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ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells

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Abstract

Increased synthesis and redistribution of the phytohormone abscisic acid (ABA) in response to water deficit stress initiates an intricate network of signalling pathways in guard cells leading to stomatal closure. Despite the large number of ABA signalling intermediates that are known in guard cells, new discoveries are still being made. Recently, the reactive oxygen species hydrogen peroxide (H_2O_2) and the reactive nitrogen species nitric oxide (NO) have been identified as key molecules regulating ABA-induced stomatal closure in various species. As with many other physiological responses in which H_2O_2 and NO are involved, stomatal closure in response to ABA also appears to require the tandem synthesis and action of both these signalling molecules. Recent pharmacological and genetic data have identified NADPH oxidase as a source of H_2O_2, whilst nitrate reductase has been identified as a source of NO in *Arabidopsis* guard cells. Some signalling components positioned downstream of H_2O_2 and NO are calcium, protein kinases and cyclic GMP. However, the exact interaction between the various signalling components in response to H_2O_2 and NO in guard cells remains to be established.

Key words: Abscisic acid, guard cells, hydrogen peroxide, nitric oxide, signalling.

Introduction

Plant growth and development are largely affected by the availability of water. Hence adaptation to water deficit stress is common to all terrestrial plants. Abscisic acid (ABA) is an endogenous anti-transpirant that reduces water loss through stomatal pores on the leaf surface. Enhanced biosynthesis of ABA occurs in response to water deficit stress, resulting in the redistribution and accumulation of ABA in guard cells surrounding the stomata. This results in the release of water, efflux and influx of ions, and loss of turgor of guard cells, causing closure of stomata (Bray, 1997).

Perception of ABA by, as yet unidentified, receptors in guard cells activates a complex web of signalling pathways leading to stomatal closure. These include the activation of ion channels and the synthesis of calcium mobilizing molecules such as cyclic ADP ribose and inositol trisphosphate, thereby elevating cytosolic calcium levels. Reversible protein phosphorylation, activation of other signalling components such as G proteins, and modulation of RNA metabolism are also placed downstream of ABA, leading to stomatal closure (Schroeder *et al*., 2001). Despite the large number of ABA signalling intermediates in guard cells, new discoveries are still being made. Recent additions include the phospholipid sphingosine-1-phosphate (SIP), phospholipase C, the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and the reactive nitrogen species nitric oxide (NO) (Hetherington, 2001; Neill *et al*., 2003*a*, *b*). In this article, the role of H_2O_2 and NO as signalling molecules in plants, particularly in guard cells, is discussed, highlighting their involvement in the ABA signalling pathway, and some future perspectives which might enhance the current understanding of guard cell signalling are considered.

H_2O_2 and NO as signalling molecules in plants

H_2O_2 and NO are forms of reactive oxygen and reactive nitrogen species, respectively, having wide-ranging effects

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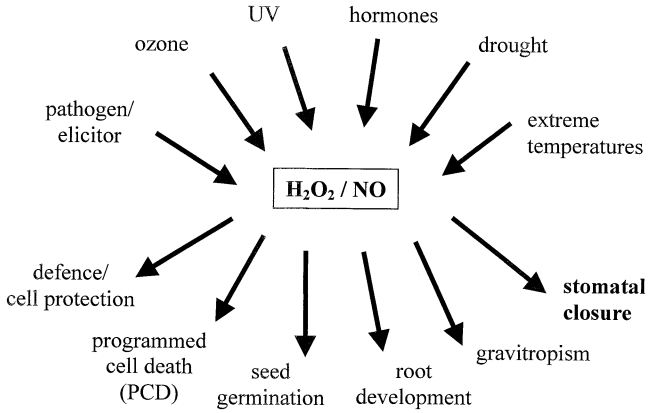

Fig. 1. Involvement of H_2O_2 and NO in cellular responses to various stresses and stimuli.

in many biological systems (Durner *et al*., 1999; Finkel and Holbrook, 2000). Although the effects of both H_2O_2 and NO on plant physiology and development have been the subject of investigation for several years, it is only relatively recently that their roles as signalling molecules have been characterized more fully. Exposure of plants to various abiotic and biotic stresses induces the generation of both H_2O_2 and NO. Such stresses and stimuli include atmospheric pollutants such as ozone and UV radiations, extremes of temperature, wounding and pathogen challenge, dehydration, and plant hormones such as ABA, auxin, cytokinin, and ethylene (Fig. 1).

