

NITRIC OXIDE AS A MEDIATOR OF ABA SIGNALLING IN STOMATAL GUARD CELLS

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Summary. Water shortage is likely to be one of the major global environmental stresses of the 21st century. A key plant response to declining soil water potential is increased biosynthesis and subsequent action of abscisic acid (ABA). ABA is an endogenous anti-transpirant that induces stomatal closure, thereby leading to water conservation. The signal transduction processes commencing with guard cell ABA recognition and culminating in transport of ions and water across the vacuolar and plasma membranes, resulting in guard cell shrinkage and stomatal closure, have not yet been completely resolved. Nitric oxide (NO) has recently been identified as a mediator of guard cell ABA signalling (Neill et al., 2002. *Plant Physiol.* 128, 13-16). ABA induces the synthesis of NO in guard cells, NO induces stomatal closure and either scavenging of NO or inhibition of NO synthesis reduces ABA-induced stomatal closure. These data indicate that NO synthesis is a critical component of ABA-induced stomatal closure. Aspects of NO synthesis and action are discussed.

Key words: abscisic acid, cyclic GMP, guard cell, nitric oxide, signalling, stomata, water deficit.1

Abbreviations: ABA – abscisic acid, cADPR – cyclic ADP ribose, cGMP – cyclic GMP, DAF-2DA – diaminofluorescein diacetate, GSNO – S-nitrosoglutathione, L-NAME – N-nitro-L-arginine methyl ester, MES – 2-(N-morpholino)ethanesulfonic acid, NO – nitric oxide, NOS – nitric oxide synthase, NR – nitrate reductase, PTIO – 2-phenyl-4,4,5,5-tetramethylimidazolinone-1-oxyl 3-oxide, ODQ – 1*H*-(1,2,4-oxadiazole-[4,3-*a*]quinoxalin-1-one, SNP – sodium nitroprusside, WUE – water use efficiency.

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Introduction

Water shortage is likely to be one of the major global environmental stresses of the twenty-first century. Reduced water availability is a major limitation to crop yield, as cell expansion requires the cell pressure potential to be sufficiently high to permit irreversible cell wall stretching. Thus, cell expansion is particularly sensitive to declining soil water availability. Reduced cell expansion means that leaf expansion will also be reduced, with a subsequent failure to utilise maximally incident irradiation. Severe water stress also has other detrimental effects on plant physiology and metabolism. It is not surprising, then, that efficient mechanisms have evolved to allow plants to combat such water stress.

More than 95% of the water that passes through plants exits via the stomatal pores, through which the vast majority of carbon dioxide required for photosynthesis enters. Stomata operate as miniature homeostatic sensory and effector systems that sense a number of stimuli to induce guard cell swelling or shrinking, resulting in stomatal opening or closing, and thus optimization of Water Use Efficiency (WUE), a measure of the efficiency with which plants facilitate CO₂ influx at the expense of water loss. Declining soil water potential is sensed by roots, and subsequently shoots, by as yet uncharacterized mechanisms. What is known, however, is that reductions in cell turgor are transduced into re-distribution and enhanced biosynthesis of the hormone abscisic acid (ABA), such that ABA is released into the transpiration stream in which it is transported to the guard cells. ABA is an endogenous anti-transpirant – when it arrives at guard cells it initiates a complex web of signalling events that culminates in release of potassium ions and water from guard cells, thereby inducing guard cell shrinkage and stomatal closure.

