own work, it has also been shown that, in *Arabidopsis* suspension cultures, H_2O_2 induced the expression of *GST* and phenylalanine ammonia-lyase (*PAL*, Desikan *et al.*, 1998*a*). GST comprises a family of enzymes involved in cellular detoxification processes following various stresses, including oxidative stress (Marrs, 1996), glutathione peroxidases scavenge H_2O_2 in the ascorbate-glutathione cycle (Foyer *et al.*, 1997), and PAL is an enzyme involved in the synthesis of defence-related compounds. Recent work also identified a tobacco gene encoding a proteasome subunit induced by H_2O_2 (Etienne *et al.*, 2000); proteasomes are involved in protein degradation, a common feature of the HR cell death process.

H_2O_2 can induce the expression of genes potentially involved in its synthesis, such as NADPH oxidase (Desikan *et al.*, 1998*b*), and also of those encoding proteins involved in its degradation, implying a complex mechanism for cellular regulation of oxidative status. H_2O_2 induced the expression of genes encoding ascorbate peroxidase in germinating rice embryos (Morita *et al.*, 1999) and in *Arabidopsis* leaves (Karpinski *et al.*, 1999), and wounding induced the expression of gene encoding a catalase via H_2O_2 in embryos and leaves of maize (Guan and Scandalios, 2000).

H_2O_2 is also involved in the regulation of gene expression by abiotic stresses. For example, UV-B-induced gene expression has been shown to occur via H_2O_2, as exposure of *Arabidopsis* plants to UV-B in the presence of antioxidants led to down-regulation of the UV-induced gene *PDF1.2* (A-H-Mackerness *et al.*, 1999).

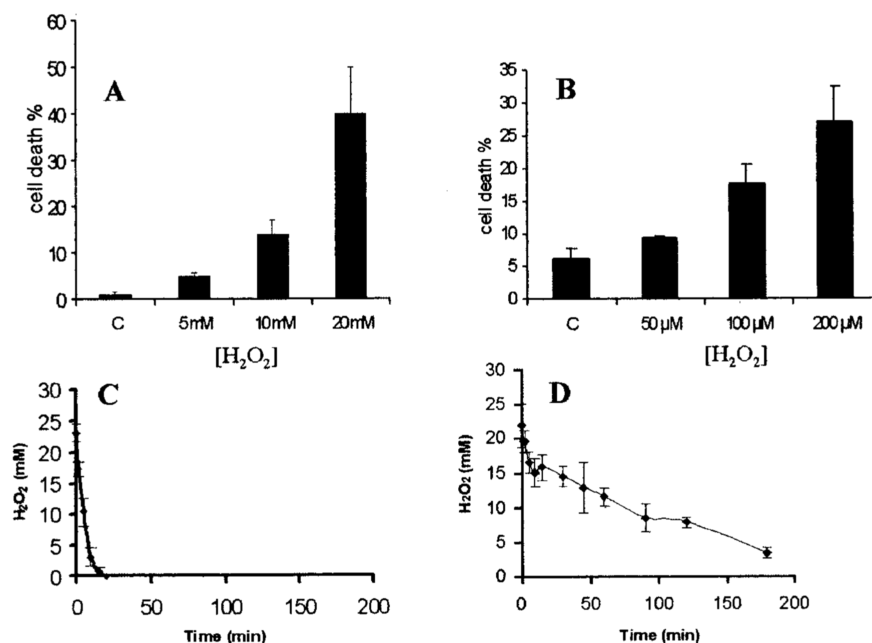

Fig. 2. Sensitivity to exogenous H_2O_2 correlates with its longevity. (A, B) Cell death induced by exogenous H_2O_2 in (A) suspension cultures, (B) protoplasts; C, control. (C, D) Degradation of exogenous 20 mM H_2O_2 by (C) *Arabidopsis* suspension cultures or (D) protoplasts prepared from such cultures.