The roles of H_2O_2 and NO in plant defence responses to pathogens have been studied in some detail. Exposure of plant cells to avirulent (non-disease inducing) pathogens or pathogen-derived elicitors induces both a rapid and a more prolonged 'burst' of H_2O_2. Generation of NO occurs within the same time frame as H_2O_2 release, and a critical balance between the two reactive species regulates cellular outcomes such as programmed cell death (Delledonne *et al*., 2001).

In addition to their role in stress responses, the involvement of H_2O_2 and NO in developmental processes such as seed germination, gravitropism and root development indicate that these signalling molecules are key regulators of plant responses to a range of endogenous signals and stimuli such as auxin and ABA (Neill *et al*., 2002*b*, 2003*b*).

Common physiological and cellular responses observed in response to these stimuli also suggest that both H_2O_2 and NO are synthesized in parallel and act in tandem in various situations. The cellular effects of increased synthesis of these molecules are thus likely to be mediated via their actions, either individually or in concert. H_2O_2 and NO have been shown to regulate the expression of a number of genes whose products are involved in limiting pathogen growth, in cellular protection or in other signalling responses (Desikan *et al*., 2001; Huang *et al*., 2002). H_2O_2 and NO also activate common cellular responses such as elevation of cytosolic calcium levels, protein kinases and phosphatases (see below). The chemical nature of H_2O_2 and NO suggests that they are also likely to have direct effects on common molecular targets such as protein thiol groups that are susceptible to oxidation or nitrosylation (Cooper *et al*., 2002). Identification of downstream targets of H_2O_2 and NO acting in parallel, together or synergistically, in various plant cells, particularly in guard cells, is clearly an important research priority.

H_2O_2 as a signal in guard cells

The effects of H_2O_2 on guard cells were first reported by McAinsh *et al*. (1996). Exposure of *Vicia faba* guard cells to exogenous H_2O_2 induced elevations of cytosolic calcium and stomatal closure. At low levels ($<10^{-5}$ M) H_2O_2 had no effect on membrane damage, whereas at higher concentrations this was apparent. The ability of guard cells to generate H_2O_2 has been demonstrated in tobacco, tomato and *Commelina* sp. (Allan and Fluhr, 1997; Lee *et al*., 1999). H_2O_2 synthesis in plant cells can easily be visualized using the cell-permeable fluorescent dye, dichlorofluorescein diacetate (H_2DCF-DA). Treatment of tobacco epidermal cells with the fungal elicitor cryptogein, induced H_2O_2 generation in guard cells and surrounding epidermal cells (Allan and Fluhr, 1997). In tomato and *Commelina*, Lee *et al*. (1999) observed H_2O_2 generation in response to an oligogalacturonide elicitor or chitosan, leading to stomatal closure. Treatment with catalase, a H_2O_2 scavenger, reduced both stomatal closure and H_2O_2 synthesis, confirming that the H_2O_2 was required to initiate stomatal closure. The discovery that ABA induces H_2O_2 synthesis in *Arabidopsis* guard cells was a significant finding that highlighted further complexities in ABA signalling. Pei *et al*. (2000) demonstrated that treatment of *Arabidopsis* guard cells with ABA induced a rapid burst of H_2O_2 production that resulted in stomatal closure. These findings have subsequently been reported in guard cells of *Vicia faba* (Zhang *et al*., 2001*c*), and observed in pea (R Desikan *et al*., unpublished data). Therefore, it appears that synthesis of H_2O_2 is essential for ABA-induced stomatal closure in various species.

H_2O_2 can be synthesized via several routes in plant cells (Neill *et al*., 2002*b*). Electron transport processes such as photosynthesis and respiration generate basal levels of H_2O_2, which increase in response to stress. Enzymatic sources of H_2O_2 include NADPH oxidase, cell wall peroxidases, amine oxidases, and other flavin containing enzymes (Neill *et al*., 2002*b*, *c*; Desikan *et al*., 2003). More than one source of H_2O_2 has also been proposed for guard cells. Allan and Fluhr (1997) suggested that, in guard cells and epidermal cells of tobacco responding to elicitor challenge, H_2O_2 was generated via intracellular flavin-

