Abscisic acid, nitric oxide and stomatal closure – is nitrate reductase one of the missing links?

Carlos García-Mata and Lorenzo Lamattina

Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, CC 1245, 7600 Mar del Plata, Argentina

Once plant endogenous nitric oxide (NO) production had been proved, NO research was directed toward both the source and the targets of this extremely bioactive molecule. As in mammals, plant NO was first thought to be generated mainly by a NO synthase-like enzymatic activity. However, nitrate reductase (NR)-dependent NO production is now receiving much of the attention because of the ubiquity of this enzyme in higher plant tissues and the precise regulation of its NO-production activity. NO has been reported to be a signal in many and diverse physiological processes, such as growth and biotic and abiotic stresses. Recently, NO has been shown to affect stomatal closure and interact with abscisic acid signaling pathways. We propose NR as a putative component in the signaling cascade of ABA-induced stomatal closure.

Unfavorable environmental conditions such as drought, salinity and low temperatures, limit crop productivity all around the world. These stresses trigger various biochemical and physiological responses, for example, water stress induces the accumulation of the plant hormone abscisic acid (ABA), which has multiple roles in the tolerance of plants to drought [1]. ABA accumulation in leaves elevates the cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in guard cells by inducing both Ca^{2+} influx from the extracellular space and Ca^{2+} release from internal stores. Elevation of $[Ca^{2+}]_i$ regulates the anion channel, resulting in a reduction of guard cell turgor and further closure of the stomatal pore [2]. In addition, it was recently reported that endogenous nitric oxide (NO) is a component of ABA-induced stomatal closure [3,4] (Fig. 1). It was suggested that ABA-mediated NO production in guard cells is generated via a nitric oxide synthase (NOS)-like activity in response to ABA [3].

NO is a bioactive molecule, first described to be a mammalian endothelial relaxing factor, but now recognized to be an active member in a myriad of physiological processes [5]. In mammals, NO is produced mainly by the enzyme NOS (EC 1.14.13.39), which catalyzes the conversion of L-arginine to L-citruline and NO in the presence of O_2. There are three different known NOS isoforms, two constitutive (neuronal [nNOS] and endothelial [eNOS]) and one inducible (iNOS) [6].

Corresponding author: Lorenzo Lamattina (lolama@mdp.edu.ar).

NOS-dependent NO production in plants

For the past decade, scientists have been trying to demonstrate the presence of a mammalian-type NOS in plants. Up until now, all evidence about NOS-like protein in plants was obtained by using two types of approaches: (1) the measurement of the passage from L-arginine to L-citruline, results that were also supported by the inhibition of the reaction with N^G-nitro-L-Arg-methyl ester (L-NAME), a mammalian NOS inhibitor [7] and (2) results obtained from immunoreactions of plant components with anti-mammalian NOS [8]. The evidence seems to be weak unless the protein purification and/or cloning of the corresponding gene could be accomplished. To date, neither cDNA, nor any protein with sequence similarity to a known NOS have been found in plants and no NOS-like gene was found in the recently sequenced *Arabidopsis thaliana* genome [9].

NOS-independent NO production in plants

More than a decade ago, it was reported that the *phaseolus* tribe of the leguminosae family was able to synthesize NO as a byproduct of NO_2^- decomposition in a reaction catalyzed by the constitutive enzyme nitrate reductase (NR; E.C. 1.6.6.1.) [10]. This enzyme is formed by two identical sub-units of ~100 kDa. Each monomer has two active sites connected by an internal electron transport pathway. Electrons are donated from NAD(P)H substrate to FAD, at one of the two active sites, and then passed through an electron chain transfer to a molybdene-molybdopterin domain (Mo-MPT) at the other active site. This results in the reduction of nitrate by Mo-MPT at the second active site [11]. Interestingly, both NOS and NR are members of the ferredoxin-$NADP^+$ reductase (FNR) family of flavoenzymes. Members of this family have a conserved structure in their FNR-like fragment [12], raising the question: could any NOS inhibitors be chemically functional to block NR activity under specific conditions?