Systemic responses to excess excitation energy stress were found to be mediated by H_2O_2, indicating that it can also function as a signal during abiotic stresses (Mullineaux *et al.*, 2000), as during pathogen-induced responses (Alvarez *et al.*, 1998). Recent work has shown that H_2O_2 induces the expression of genes encoding proteins required for peroxisome biogenesis (Lopez-Huertas *et al.*, 2000). Peroxisomes are important sources of ROS, as well as antioxidants and NO (see below), and are thus important regulators of the cellular redox state. Induction of peroxisome biogenesis genes by various stresses such as pathogen challenge and wounding (which also generates H_2O_2), and exogenous H_2O_2 (Lopez-Huertas *et al.*, 2000) places H_2O_2 as a key signal molecule mediating cellular responses to stress.

In this laboratory, differential mRNA display analysis was used to identify several H_2O_2-induced genes in *Arabidopsis* suspension cultures. Up-regulated genes included those encoding a sensecence-related protein, a protein kinase and a DNA damage repair protein (Desikan *et al.*, 2000). This work has been extended to a microarray analysis using the *Arabidopsis* Functional Genomics Facility (AFGC, Desikan *et al.*, 2001*a*). This experiment identified a large number of up-regulated genes. As might be expected from previous work, some of these genes encode antioxidant enzymes, defence and stress-related proteins. Interestingly, genes encoding signalling proteins such as transcription factors, protein kinases and protein phosphatases were also up-regulated by H_2O_2; these genes were similarly induced by other stresses such as wilting, UV challenge and elicitor treatment of cells. Several genes down-regulated by H_2O_2 have also been identified in this work: these include genes encoding cysteine proteases, a protein kinase and photosystem-related proteins. A tobacco protein phosphatase 2C (NtPP2C1) gene is down-regulated by H_2O_2 and heat shock, but up-regulated by drought stress (Vranova *et al.*, 2000). Such data highlight the complexity of signalling responses likely to be activated by H_2O_2.

A physiological response induced by H_2O_2 that has recently received much attention is stomatal closure in response to abscisic acid (ABA) and elicitors (McAinsh *et al.*, 1996; Allan and Fluhr, 1997; Lee *et al.*, 1999; Pei *et al.*, 2000). Oxidative stress/H_2O_2 was shown to induce stomatal closure (McAinsh *et al.*, 1996), Allan and Fluhr showed that elicitors could induce H_2O_2 production (via two distinct sources) (Allan and Fluhr, 1997), and Lee *et al.* demonstrated that both these responses were linked: elicitors caused H_2O_2 production which, in turn, caused stomatal closure (Lee *et al.*, 1999). An exciting new development in this area is the recent demonstration that ABA-induced stomatal closure in *Arabidopsis* requires H_2O_2 (Pei *et al.*, 2000). ABA induced H_2O_2 generation, H_2O_2 caused stomatal closure, and pre-treatment with diphenylene iodonium, a potential inhibitor of NADPH oxidase (Cross and Jones, 1986) and therefore H_2O_2 production, inhibited ABA-induced stomatal closure (Pei *et al.*, 2000). It may be that other ABA responses are mediated, at least partly, by H_2O_2, for example, ABA-induced catalase gene expression in maize cells occurs via H_2O_2 (Guan *et al.*, 2000). ABA-induction of guard cell H_2O_2 generation has also been reported for *Vicia faba* (Miao *et al.*, 2000), and the authors too have found that ABA-mediated stomatal closure in pea involves H_2O_2 synthesis. ABA-inhibition of stomatal opening similarly seems to involve H_2O_2, but inhibitor work suggests that there are different H_2O_2 signalling pathways during closure and inhibition of opening (SJ Neill, unpublished results).