containing enzymes, apoplastic peroxidases, and amine oxidase-type enzymes. In *Arabidopsis*, ABA-induced stomatal closure was inhibited by the flavin analogue diphenylene iodonium (DPI), used widely as an inhibitor of NADPH oxidase (Cross and Jones, 1986). Together with NADPH-dependence of H_2O_2-induced calcium channel activation, this implies a role for a NADPH oxidase-like enzyme mediating H_2O_2 synthesis in response to ABA in *Arabidopsis* guard cells (Pei *et al*., 2000; Murata *et al*., 2001). Zhang *et al*. (2001*c*) proposed two different sources of H_2O_2 in *Vicia faba* guard cells in response to ABA, one chloroplastic and another via a plasma membrane-located enzyme (potentially NADPH oxidase). In this laboratory, it has been shown that exposure of pea guard cells to catalase or DPI reduced ABA-induced H_2O_2 synthesis and stomatal closure (R Desikan *et al*., unpublished data), also suggesting NADPH oxidase as a potential source of H_2O_2.

Until recently, there has been little molecular evidence to support the pharmacological data indicating a role for NADPH oxidase in guard cell functioning. Genes encoding the large subunit of the NADPH oxidase (respiratory burst oxidase homologue, *rboh*) have been cloned from different species (Desikan *et al*., 1998; Keller *et al*., 1998; Torres *et al*., 1998, 2002; Amicucci *et al*., 1999; Yoshioka *et al*., 2001). Reverse genetic approaches have been used to establish a functional role for NADPH oxidase in plant responses to pathogens (Simon-Plas *et al*., 2002; Torres *et al*., 2002; Yoshioka *et al*., 2003). In exciting new developments, the *rboh* transposon-insertion mutants of *Arabidopsis* have been used to provide unequivocal evidence that NADPH oxidase-mediated H_2O_2 synthesis is required for ABA-induced stomatal closure (Kwak *et al*., 2003). Two homologues of the *rboh* gene family (*AtrbohD* and *AtrbohF*) were found to be expressed in guard cells, and treatment with ABA increased the expression of *AtrbohD* in guard cells. *atrbohD/F* double mutants were impaired in stomatal closure and activation of calcium channels in response to ABA, and H_2O_2 application rescued these responses. These data provide direct molecular genetic evidence that NADPH oxidase homologues function in guard cell ABA signal transduction. These mutants will no doubt be an important research tool to dissect downstream responses to ABA, ROS and other signalling molecules likely to interact with ROS.

Nitric oxide as a signal in guard cells

The above findings linking ABA and H_2O_2 in guard cells were soon followed by the discovery that NO is also an essential signal mediating ABA-induced stomatal closure (Neill *et al*., 2002*a*). Exogenous NO (applied as NO donors) induced stomatal closure and reduced transpiration in *Vicia faba, Salpichroa organifolia* and *Tradescantia* sp. (Garcia-Mata and Lamattina, 2001). In this laboratory, it has been shown that different NO donors induce stomatal closure in pea, *Arabidopsis*, tomato, barley, and wheat (Desikan *et al*., 2002; Neill *et al*., 2002*a*; SJ Neill, unpublished data), a process that is time- and dose-dependent, and fully reversible (Neill *et al*., 2002*a*).

The discovery that NO is a key signal mediating ABA responses in pea guard cells (Neill *et al*., 2002*a*), has since been confirmed for *Vicia faba* (Garcia-Mata and Lamattina, 2002) and *Arabidopsis* (Desikan *et al*., 2002). Application of PTIO (or cPTIO), a NO scavenger, inhibited ABA-induced stomatal closure, indicating the involvement of endogenous NO (Desikan *et al*., 2002; Garcia-Mata and Lamattina, 2002; Neill *et al*., 2002*a*). NO synthesis was monitored in guard cells using the NO-fluorescent dye diaminofluorescein diacetate (DAF-2DA), previously used to monitor NO synthesis in guard cells and epidermal cells of tobacco in response to elicitor treatment (Foissner *et al*., 2000). ABA-induced increases in NO synthesis have been observed in guard cells of *Arabidopsis*, pea and *Vicia faba* (Desikan *et al*., 2002; Neill *et al*., 2002*a*, 2003*a*; Garcia-Mata and Lamattina, 2002). Increased DAF-2DA fluorescence was observed in the cytoplasm, and in some cases around the chloroplasts, of ABA-treated guard cells. This fluorescence was NO-specific, as no increase in fluorescence was observed using the NO-inactive dye 4AF-DA or by co-incubation of ABA with NO scavengers (Desikan *et al*., 2002; Garcia-Mata and Lamattina, 2002). These studies demonstrate that NO synthesis is essential for ABA-induced stomatal closure in various species.