The mechanisms by which ABA initiates stomatal closure are complex. ABA probably interacts with cellular receptors to induce rapid responses that involve cytosolic alkalisation and activation of anion and calcium channels. Enhanced cytosolic calcium levels are achieved via both influx from the extracellular milieu and by release from intracellular stores. Such intracellular release is probably effected via rapid, transient synthesis and action of calcium-mobilising molecules such as inositol trisphosphate and cyclic ADP ribose (cADPR). ABA responses also require reversible activation and inactivation of phospholipase D, farnesyltransferase, protein kinases and protein phosphatases; modulation of the actin cytoskeleton and activation of a small G protein; and effects on RNA metabolism (Hetherington, 2001; Schroeder et al., 2001). Recent research from a number of laboratories has demonstrated the involvement of hitherto-unsuspected intermediates in ABA signalling, including the unusual phospholipid sphingosine-1-phosphate, hydrogen peroxide (H₂O₂) and, in our laboratory, nitric oxide, NO (Neill et al., 2002b).

NO is a well-characterised signalling molecule in mammalian cells that increasingly appears to be used by all organisms. Two seminal papers in 1998 demon-

strated a key role for NO during plant defence responses to pathogen challenge (Delledonne et al., 1998; Durner et al., 1998). NO is synthesized rapidly following pathogen challenge, with similar kinetics to the oxidative burst, during which large amounts of H₂O₂ are synthesized and released. H₂O₂ and NO interact to induce programmed cell death and other defence responses (Delledonne et al., 1998; 2001; Torres et al., 2002). There is now a growing list of plant processes that are seemingly regulated by NO and thus considerable interest in determining how NO synthesis is effected and regulated, and the mechanisms by which NO exerts its effects.

NO is an endogenous signalling intermediate in ABA-induced stomatal closure

NO is a short-lived free radical gas, and so is typically applied to plants via the administration of NO-releasing compounds such as GSNO (S-nitrosoglutathione) or SNP (sodium nitroprusside). Mata and Lamattina (2001) showed that exogenous NO (applied as SNP) reduced transpiration and induced stomatal closure in several species such as *Vicia faba*, *Salpichroa organifolia* and *Tradescantia spp.* NO-induced stomatal closure is likely to be a common, if not universal response, as it is seen in other species such as barley, tomato and *Arabidopsis* (Neill et al., unpublished).

We have been studying the role of NO as an endogenous intermediate in ABA signalling in pea, *Pisum sativum* L. (Neill et al., 2002b) and other species such as *Arabidopsis* (unpublished). In order to determine if ABA-induced stomatal closure involves endogenous NO as a signalling intermediate, epidermal peels prepared from pea leaves were incubated in ABA and endogenous NO removed by treatment with the NO-scavenger PTIO (2-phenyl-4,4,5,5-tetramethylimidazolinone-1-oxyl 3-oxide). Such treatment clearly inhibited ABA-induced closure (Fig. 1). Exposure to exogenous NO via incubation in either of two different NO donors, GSNO and SNP, induced stomatal closure, confirming the effects of NO. Inhibition of these effects by co-incubation in PTIO demonstrated that the effects of the two donors were indeed due to NO release (Fig. 1). Stomatal closure induced by SNP was both dose- and time-dependent (Fig. 1), similar to stomatal closure induced by ABA (data not shown). These data demonstrating the role of NO as an endogenous signalling intermediate in ABA-induced stomatal closure have been confirmed for *V. faba* (Mata and Lamattina, 2002) and *Arabidopsis* (Neill et al., unpublished).

ABA induces NO synthesis in stomatal guard cells

In order to determine if ABA induces the synthesis of NO in guard cells, pea epidermal peels were loaded with the NO-indicator dye DAF-2DA (diaminofluorescein