H_2O_2 signal transduction

Calcium mobilization and reversible protein phosphorylation are ubiquitous components of eukaryotic signalling cascades. Elevations of cytosolic calcium concentrations have been shown to occur during most abiotic stresses, including oxidative stress (Knight and Knight, 2001). Oxidative stress increased cytosolic calcium concentrations in tobacco (Price *et al.*, 1994), and H_2O_2-induced calcium influx mediated stomatal closure in *Commelina communis* and *Arabidopsis* (McAinsh *et al.*, 1996; Pei *et al.*, 2000). Stomata of the ABA-insensitive *gca* mutant of *Arabidopsis* that do not close in response to ABA, also failed to respond to H_2O_2, suggesting that the mutation may be in a gene encoding a protein required for H_2O_2 signal transduction (Pei *et al.*, 2000). It is likely that different 'calcium signatures' are invoked by different stimuli (McAinsh and Hetherington, 1998) and it may be that differing sources of H_2O_2 induce specific calcium responses. Previous exposure to oxidative stress altered subsequent calcium responses to drought and cold (Knight and Knight, 2001), suggesting that the intracellular calcium responses are involved in mediating cross-tolerance.

Calcium has also been implicated as an important signal following the oxidative burst in response to pathogen challenge. It was reported that H_2O_2 generated following pathogen challenge induces a rapid influx of calcium ions leading to apoptosis in soybean cells (Levine *et al.*, 1996). In *Arabidopsis* plants infiltrated with avirulent bacteria, specific calcium 'waves' were found to occur concurrent with the oxidative burst, leading to the HR (Grant M *et al.*, 2000). Calcium influx also stimulates the oxidative burst in soybean (Chandra and Low, 1997) and in tobacco cells in response to elicitor or pathogen challenge (Baker *et al.*, 1993). The enzyme NADPH oxidase, one of the potential sources of H_2O_2 in plants, also has calcium binding domains (Desikan *et al.*, 1998*b*; Keller *et al.*, 1998; Torres *et al.*, 1998). Moreover,

a calcium binding protein, calmodulin, links calcium and H_2O_2: tobacco cells expressing a constitutively active calmodulin showed enhanced HR cell death in response to an incompatible pathogen (Harding *et al.*, 1997). Calmodulin regulates NAD kinase activity, which generates NADPH for NADPH oxidase activity. Thus, cross-talk between H_2O_2 and calcium could regulate specificity and/or cross-tolerance towards various stresses (Bowler and Fluhr, 2000).

Early pharmacological data from several research groups suggested that reversible protein phosphorylation is a key event regulating the oxidative burst in response to pathogen challenge (Schwacke and Hager, 1992; Baker *et al.*, 1993; Levine *et al.*, 1994; Chandra and Low, 1995; Desikan *et al.*, 1996). There are also pharmacological data to show that reversible protein phosphorylation is similarly involved in downstream signalling following H_2O_2 generation and/or perception (Levine *et al.*, 1994; Rajasekhar *et al.*, 1999; Grant JJ *et al.*, 2000). Given the large number of protein kinases and phosphatases in plant genomes (The Arabidopsis Genome Initiative, 2000) and the complexity of signal transduction, it is likely that an interconnecting network of protein kinases and phosphatases (and other signalling components) will eventually be characterized. Moreover, it is also likely that the intracellular location of these components will be of critical importance in determining the specific outcomes of the signalling pathways that are activated by specific stimuli. As cytosolic calcium elevation is a common, early response to H_2O_2, it is likely that activation of calcium-dependent protein kinases and phosphatases will be an early step, with some enzymes potentially mediating downstream signalling components such as other protein kinases/phosphatases and other effector proteins. To date, though, no calcium-dependent protein kinases have been shown to be regulated by H_2O_2, although H_2O_2-regulated genes encoding protein kinases and phosphatases have been discovered (see earlier). However, it is of course possible that constitutively active calcium-dependent protein kinases are involved in H_2O_2 signalling.