Recently, both *in vitro* and *in vivo* NR-dependant NO production was also found in other plant species such as maize, sunflower and spinach [13,14]. In addition, it has been shown that NO production rates by NR *in vivo* not only are in the range of NOS activity reported in tobacco plants but also can exceed it by up to 100-fold under certain conditions [14]. Besides NR, there are other ways by which plants can generate endogenous NO. For example, under

 1360-1385/02/$ - see front matter PII: S1360-1385(02)00009-2

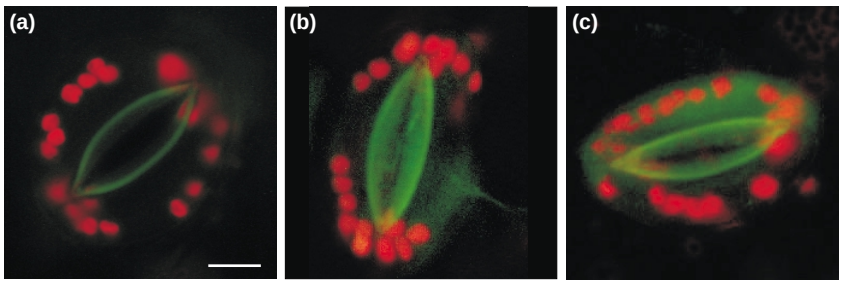

Fig. 1. Nitric oxide (NO) accumulation in *Vicia faba* guard cells. Exogenous addition of abscisic acid (ABA) to epidermal strips of *Pisum sativum* and *V. faba* induces an increase in the level of endogenous NO in guard cells, similar to levels observed when epidermal strips are treated with the specific NO donor sodium nitroprusside (SNP) [3,4]. Epidermal strips of *V. faba* were treated with: (a) opening buffer; (b) 150 μM of the NO donor SNP or (c) 10 μM abscisic acid; and then loaded for 30 min with the NO-specific fluorescent probe 4,5-diamino-fluorescein diacetate (DAF-2 DA). Green fluorescence (505–530 nm) corresponds to DAF-2 DA, red fluorescence corresponds to chlorophyll autofluorescence. Scale bars = 10 μm.

acidic conditions, NO can be produced from HNO_2 in the presence of a reductant such as ascorbate or glutathion [5]. NO can also be generated as a byproduct of chemical reactions between NO_x and plant metabolites [5].

Here we explore the available evidence supporting the involvement of NR as a link between NO and ABA-induced stomatal closure. Fig. 2a integrates some of the known second messengers that take part in the ABA signaling cascade leading to stomatal closure. We propose to introduce NR within this picture to link different results reported in the literature.

Some convergent evidence supporting NR as a link in NO-mediated ABA-induced stomatal closure

First line of evidence: NO production correlates with the NR activity in vivo and in vitro

Peter Rockel and colleagues [14] have recently presented evidence for the regulation of NO-production by plant NR. They showed that the rate of NO produced by NR is highly regulated by nitrite levels, and that nitrate acts as a competitive inhibitor. Under their experimental conditions, NO production was unaffected by NOS inhibitors but blocked by okadaic acid (OA), an inhibitor of NR activity [14,15].

In healthy plants, nitrite is rapidly converted to ammonium by nitrite reductase (NiR) in the chloroplast. Translocation of nitrite from the cytosol into the chloroplast requires a ΔpH across the envelope and the activity of the photosynthetic active transport mechanism at the thylakoid membranes. Therefore, nitrite accumulation followed by NO production could be detected when the redox potential generated by the chloroplast is altered. There are reports that show high NO emissions from plants when in the dark [16] or when treated with photosynthetic electron transport inhibitors [16,17]. In this sense, two facts must be considered when discussing the apparent contradiction between a high diurnal NR activity and a nocturnal stomatal closure: (1) a high and transient NO production has been detected in sunflower, tobacco and spinach leaves when the light was switched off [14] and (2) when epidermal *Vicia faba* strips were placed under dark conditions, stomatal closure could be partially prevented by treating them with the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [18]. These results also support the idea that NO has an active role during nocturnal induction of stomatal closure, in addition to NO-mediated stomatal closure in ABA-treated epidermal strips.