There are two main candidates for enzymes of NO synthesis in plant cells, nitric oxide synthase (NOS) and nitrate reductase (NR), although other routes, both enzymatic and non-enzymatic, may exist (Neill *et al*., 2003*b*). In mammalian cells, a family of NOS enzymes catalyses the conversion of L-arginine to citrulline, with the simultaneous release of NO. This NO synthesis can be monitored by measuring the conversion of radiolabelled arginine to citrulline, an assay that has been widely used in plants. Inhibition of NO synthesis, and arginine to citrulline conversion by arginine analogues such as L-NAME (*N*-nitro-L-arginine methyl ester), have provided some evidence for the presence of NOS in plants (Wendehenne *et al*., 2001; Neill *et al*., 2003*b*). In pea guard cells it has been observed that ABA-induced NO synthesis and stomatal closure can be partially inhibited by L-NAME (Neill *et al*., 2002*a*), suggesting the involvement of a NOS-type enzyme. Until very recently, there had been no molecular evidence for NOS-like genes in plants. In an exciting new development, the Klessig group has shown that a pathogen-inducible NOS (iNOS) in tobacco and *Arabidopsis* is actually a variant of the P protein of the glycine decarboxylase complex (Chandok *et al*., 2003). This protein catalyses the conversion of arginine to citrulline, an activity that was inhibited by NOS inhibitors. Purification and subsequent sequence analysis of this

protein revealed only very limited similarity with mammalian NOS enzymes, although the requirements for activation or induction were similar. This is a major discovery that will no doubt lead to further insight into the role of NOS-like proteins in NO signalling.

Nitrate reductase (NR) is an enzyme important for nitrogen assimilation in plants, where its primary function is to convert nitrate to nitrite via a NAD(P)H-dependent electron transfer reaction (Crawford and Forde, 2002). In addition, NR also catalyses the NAD(P)H-dependent reduction of nitrite to NO, originally demonstrated in soybean (Dean and Harper, 1988), but more recently shown both *in vitro* and *in vivo* in maize, sunflower and spinach (Yamasaki *et al*., 1999; Yamasaki and Sakihama, 2000; Kaiser *et al*., 2002; Rockel *et al*., 2002). A physiological role for NR-mediated NO synthesis was established in the author's laboratory (Desikan *et al*., 2002). Treatment of *Arabidopsis* guard cells with nitrite induced NO synthesis and stomatal closure. Pre-treatment with PTIO inhibited NO accumulation and stomatal closure in response to nitrite (Desikan *et al*., 2002). Moreover, tungstate, an inhibitor of NR activity (Notton and Hewitt, 1971; J Bright, unpublished data) inhibited ABA and nitrite-induced NO synthesis and stomatal closure in *Arabidopsis* guard cells (J Bright, R Desikan, unpublished data). In addition, the NOS inhibitor L-NAME did not inhibit ABA-induced NO synthesis or stomatal closure in *Arabidopsis* epidermal peels (Desikan *et al*., 2002). Together, these data suggest that NR acts as a source of ABA-induced NO synthesis in *Arabidopsis* guard cells. To confirm this, the NR-deficient *nia1, nia2* mutant of *Arabidopsis* was used which exhibits less than 5% NR activity of wild-type plants (Wilkinson and Crawford, 1993). In epidermal peels of *nia1, nia2*, neither ABA nor nitrite induced stomatal closure or NO synthesis (Desikan *et al*., 2002). Importantly, it was found that guard cells of *nia1, nia2* do respond to other closing stimuli such as darkness, H_2O_2 or a NO donor, implying that they do possess functional guard cells (Desikan *et al*., 2002). A stomatal closing response to NO also suggests that the deficiencies caused by the *nia1, nia2* mutations lie upstream of NO in the signalling pathway. Together, these data provide firm genetic evidence for NR as a source of NO during ABA-induced stomatal closure in *Arabidopsis*. The significance of the apparently different sources of guard cell NO in pea and *Arabidopsis* and the potential contribution to NO synthesis by iNOS remain to be determined.