A protein phosphorylation cascade that has been shown to be activated by H_2O_2 is a mitogen activated protein kinase (MAPK) cascade. MAPK cascades are evolutionarily conserved in all eukaryotes and have the typical organization shown in Fig. 3. Perception of an extracellular signal activates a MAP kinase kinase kinase (MAPKKK). This kinase then phosphorylates a MAPKK, which in turn activates a MAPK by dual phosphorylation on both threonine and tyrosine residues in a conserved T-X-Y motif (Fig. 3). Activation of the MAPK can facilitate its translocation to the nucleus where it can phosphorylate and activate transcription factors, thereby modulating gene expression (Hirt, 1997). In parsley cells, an elicitor-activated MAPK translocates to the nucleus, leading to subsequent defence responses (Ligterink *et al.*, 1997). In plants, MAPKs can be activated in response to extracellular signals such as drought, cold, phytohormones, pathogen challenge, and osmotic stress, that lead to the activation of signal transduction pathways resulting in nuclear gene expression (Hirt, 1997). It was shown that H_2O_2 induces the activation of a MAPK in *Arabidopsis* suspension cultures (Desikan *et al.*, 1999), and H_2O_2 has been shown to activate two MAPKs in *Arabidopsis* plants, at least one of which is activated independently of salicylic acid, jasmonate and ethylene signalling pathways (Grant JJ *et al.*, 2000). The H_2O_2-activated MAPK in suspension cultures has now been identified as AtMPK6 and shown that it is activated in *Arabidopsis* leaves and protoplasts (Fig. 4; Desikan *et al.*, 2001*b*). H_2O_2 also activates AtMPK6, and the related AtMPK3, in *Arabidopsis* leaf protoplasts (Kovtun *et al.*, 2000). A similar activation of two MAPKs was found in suspension culture protoplasts, compared to the activation of AtMPK6 alone in cells (Fig. 4). This probably reflects the increased sensitivity of protoplasts due to their reduced H_2O_2-scavenging capacity (Fig. 2).

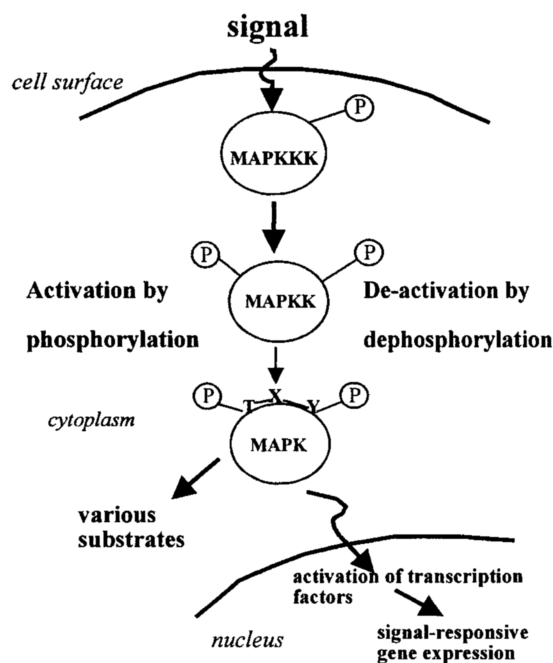

Fig. 3. Mitogen-activated protein kinase (MAPK) signalling cascade. Schematic representation of a MAPK cascade, which involves activation of a MAPK kinase kinase (MAPKKK) by an extracellular stimulus leading to the sequential phosphorylation of a MAPK kinase (MAPKK) and a MAPK, the latter being dually phosphorylated on conserved threonine (T) and tyrosine (Y) residues.

AtMPK6 is also activated in response to elicitor challenge and cold stress (Nuhse *et al.*, 2000; Ichimura *et al.*, 2000; Desikan *et al.*, 2001*b*). Furthermore, ozone and H_2O_2 treatment induced the activation of the tobacco orthologue of AtMPK6, SIPK (Samuel *et al.*, 2000).