Second line of evidence: NAD(P)H mediates both ABA-induced stomatal closure and NR activity

Membrane hyperpolarization activates calcium-permeable channels, this process is strictly linked to the prevailing membrane voltage in such way that a slight variation beyond a narrow threshold is enough to trigger a $[Ca^{2+}]_i$ rise [19]. However, even though ABA induces a depolarization of the plasma membrane of guard cells (primarily via regulation of K^+ and Cl^- fluxes), it has been also proved to displace the membrane voltage threshold, inducing the activation of Ca^{2+} channels and a consequent rise in $[Ca^{2+}]_i$ [19,20]. In addition, ABA not only triggers a $[Ca^{2+}]_i$ rise, but it also strengthens the Ca^{2+} signal [19], which requires the synthesis of NAD(P)H and reactive oxygen species (ROS) [21]. The role of ROS as signal transducers in promoting stomatal closure is still under debate [22] because the specificity and side-effects of these compounds are almost completely unknown in plants.

It has been reported that ABA can activate Ca^{2+} channels in isolated membrane patches, indicating a close physical association between the site of ABA perception and the Ca^{2+} channel [20]. Interestingly, the presence of an NR-like plasma membrane-bound enzyme might also contribute to NO production [23]. Thus, these membrane patches could contain sites for: (1) ABA perception, (2) NO production and (3) Ca^{2+} channels. This putative tight association could allow a limited and rapid way to control an integrated physiological response of discrete plasma membrane patches in guard cells. However, there are two other relevant facts playing against the above described scenario: (1) No NR has been reported in guard cells to date. But, in a reasonable conceptual framework, NO could freely diffuse from its generation in mesophyll cells to vascular tissues and across epidermal cells to evoke biological responses. (2) In vascular smooth muscle, NO directly activates Ca^{2+}-dependent K^+ channels through the *S*-nitrosylation of a thiol-containing domain of the protein [24]. Given that none of them eliminates a putative function for NO and NR in stomatal movement, it would be useful to test whether the K^+ channels of guard cells are also sensitive to NO action and,