Stomatal opening responses to ABA, H_2O_2 and NO

ABA inhibition of stomatal opening is a process distinct from ABA-induced stomatal closure (Schroeder *et al*., 2001). Although it is now clear that both H_2O_2 and NO are essential signals required to mediate ABA-induced stomatal closure, there is little information on whether these signalling molecules mediate ABA-inhibition of stomatal opening. In pea guard cells, treatment with catalase inhibited ABA-inhibition of stomatal opening, suggesting the involvement of H_2O_2 (R Desikan *et al*., unpublished data). Treatment of pea epidermal peels with exogenous H_2O_2 alone also inhibits stomatal opening. Preliminary pharmacological data indicate that a MAPK pathway is involved in H_2O_2-induction of stomatal closure, but not in H_2O_2 inhibition of stomatal opening (data not shown). Similar divergence of signalling pathways downstream of ABA has been reported by others. *Arabidopsis* knock-out mutant plants lacking functional G proteins show altered responses to ABA-inhibition of stomatal opening, but not ABA-induced closure (Wang *et al*., 2001). Interestingly, for NO, the data obtained with the *nia1, nia2* double mutant indicate that although guard cells of these mutants do not close in response to ABA, inhibition of stomatal opening in response to ABA is not compromised (Desikan *et al*., 2002), suggesting that NR-mediated NO synthesis is not required for this response.

A recent publication describes the role of NO in promoting stomatal opening in *Vicia faba* (Sakihama *et al*., 2003). However, these data contradict those reported by Garcia-Mata and Lamattina (2001) from the same species, where NO was found to induce stomatal closure. These discrepancies could possibly be accounted for by the relative concentrations of the NO donor (SNAP) used. Whilst the Lamattina group used micromolar concentrations, at least 10-fold higher concentrations were used by Sakihama *et al*. (2003). This concentration dependence has also been observed in the author's laboratory in both pea and *Arabidopsis* (Neill *et al*., 2003*a*). NO (administered via SNP) in the range of 10–200 μM causes stomatal closure whilst at higher concentrations of SNP (0.5–2 mM), stomata remain open. The physiological relevance for this phenomenon is not known, particularly as endogenous NO concentrations in and around guard cells have not yet been determined.

H_2O_2 and NO signalling and cross-talk in guard cells

It is clear that both H_2O_2 and NO regulate stomatal movements. However, there is little information on the cellular processes by which H_2O_2 and NO act in guard cells. An understanding of the signalling events that occur downstream of H_2O_2 or NO may determine any convergence/divergence between stomatal closure (or opening) responses.

Pharmacological data from the author's laboratory suggest that MAPK(s) mediate both ABA and H_2O_2-induced stomatal closure (R Desikan *et al*., unpublished data; Burnett *et al*., 2000). In previous work it was shown

that H_2O_2 induces the activation of a MAPK in cell cultures, leaves and protoplasts of *Arabidopsis* (Neill *et al*., 2002*b*). In pea epidermis, H_2O_2 induces the transient activation of a MAPK-like enzyme (R Desikan *et al*., unpublished data), possessing properties similar to those of the ABA-activated kinase (Burnett *et al*., 2000). Interestingly, treatment with the NO donor SNP also induces the transient activation of a similar kinase (data not shown). These data suggest that ABA, H_2O_2 and NO may converge on MAPK signalling pathways that are involved in regulating stomatal closure. Recent data from Giraudat's laboratory identified an ABA-activated protein kinase, OST1, that lies upstream of H_2O_2 production in guard cells (Mustilli *et al*., 2002). It remains to be determined whether the ABA-activated kinase in pea also regulates H_2O_2 or NO production.

Protein dephosphorylation also plays an important role in ABA-induced stomatal movements (Schroeder *et al*., 2001). The protein phosphatase 2C enzymes ABI1 and ABI2 are negative regulators of ABA signalling (Merlot *et al*., 2001). Recent data from the Schroeder laboratory indicate that ABI1 lies upstream, and ABI2 downstream, of H_2O_2 synthesis in *Arabidopsis* guard cells (Murata *et al*., 2001). Further complexities in this scheme are likely to become apparent, as *in vitro* studies have revealed that ABI1 and ABI2 activities can be inhibited by H_2O_2 (Meinhard and Grill, 2001; Meinhard *et al*., 2002). Whether or not this occurs *in vivo* is not yet known. A recent addition to this proposed signalling scheme is NO. It has been shown in the author's laboratory that, in the *abi1-1* and *abi2-1* mutants that are ABA insensitive in their response to stomatal closure, NO synthesis still occurs in response to ABA. However, treatment with the NO donor SNP did not induce stomatal closure in these mutants, indicating that the action of both the phosphatase enzymes occurs downstream of NO synthesis (Desikan *et al*., 2002). Whether these enzymes can be modified directly by NO, as with H_2O_2 (Meinhard *et al*., 2002; Meinhard and Grill, 2001) is an interesting question.