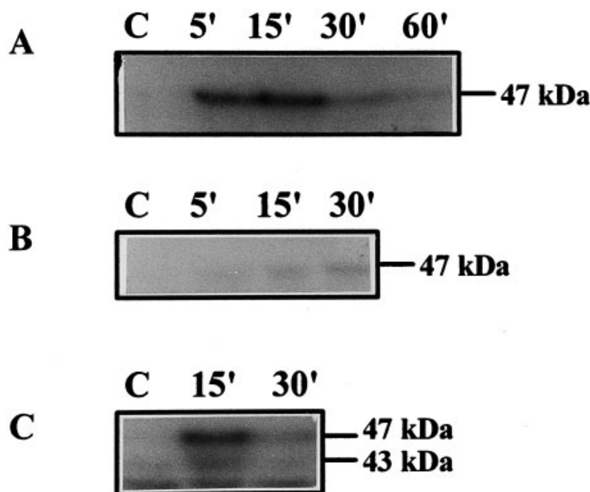

Fig. 4. H_2O_2 activation of MAPKs in *Arabidopsis*. H_2O_2 (20 mM) was added to either *Arabidopsis* cells (A) or protoplasts (C) or vacuum-infiltrated into leaves (B) for various times (min), proteins extracted and in-gel kinase assays performed using myelin basic protein as a substrate. Controls (C) were mock-treated with water. The molecular masses of the MAPK(s) are indicated.

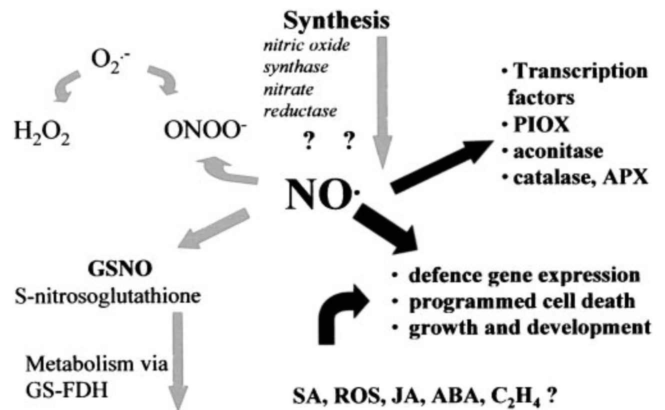

Fig. 5. NO signalling in plants. Grey arrows represent potential synthesis and biochemical interactions, black arrows represent potential cellular effects and targets of NO.

Using a transient gene expression system, it was demonstrated that H_2O_2 induced transcription driven by the specific stress-responsive promoters, *GST6* and *HSP18.2* (Kovtun *et al*., 2000). It was also shown that H_2O_2 activates AtMPK3/6 via ANP1, the MAPKKK at the head of the cascade. Furthermore, constitutive expression of ANP1 also activated *GST6* and *HSP18.2* expression. In an extension of this work, it was shown that transgenic tobacco plants over-expressing a tobacco MAPKKK orthologous to ANP1, possessed enhanced tolerance to heat shock, freezing, and salt stress, thereby demonstrating that manipulation of a key signalling component responsive to H_2O_2 can protect plants against various environmental stresses (Kovtun *et al*., 2000).

Thus, various observations indicate that H_2O_2-activation of a MAPK cascade is a central response mediating tolerance of various stresses: firstly, that H_2O_2 generation occurs in response to diverse biotic and abiotic stresses; secondly, that exposure to one stress offers cross-tolerance towards another; thirdly, that there exist commonalities in defence responses to various stresses (such as MAPK activation), and, fourthly, that activation of a H_2O_2-regulated MAPK pathway mediates multiple stress tolerance. It is quite likely that other stress-related MAPK signalling pathways are also involved.