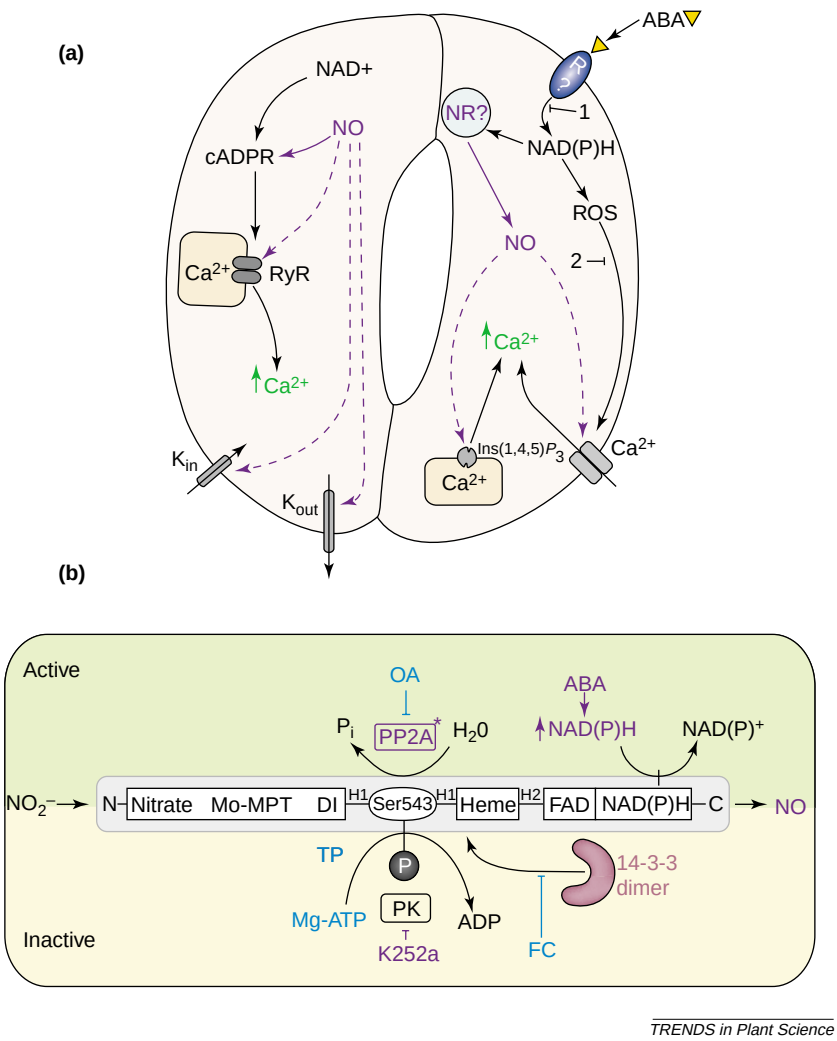

Fig. 2. Model of a link between the regulation of nitrate reductase activity and guard cell abscisic acid (ABA)-signalling cascade. Regulation models: (a) Model of ABA-induced stomatal closure. Purple broken arrows indicate nitric oxide (NO) effects previously reported in guard cells; purple unbroken arrows indicate effects of NO reported in different animal signaling pathways, but not yet described in plants. (b) Nitrate reductase (NR) and its cofactors. The gray box represents a NR protein with its eight sequence domains: (i) N-terminal; (ii) molybdene-molybdopterin (Mo-MPT); (iii) dimer interface (DI); (iv) Hinge 1 (H1) where the phosphorylated Ser543 is located; (v) Heme; (vi) Hinge 2 (H2); (vii) flavin adenine dinucleotide (FAD) binding, and (viii) NAD(P)H binding at the C-terminus [47]. The upper green half of the figure contains those components that raise NR activity and okadaic acid (OA), a protein phosphatase type-2 A inhibitor (PP2 A). The lower cream half contains those effectors that inactivate NR and K252a, a protein kinase (PK) inhibitor. Purple components denote elements that positively regulate the ABA-dependent stomatal closure (an asterisk indicates conditional). Blue components negatively regulate ABA-induced stomatal closure. Abbreviations: 1, *abi1–1*; 2, *abi2–1*; cADPR, cyclic ADP ribose; FC, fusicoccin; ⊤, inhibition; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; K252a, protein kinase inhibitor; NR, nitrate reductase; R, ABA receptor; ROS, reactive oxygen specie; RyR, ryanodine sensitive channels.

as a consequence, to rethink the targets for NO-mediated signaling events in stomatal guard cells.

Although NAD(P)H participation is poorly understood in ABA-mediated stomatal closure, it was recently proposed to be the linking point between guard cell metabolism and ion channel regulation [22]. NR significantly increases NO production, both *in vitro* and *in vivo*, after NAD(P)H addition in a nitrite-dependent reaction [13,14]. Therefore, we propose that in the guard cell ABA signaling cascade, a physiological role of NAD(P)H could be to activate NR-dependent NO production.

Nonetheless, one separate argument against NR and NO function comes from the description of cyclic ADP ribose (cADPR)-independent pathways that release Ca^{2+} from endoplasmic reticulum vesicles, which are activated by nicotinic acid adenine dinucleotide (NAADP) [25]. Thus, the NADP metabolite itself possesses the capacity to mobilize Ca^{2+} and modulate stomatal movement.

Third line of evidence: phosphorylation–dephosphorylation events regulate the ABA signaling cascade and NR activity

It is widely accepted that ABA-induced stomatal closure is regulated by phosphorylation–dephosphorylation events. Several protein kinases (PK) were characterized and found to mediate guard cell ABA responses. Among them: (1) a 57 kDa Ca^{2+}-dependent PK – a member of the calcium-dependent protein kinase (CDPK) family – which is a positive regulator of the KAT1 K^+ channels, and (2) two Ca^{2+}-independent PKs, a mitogen-activated PK (MAPK) called AMBPK and a non-MAPK called AAPK, which activates slow anion channels [21,26]. These studies

linking the regulation of PKs, protein phosphatases (PPs) and ion channels were described from pharmacological experiments but are poorly understood [27]. Dephosphorylation events are also important in ABA signaling during stomatal closure. *Arabidopsis* ABI1 and ABI2 genes encode homologous proteins that belong to the type-2C class of serine/threonine protein phosphatases (PP2C). The *abi1-1* and *abi2-1* mutants have a low responsiveness to ABA, which affects stomatal movement owing to their incapacity to activate membrane slow anion channels [28,29]. In addition, it was reported that another PP is also involved that is different from the PP2C, and which is OA sensitive [30,31]. Intriguingly, OA inhibition of this PP reduces ABA activation of slow anion channels during ABA-induced stomatal closure in *Arabidopsis*, but has the opposite effect in other plant species [29].