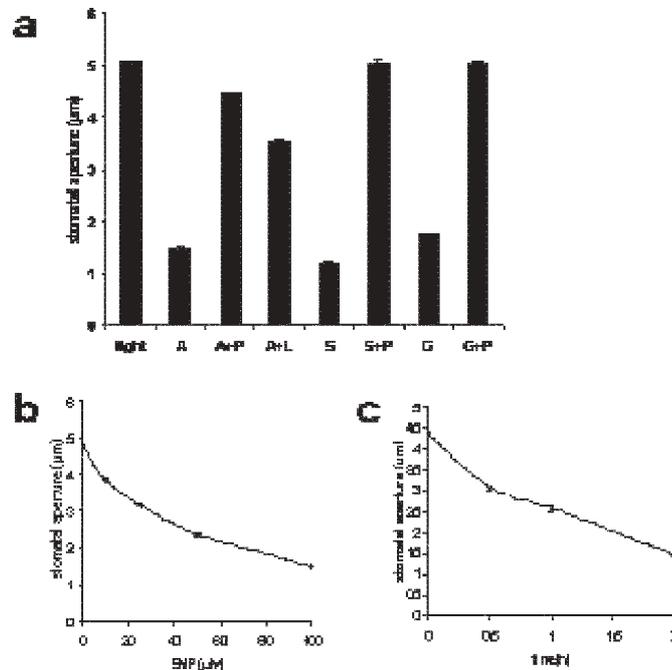


Fig. 1. Effects of ABA and NO on stomatal closure in pea. A, Epidermal peels, prepared from *Argenteum* pea, were incubated in the light in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.01 M MES-KOH, 0.05 M KCl, pH 6.15) to induce stomatal opening and then: incubated for 2 h in buffer alone (light), 10 µM ABA (A), ABA+200 µM PTIO (A+P), ABA+25 µM L-NAME (A+L), 100 µM SNP (S), SNP+200 µM PTIO (S+P), 500 µM GSNO (G), and GSNO+200 µM PTIO (G+P). B, Dose response for SNP, after incubation for 2 h. C, Kinetics of SNP-induced stomatal closure (100 µM SNP). Bars = SE (n=180). Reproduced with permission from the American Society of Plant Biologists.

diacetate), exposed to ABA and imaged using confocal microscopy. DAF-2DA is a dye that reacts specifically with NO and subsequently fluoresces (Kojima et al., 1998) and has been used to demonstrate NO generation in plant cells (Foissner et al., 2000). In untreated pea guard cells, little fluorescence was apparent in the cytoplasm of most cells, but there was some autofluorescence associated with the guard cell lips. ABA caused a rapid and striking increase in fluorescence in guard cells. Fluorescence was visible throughout the cytoplasm and in some cells particularly intense in the chloroplasts (Fig. 2). Co-incubation with the NO scavenger PTIO greatly reduced the ABA-induced fluorescence (Fig. 2), confirming that the fluorescence was due to increased amounts of cellular NO. Mata and Lamattina (2002) have confirmed the effects of ABA on guard cell NO synthesis in *V. faba*. These workers also used a NO-inactive dye 4-AF DA to confirm that the fluorescence was indeed due to NO. We

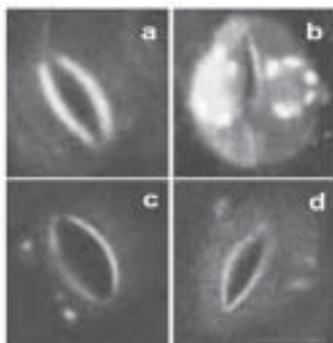


Fig. 2. ABA induces NO synthesis in *Argenteum* pea guard cells. Epidermal peels were floated in MES buffer in the light for 1 h and then loaded with DAF-2DA ($10\mu\text{M}$ in MES, 10 min in the dark, 20 min wash in MES). Following treatments, peels were observed with a laser confocal scanning microscope (Nikon PCM2000, Nikon Europe B.V. Badhoevedorp, The Netherlands; excitation 495 nm, emission 515–560 nm). Acquired images were processed using Adobe Photoshop (Adobe Systems, Mountain View, CA). Images are shown after a 30 min treatment. PTIO and L-NAME treatments reduced both the intensity and the number of guard cells visibly fluorescing; figure shows those cells in which fluorescence was still visible. a) Control (buffer only). b) $10\mu\text{M}$ ABA. c) ABA+ $200\mu\text{M}$ PTIO. d) ABA+ $25\mu\text{M}$ L-NAME. Reproduced with permission from the American Society of Plant Biologists.

have now extended our studies to show that ABA similarly induces NO synthesis in *Arabidopsis* (unpublished).