NO responses

It has been known for some time that plants synthesize and release the gaseous molecule nitric oxide (NO) (Fig. 5; Wildt *et al*., 1997) and early work suggested roles for NO in the control of root growth and as an endogenous maturation and senescence factor (Leshem *et al*., 1998; Ribiero *et al*., 1999). In animals, NO is synthesized via the enzyme nitric oxide synthase (NOS), although there may be other biochemical routes. NOS activity has been found in plants, and inhibitors of mammalian NOS shown to inhibit NO generation (Cueto *et al*., 1996; Ninnemann and Maier, 1996; Delledonne *et al*., 1998; Foissner *et al*., 2000; Pedroso *et al*., 2000). In addition, antibodies raised against mammalian NOS enzymes cross-react with plant proteins (Huang and Knopp, 1998; Barroso *et al*., 1999; Ribiero *et al*., 1999). However, no full-length NOS gene sequence has yet been isolated, and there appear to be no easily discernible NOS sequences in the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000). It is possible though, that plant NOS-like enzymes do exist, with only limited sequence homology to their mammalian counterparts. An NOS enzyme activity has been purified from pea peroxisomes (Barroso *et al*., 1999), and a recent report that a partial cDNA clone for pea NOS has been obtained is particularly exciting (Corpas *et al*., 2001). Alternative sources of NO include nitrate reductase (Yamasaki and Sakihama, 2000; Wojtaszek, 2000), and there may be other, non-enzymatic routes to NO synthesis involving, for example, nitrite/ascorbate interaction or the light-mediated conversion of nitrogen dioxide to NO via carotenoids (Wojtaszek, 2000). Whatever the source of NO, it is clear that the NO-synthesis activity can be rapidly activated. Bacterial challenge induced rapid NO synthesis in soybean and *Arabidopsis* suspension cultures (Delledonne *et al*., 1998; Clarke *et al*., 2000) and elicitation of tobacco epidermal peels resulted in NO generation within minutes (Foissner *et al*., 2000).

So far, NO generation has been detected under conditions in which H_2O_2 generation is also stimulated (Delledonne *et al*., 1998; Clarke *et al*., 2000) and it may

well turn out that these two molecules are commonly present during various stresses. Thus, stress responses may reflect responses to both H_2O_2 and NO. In fact, bacterially-induced PCD has been reported to involve both these signals in soybean (Delledonne *et al.*, 1998) and *Arabidopsis* (Clarke *et al.*, 2000), although in the former situation the effects of NO and H_2O_2 were synergistic, but additive in the latter. H_2O_2 formation may, in some cases, occur via the superoxide radical ($O_2^{\cdot-}$). It is then possible that NO, itself a free radical ($NO^{\cdot}$) can react with $O_2^{\cdot-}$ to form the highly reactive peroxynitrite anion, $ONOO^-$. Subsequent cellular effects may then be induced by peroxynitrite.

In mammals, NO has been shown to react with glutathione to form *S*-nitrosoglutathione (GSNO) which can serve as a systemic source of NO, and a similar situation has been suggested for plants (Fig. 5; Durner and Klessig, 1999). Interestingly, a GSNO-catabolizing enzyme (a glutathione-dependent formaldehyde dehydrogenase GS-FDH), and its encoding gene have recently been characterized (Liu *et al.*, 2001). Mutant yeast which lacked this gene showed enhanced susceptibility to nitrosative challenge, indicating an important biological role for this enzyme (Liu *et al.*, 2001). This gene also exists in plants—it has been cloned from pea (Shafqat *et al.*, 1996)—so it will be of interest to determine what function, if any, this enzyme has in plants relevant to NO signalling.

A further level of complexity of H_2O_2 and NO signalling is clear when one remembers that they are not working alone, but in concert (or several concerts!) with other signalling molecules. Such molecules may be constitutively present, or increase in concentration/activity (e.g. via altered cellular sensitivity) during stress, and include compounds such as ABA, jasmonic acid, ethylene, and salicylic acid (Fig. 5).