Phosphorylation events also play a key role in the regulation of NR activity. There are two types of protein kinases (PKs) found to phosphorylate NR, and one of them also belongs to the CDPK family [11]. Both PKs depend on sub-micromolar concentrations of Ca^{2+} for their activation, and both PKs target the same sequence motif that in most plant NRs flanks the phosphorylated Ser543 (Fig. 2b). NR phosphorylation seems to be necessary but not sufficient to inactivate the enzyme. Phospho-NR is the target of an inhibitor protein identified as a member of the 14-3-3 binding protein family that appears to block electron transfer to the Mo-MPT. Inactivation of phospho-NR requires the binding of 14-3-3 proteins in the presence of millimolar concentrations of divalent cations (Ca^{2+}, Mg^{2+} or Mn^{2+}) and is sensitive to ionic strength [17]. In addition, polyamines can substitute divalent cations and make the binding of the 14-3-3 to NR more efficient [32]. Interestingly, changes in either divalent cation concentrations or in ionic strength, affect both NR activation state and guard cell response [17,21]. Moreover, fusicoccin, a toxin produced by the fungus *Fusiccocum amygdali*, can bind to 14-3-3 and deregulates the activity of NR [11]. Fusicoccin was also reported to trigger the activation of a H^+-ATPase at the guard cell plasma membrane leading to an irreversible opening of the stomatal pore [33–35]. The inactive NR-phosphate is dephosphorylated by an OA-sensitive PP, which corresponds to the type-2 A group. Alternatively, NR could also be dephosphorylated, at least *in vitro*, by a Mg^{2+}-dependent PP [36], which is similar to the 2C-type PPs involved in *ab1-1* and *abi2-1* mutations. Moreover, the kinase inhibitor K252a not only prevents NR inactivation [37] but it is also able to reverse the *abi1-1* mutation [22].

Because protein phosphorylation–dephosphorylation and the transition from one state to another is a central feature of signal transduction pathways, it is clear that we are describing a highly pleiotropic mechanism that affects many cellular targets and physiological responses. For example, the voltage-dependent outward-rectifying K^+ channels are also targets for modulation by 14-3-3 proteins [38]. Therefore, even though we still have limited knowledge about the specific regulation of this relevant signaling pathway in guard cells, experimental results support the hypothesis presented here.

pH variations: another intriguing and controversial regulator of ABA signaling leading to stomatal closure and NR activation

Once ABA is synthesized and transported into leaves, it has been postulated that an increased sap pH enhances the ABA signal received at the guard cells [39]. Even though the effect of pH on stomatal closure has been extensively studied during the past decade, there are no precise data on the way it acts or on the targets.

Concerning apoplastic pH (pH_{ext}), it is widely accepted that a 0.2 unit increase in pH_{ext} is strong enough to close stomata in a whole turgid leaf. However, it was also reported that (1) acidic pH reduces stomatal aperture compared with the effect of a more alkaline pH treatment [40]. (2) In potato leaves, a 10^{-4} M ABA-treatment induces stomatal closure with a simultaneous and transient apoplastic alkalinization [41]. (3) In isolated epidermal strips, an increasing pH_{ext} induces stomatal aperture [39]. So, a unique response has yet to be established.

With regard to the internal pH (pH_i), ABA treatment of guard cells induces a 0.1–0.3 unit rise of pH_i [42]. ABA-stimulated pH_i changes account for the activation of those K^+ channels that give rise to the outward rectifying current ($I_{k,out}$). Evidence for a pH_i rise without observed changes in $[Ca^{2+}]_i$ indicate that this pH_i increase cannot be explained by a Ca^{2+}–H^+ exchange. The lowering of pH_i to values near 7.0 promotes a rise of $[Ca^{2+}]_i$, conversely, increasing $[Ca^{2+}]_i$ favors a pH_i alkalinization [42]. Consequently, it was proposed that pH_i and $[Ca^{2+}]_i$ do not interact reciprocally. Although the interaction between $[Ca^{2+}]_i$ and pH_i has been proved, the picture is not clearly understood yet.