Biosynthesis of NO

As ABA induces NO synthesis very rapidly in guard cells, it is likely that ABA in some way activates the enzymes of NO synthesis (if synthesis is indeed enzyme-mediated). In mammalian cells, the biosynthetic origins of NO have been well-characterised. NO is generated from arginine by nitric oxide synthase (NOS), the activity of which can be inhibited by arginine analogues such as L-NAME (N-nitro-L-arginine methyl ester). However, the situation is undoubtedly more complex in plant cells. Several reports have provided biochemical and immunological evidence for NOS activity but no plant NOS protein or gene has yet been sequenced (Wendehenne et al., 2001; Neill et al., 2002c). Alternative sources of NO include NAD(P)H-dependent nitrate reductase (NR). NO synthesis by NR *in vitro*, using nitrite as a substrate, was originally demonstrated for soybean NR (Dean and Harper, 1988) and has subsequently been confirmed for other sources of NR (Yamasaki and Sakihama, 2000). *In vivo* synthesis of NO by NR has now been demonstrated (Rockel et al. 2002; Kaiser et al., 2002), and genetic evidence for the requirement for NR for NO synthesis provided by use of a NR-deficient mutant of *Chlamydomonas* (Sakihama et al., 2002). In addition to enzyme-mediated synthesis, NO can also be generated by the reduction of nitrite at low pH in the presence of ascorbate (Wojtaszek, 2000).

Treatment of pea epidermal peels with L-NAME partially inhibited ABA-induced stomatal closure (Fig. 1), suggesting that NO biosynthesis in pea guard cells might proceed via NOS. Such a suggestion is reinforced by the observation that the inhibitory effect of L-NAME on ABA-induced closure correlated with its inhibition of ABA-induced NO synthesis as visualized by DAF-2DA staining (Fig. 2). Interestingly, NOS-

like enzyme activity, inhibited by L-NAME, has been identified and partially purified from pea leaves (Barroso et al., 1999). The mechanisms by which ABA induces guard cell NO synthesis appear to differ between species. Although L-NAME inhibits ABA-induced stomatal closure and NO synthesis in pea, it has a greatly reduced effect in *Arabidopsis*, in which NR may be the generator of guard cell NO. ABA-induced stomatal closure and NO synthesis are both inhibited in *Arabidopsis* by treatment with the NR-inhibitor tungstate, which has no effect in pea. Moreover, the effects of L-NAME and tungstate on ABA-induced stomatal closure in *V. faba* and *Brassica nigra* are the same as in pea and *Arabidopsis* respectively, suggesting that there may indeed be real differences between species (unpublished data). Clearly, identification of the origins of NO in guard cells is a research priority.

ABA and NO signalling

If NO is a component of ABA signalling during stomatal closure, NO must induce at least a sub-set of the intracellular processes required to effect guard cell shrinkage. ABA induces closure via both calcium-dependent and independent pathways (Assmann and Shimazaki, 1999) but the effects of NO on guard cell calcium oscillations are not yet known. However, cyclic nucleotide-gated ion channels have been cloned from plants (Trewavas et al., 2002) and modulation of the activity of such channels may well be one mechanism by which NO effects stomatal closure.