The cellular targets for NO have not been well characterized. NO can react directly with proteins via nitrosylation (Durner and Klessig, 1999). NO has been shown to inhibit the activity of tobacco aconitase, an iron–sulphur containing enzyme that regulates iron homeostasis, suggesting a role for NO in modulating iron levels in plants (Navarre *et al.*, 2000). NO also inhibits catalase and ascorbate peroxidase activity (Clark *et al.*, 2000). NO activates the expression of the defence-related genes *PAL1*, *PR-1* and *GST* during plant–pathogen interactions (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Another potential NO target gene is PIOX (pathogen-induced oxygenase), which is involved in redox signalling during plant defence responses (Sanz *et al.*, 1998). This enzyme is a homologue of cyclo-oxygenases, major targets of NO in mammals (Nogawa *et al.*, 1998). The involvement of NO in mediating UV-B induction of CHS in *Arabidopsis* has also been reported recently (A-H-Mackerness *et al.*, 2001).

NO signal transduction

NO signalling in mammalian cells typically involves cyclic GMP (cGMP)-dependent and independent pathways, such as protein nitrosylation (Fig. 6). It was shown that NO signalling in tobacco required cGMP synthesis (Durner *et al.*, 1998). NO challenge induced a transient increase in cGMP content and inhibitors of cGMP synthesis via guanylate cyclase inhibited NO-induced activation of *PAL*. cGMP synthesis is also required during NO-induced PCD in *Arabidopsis*. Inhibition of guanylate cyclase prevented NO-mediated PCD, and such inhibition could be relieved by the addition of a cell-permeable cGMP analogue, 8-bromo cGMP (Clarke *et al.*, 2000). However, treatment with 8-bromo cGMP alone did not induce PCD, indicating that cGMP synthesis was required, but not sufficient, for the NO response. Neither guanylate cyclase nor a cGMP-dependent protein kinase (a potential target for cGMP) have yet been isolated and cloned from plants. cGMP also acts in mammalian cells via cyclic ADP ribose (cADPR). It was shown that cADPR is involved in NO-induced activation of *PAL* and *PR-1* (Durner *et al.*, 1998). cADPR regulated calcium levels in stomatal guard cells in response to ABA (Leckie *et al.*, 1998), and a calcium channel blocker inhibited cADPR- and cGMP-induced responses in tobacco (Durner *et al.*, 1998). In addition, NO activates MAP kinases in both tobacco (Kumar and Klessig, 2000) and *Arabidopsis* (Clarke *et al.*, 2000). The NO-activated MAPK in tobacco can also be activated by other signals such as salicylic acid (Kumar and Klessig, 2000), and H_2O_2 (Samuel *et al.*, 2000). Thus, activation of a central MAPK cascade could be a focal point of convergence of both H_2O_2 and NO signalling pathways activated in response to various stresses.

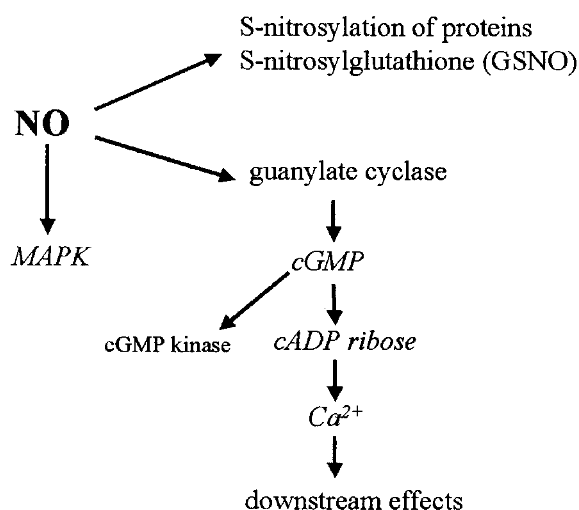

Fig. 6. NO signal transduction. The targets of NO in plants that have been identified are indicated in italics. Other potential targets have been identified in animals, but not yet in plants.