In parallel, cytosolic pH has been also reported to modulate NR activity, but the mechanism is poorly understood. In most higher plants, cytosolic pH acidification seems to induce an activation of NR [36]. This observation comes mainly from measurements made on NR activation under anoxia or artificial tissue acidification [36]. Variations on NR activity can also be observed by slight pH variations [43]. All these observations relate to the nitrate reduction activity of NR. In addition, pH regulation of NR-dependent NO production activity (i.e. nitrite reduction activity) shows that the pH range at which NO production occurs is between 6.50 and 7.25, with an optimum pH of 6.75 [44]. It has also been reported that a 50% increase in NR-dependent NO production can be obtained by a 0.20–0.30 rise of pH, from 6.50 to 6.75, remaining almost constant up to pH 7 [44]. Thus, the 0.10–0.30 unit rise of pH_i observed after leaf ABA accumulation is compatible with the NO production activity by NR.

Slight changes of the cytosolic pH can profoundly affect the redox state of sensitive guard cell components. In this scenario, an interrelationship between the three possible redox forms of NO: nitrosonium cation (NO^+), nitric oxide radical ($NO^{\cdot}$) and nitroxyl anion (NO^-) would provide a means of modulating chemical signals of redox active biomolecules under the control of either thiol- or metal-functional groups that sense the changes in redox state within the guard cell and transduce it into physiological responses (Box 1). Thus, the interconversion of the various

Box 1. Nitric oxide chemistry

In plants, nitric oxide (NO) can be enzymatically generated from nitrite (NO_2^-) by the enzyme nitrate reductase (NR), or from L-arginine by a nitric oxide synthase (NOS)-like activity. The enzyme NR, present in all higher plant species, is activated by high concentrations of NO_2^- and requires NAD(P)H to generate NO + NAD(P) in the presence of the cofactors FAD and Mo-Molybdopterin [a] (Fig. I). NOS-like activity was reported *in vitro* and *in vivo*, but neither NOS protein nor cDNA has been purified yet in plants [b].

Although NO biochemistry focuses on the NO free radical ($NO^{\cdot}$), under physiological conditions $NO^{\cdot}$ can be interconverted to different redox forms with distinctive chemistries. $NO^{\cdot}$ is rapidly oxidized by the removal of one electron to give a nitrosonium cation (NO^+) or reduced by adding one electron to form a nitroxyl anion (NO^-), which are important intermediates in the chemistry of NO [c]. Interconversion of this redox form can be reached by charge transfer between $NO^{\cdot}$ electron acceptors, or by interaction between $NO^{\cdot}$ and transition metals that have stable oxidation states differing by one electron transfer. NO is special in that it reacts with both the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms of iron. Complexes of ferric iron with $NO^{\cdot}$ are called nitrosyl compounds and will nitrosate (add a NO^+ group) to other compounds while reducing the iron to the ferrous state [d]. There are many compounds that are reported to be susceptible to nitrosative attack. One-electron transfer processes such as this might provide a means, analogous to metal nitrosilation, to interconvert redox-related forms [c].