NO signalling in mammalian cells typically involves activation of the enzyme guanylyl cyclase, with a transient increase in the concentration of the second messenger molecule cyclic GMP (cGMP; Neill et al., 2002c; Trewavas et al., 2002). Pharmacological and biochemical studies indicate that NO signalling in plants also involves cGMP. Inhibition of cGMP synthesis inhibited NO responses, and exposure to NO induced a transient increase in the endogenous cGMP content of tobacco cells (Durner et al., 1998). Here, in pea guard cells, inhibition of cGMP synthesis with the guanylyl cyclase inhibitor ODQ (1*H*-(1,2,4-oxadiazole-[4,3-*a*]quinoxalin-1-one) inhibited stomatal closure induced by either ABA or NO (Fig. 3). This inhibition could be relieved by co-incubation with a cell-permeable analogue of cGMP, 8-bromo-cGMP (Fig. 3), although 8-bromo-cGMP alone had no effect (data not shown), indicating that cGMP is required, but not sufficient, for ABA-induced stomatal closure. These data suggest that ABA activates the enzyme responsible for cGMP synthesis - thus it will be important to quantify cGMP in guard cells, and to isolate and characterize the enzymes that regulate cGMP turnover.

One downstream signalling response to NO and cGMP is intracellular generation of cADPR, a calcium-mobilising compound (Neill et al., 2002c). cADPR involvement in ABA responses has already been demonstrated (Wu et al., 1997; Leckie et al., 1998; MacRobbie, 2000). Treatment with nicotinamide, an antagonist of cADPR production,

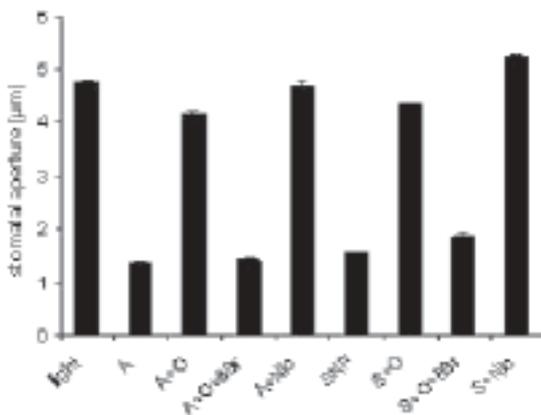


Fig. 3. ABA- and NO-induced stomatal closure requires cGMP and cADPR. Epidermal peels were incubated in the light to induce stomatal opening and then incubated for 2 h in the buffer alone (light), 10 µM ABA (A), ABA + 2 µM ODQ (A+O), ABA + ODQ + 50 µM 8Br-cGMP (A+O+8Br), ABA + 5 mM nicotinamide (A+Nic), SNP (100 µM), SNP + 2 µM ODQ (S+O), SNP + ODQ + 100 µM 8Br-cGMP (S+O+8Br), and SNP + 5 mM nicotinamide (S+Nic). Bars = SE (n=180). Reproduced with permission from the American Society of Plant Biologists.

inhibited the effects of both ABA and NO (Fig. 3), suggesting that NO does indeed activate the synthesis of cGMP and subsequently cADPR.

NO interaction with other signals

It is abundantly clear that guard cells are highly complex, and that the signalling mechanisms by which ABA exerts its effects represent a complicated web of inter-connecting processes, rather than a collection of parallel but separate pathways. Consequently, it is to be expected that NO is but one of several signals to which guard cells respond, and that NO will interact in various ways with other signals to fine-tune stomatal movements and metabolism. One example of an associated signal is H_2O_2 , another molecule generated in guard cells in response to ABA (see Neill et al., 2002a). NO and H_2O_2 appear to be made and to operate in tandem during plant defence responses as well as during stomatal responses to ABA (Neill et al., 2002a).

Conclusions

The elucidation of novel signalling pathways mediating water stress tolerance provides new opportunities to enhance WUE of plants. Consequently, the involvement of NO signalling during stomatal responses to ABA may provide new approaches to manipulating plant water relations to improve agricultural productivity. Advances in genomic and post-genomic technologies will facilitate further exploration of the mechanisms by which guard cells recognize and respond to signal molecules such as ABA and NO. It will be necessary to identify target molecules for NO as well as the downstream signal cascades by which such perception of NO is transduced into altered stomatal apertures. This information may then be used to develop plants better able to withstand reduced water supplies and to design chemicals that can modify WUE at key stages of the crop growth cycle.

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