Stomatal closure in response to ABA typically requires elevated cytosolic calcium (Allen *et al*., 2000). Synthesis and action of calcium-mobilizing molecules such as inositol trisphosphate and cADPR regulate these elevations in calcium (Leckie *et al*., 1998). There is some evidence that both H_2O_2 and NO actions in guard cells require calcium. H_2O_2 activates calcium channels in *Arabidopsis* guard cells (Pei *et al*., 2000; Kohler *et al*., 2003). In conjunction with this, a role for phosphatidylinositol 3-phosphate in ABA-induced H_2O_2 generation was recently demonstrated in *V. faba* guard cells (Park *et al*., 2003). A requirement for calcium in NO-mediated stomatal closure has also been demonstrated (Garcia-Mata and Lamattina, 2001; R Desikan *et al*., unpublished data). In mammalian cells, NO acts via enhanced synthesis of cADPR. Using a pharmacological approach it was

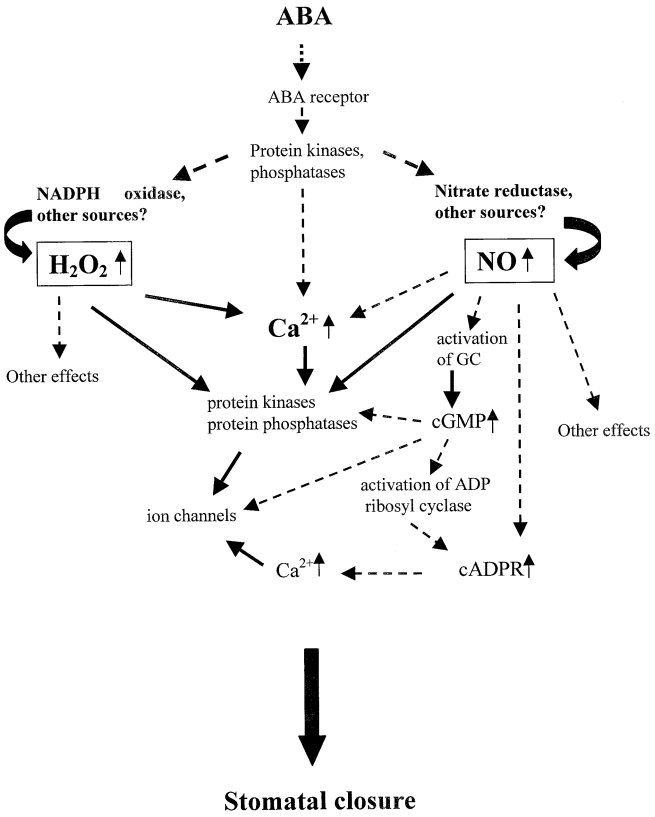

Fig. 2. Schematic representation of ABA, H_2O_2 and NO signalling cross-talk in stomatal guard cells. Solid lines represent those signalling pathways for which experimental evidence is available; broken lines indicate predicted pathways.

shown that ABA and NO-induced stomatal closure can be inhibited with nicotinamide, an antagonist of cADPR synthesis (Neill *et al*., 2002*a*), implying that ABA and NO-induced stomatal closure occur via synthesis of cADPR. However, NO-induced increases in cADPR synthesis (leading to elevations of cytosolic calcium) have not been demonstrated in guard cells or any other plant cell; clearly this is an important research priority that needs to be addressed.

In addition to regulating calcium channels, H_2O_2 also inhibits K^+ channel activity and induces cytosolic alkalinsation in guard cells (Zhang *et al*., 2001*a*, *b*; Kohler *et al*., 2003). However, it is not known whether H_2O_2 acts directly on such ion channels via conformational changes to protein structure or via an intermediate mechanism. The role of NO in activating ion channels is not yet known, although it is quite likely that this will be a research area gaining much interest in the future.