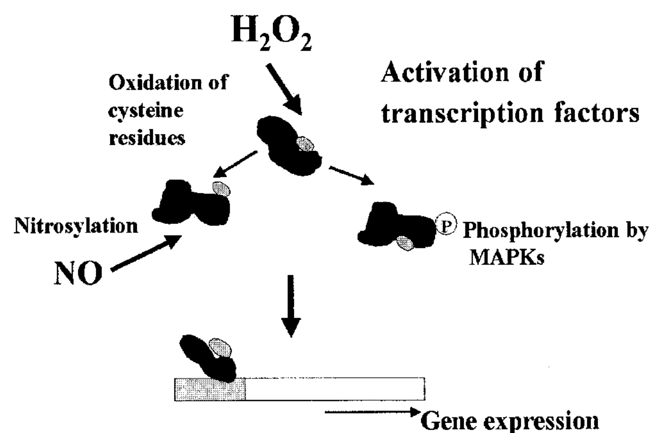

Fig. 7. H_2O_2, NO and gene expression. H_2O_2 and NO might regulate the activity of transcription factors directly via nitrosylation (NO) or oxidation of cysteine residues (H_2O_2). Activation of transcription factors can also occur via activation of a MAPK cascade which leads to phosphorylation of the transcription factor.

H_2O_2, NO and gene expression

It is clear that both H_2O_2 and NO can mediate the transcription of specific genes. However, the exact mechanisms by which this process occurs are not yet known. It could be that H_2O_2 and NO have direct effects on transcription factors, for example via oxidation of cysteine residues (for H_2O_2) or *S*-nitrosylation (for NO) (Fig. 7). Recently, a redox-sensitive transcription factor was characterized in yeast (Delaunay *et al.*, 2000). This protein is a direct target for H_2O_2: oxidation by H_2O_2 modified its conformation and thereby activity. It is also possible that activation of transcription factors occurs via a phosphorylation cascade such as the MAPK cascade. With both H_2O_2 and NO activating MAPKs, this seems a likely mechanism of activating gene expression (Fig. 7).

Conclusions

From being molecules of somewhat novelty interest, in the last few years H_2O_2 and NO have emerged to be central players in the world of plant cell signalling, particularly under various stressful situations. The full range of biological functions for these two signalling molecules remains to be catalogued, and determining the ways in which they interact, both together and with the ever-increasing array of signals known to be recognized by plants, will need to be elucidated. Other research priorities must include full characterization of the enzymes through which the intracellular concentrations of H_2O_2 and NO are regulated, and where these enzymes are located in different cells and tissues. The intracellular signalling cascades that transduce H_2O_2 and NO perception into cellular responses have so far been characterized only superficially. Finally, there arises the question of how H_2O_2 and NO are detected by cells. Such perception could conceivably involve direct interaction of H_2O_2 and NO with various cellular proteins, such as transcription factors, ion channels or enzymes. H_2O_2- and NO-sensitive enzymes could include signalling enzymes such as protein kinases and phosphatases. In mammalian cells, H_2O_2 modulates MAPK activity by interacting with a protein tyrosine phosphatase (Wu *et al.*, 1998). Given the complexity of plant cell signalling and the plethora of protein kinases and phosphatases, it will be surprising if similar examples do not exist in plants. Recent work with yeast has identified the plasma membrane-located hybrid histidine kinase SLN1 as a direct 'peroxisensor' that detects H_2O_2 (Singh, 2000). Such hybrid histidine kinases are common in plants. For example, there are eight in the *Arabidopsis* genome and some of these have already been identified as sensors for ethylene, cytokinin and osmotic stress (Urao *et al.*, 2000). Thus it may well be that a hybrid histidine kinase can function as a peroxisensor in *Arabidopsis*—in this context it is interesting that a hybrid histidine kinase gene whose expression is increased by H_2O_2 has been identified (Desikan *et al.*, 2001*a*).

In summary, both H_2O_2 and NO have now been shown to function as stress signals in plants, mediating a range of responses to environmental stress. Given that such stresses impose considerable constraints on crop production, there is a real need for continued research in this area.

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