NO action requires the synthesis of the cyclic nucleotide cyclic GMP (cGMP). Although the existence of this molecule in plants has been speculated for several years, recent data indicate that cGMP is indeed synthesized in plant cells, and enhanced in response to NO (Newton *et al*., 1999; Neill *et al*., 2003*b*). In guard cells, cGMP was

highlighted as a likely target of NO signalling. Treatment of pea guard cells with with ODQ, an inhibitor of cGMP synthesis, attenuated ABA and NO-induced stomatal closure, processes that were reversed by co-incubation with 8Bromo-cGMP (8Br-cGMP, a cell-permeable analogue of cGMP). However, treatment with 8Br-cGMP alone did not induce stomatal closure, implying that cGMP synthesis is required, but not sufficient, for stomatal closure (Neill *et al*., 2002*a*). Interestingly, H_2O_2-induced closure is not inhibited by ODQ (R Desikan, unpublished data), suggesting a branch in the ABA-H_2O_2/NO signalling pathway (Fig. 2).

From the findings described above, it is not yet clear whether signalling pathways unique to either H_2O_2 or NO exist in guard cells. Given the convergence, divergence and network of signalling pathways that occur in response to ABA in guard cells, it may be that ABA-H_2O_2 and ABA-NO guard cell signalling are not singular entities and, instead, they are networked so that they function together to effect co-ordinated stomatal responses. It is now imperative to undertake further studies to dissect the complexities underlying H_2O_2 and NO signalling responses in guard cells.

Conclusions and future perspectives

Guard cells are a unique signal transduction research tool that provide an elegant system to dissect an intricate network of signalling pathways. Clearly, both H_2O_2 and NO play a central role in the guard cell ABA signalling network. Both H_2O_2 and NO are synthesized in response to ABA, perhaps in parallel, and both control a single response—a reduction in stomatal aperture. NADPH oxidase is a source of H_2O_2 biosynthesis in guard cells, although other sources could also exist. NO synthesis occurs via NR, and/or possibly other enzymes with NOS-like activity. Lack of either NADPH oxidase or NR activities reduces stomatal responses to ABA, but there is no indication that it results in plants with a wilty phenotype under normal growth conditions. Clearly, further evaluation of the mechanisms of synthesis of H_2O_2 and NO is required, in order to elucidate the exact function of their biosynthetic enzymes in response to ABA and drought stress in guard cells. Genetic ablation of both NADPH oxidase and NR, for example, will be useful to study the role of both H_2O_2 and NO in response to drought stress or ABA treatment. In the NR-deficient mutant, although the guard cells do not synthesize NO in response to ABA, they exhibit enhanced synthesis of H_2O_2 (R Desikan *et al*., unpublished data), perhaps reflecting a cellular capacity to compensate for the chronic lack of a key enzyme. Would plants lacking both NR and NADPH oxidase be able to retain functional guard cell ABA responses through some compensatory mechanism?

Development of techniques to monitor simultaneously the generation of H_2O_2 and NO in response to ABA, will be essential to discern the spatial and temporal co-ordination of events leading to stomatal closure. Investigating the position of different signalling components and their relative importance in this signalling scheme will allow this complex system to be understood more clearly. The use of various ABA signalling mutants defective in stomatal responses has already proved useful to position H_2O_2 and NO in the signalling scheme (Pei *et al*., 2000; Murata *et al*., 2001; Desikan *et al*., 2002). Application of recently developed genetic screens to monitor water loss, such as thermo-imaging (Mustilli *et al*., 2002) may be useful in revealing novel targets of H_2O_2 and NO in guard cells. Identification of downstream targets of H_2O_2 and NO should be advanced using proteomics analysis of guard cells from the wild type, and from *rboh* or *nia* mutants. This technology should reveal novel targets for both H_2O_2 and NO in guard cells, for example, thiol-modified proteins, or newly synthesized proteins, and whether they are common or unique to H_2O_2 or NO. It has already been established that MAPKs, cGMP, cADPR, and protein phosphatases are likely targets downstream of H_2O_2 or NO. Further work is required here to measure, quantify and determine accurately the concentrations of cGMP and cADPR specifically in guard cells. Functional genomic studies have already proved useful to identify a source of H_2O_2 synthesis in guard cells (Kwak *et al*., 2003). This approach could also be utilized to determine the role of other components of guard cell signalling, such as MAPKs or cGMP-synthases, that are activated by H_2O_2 and/or NO, respectively. Guard cell research in the forthcoming years will no doubt remain exciting, with the prospect of developing plants better able to tolerate water stress, and thus impact on global agriculture.

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