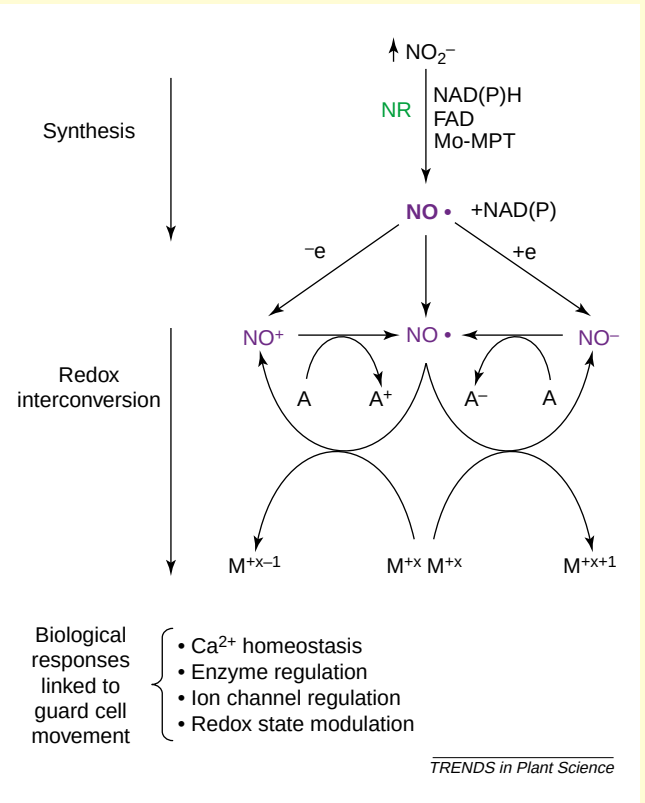

Fig. I. Enzymatic synthesis and redox chemistry of nitric oxide (NO) in plants. Nitrate reductase (NR) converts NO_2^- in the presence of NAD(P)H, FAD and molybdene-molybdopterin (Mo-MPT), to a radical form of NO ($NO^{\cdot}$). Two alternative pathways of interconvertion between the possible NO redox species NO^+ and NO^- are shown: (i) metal (M) nitrosil complexes and (ii) charge transfer to electron acceptor (A).

References

a Campbell, W.H. (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 277–303

b Beligni, M.V. and Lamattina, L. (1999) Nitric oxide in plants: the history is just beginning. *Plant Cell Environ.* 224, 267–278

c Stamler, J.S. *et al.* (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science* 258, 1898–1902

d Beckman, J.S. (1996) The physiological chemistry of nitric oxide. In *Nitric Oxide – Principles and Actions* (Lancaster, J., ed.), pp. 1–83, Academic Press

redox forms of NO could trigger different biological responses through specific interactions with different targets, as already described for hemoglobin [45]. Both NOS and hemoglobin posses a heme group that is a potential target for NO regulation. Given that NR also posses a heme group, it will be interesting to determine how different redox forms of NO can modulate NR activity.

Conclusions and future approaches

As described for many other signaling systems, the ABA-mediated stomatal response depends on coordinated interactions (either direct or indirect) between positive and negative regulators. Many cellular parameters are able to change and interplay in such a complex way that no single factor can be identified as being responsible for guard cell movement *in situ*. Moreover, the interplay of the different actors probably involves activation and simultaneous inhibition between themselves, allowing the rapid and characteristic oscillations needed for guard cell responses.

The available data show that NR possesses a NO-producing ability, assigning a role to NR in plant cells similar to that of NOS in mammalian cells. NR activity is highly regulated by transcriptional and post-translational mechanisms in response to many environmental conditions, including NO_3^- supply, light, temperature, CO_2 and O_2 availability, as well as changes in cytosolic pH [36]. Coincidentally, all these factors are implicated in the regulation of stomatal movement.

The inferred involvement of NR in the ABA signaling pathway highlights the complexity of the interaction between metabolism and signal transduction in guard cells. Thus, NR seems to be suitable to participate, not only as a key enzyme in the coordination of N and C assimilation, but also as an NO source in the signal transduction network of the ABA-induced stomatal closure.

Overall, stomatal movement is controlled by a tuned mechanism in which specific and redundant functions seem to converge in a wide range of inputs that are integrated into a small number of outputs [46]. This issue opens up one of the fundamental areas for future research in this field. Thus, an important challenge will be to differentiate between the specific and redundant functions operating in guard cells. In other words: are the stomatal control mechanisms triggered by NO (and ROS) the same mechanisms that mediate ABA effects? Do they share some common intermediates and/or downstream targets? Or, do the two processes operate in parallel? Experiments to discriminate between signal intermediates that operate in parallel versus in series are needed, for instance, to measure the NR activity and NO production in ABI1 and ABI2. Among crucial

experiments that could help to test the hypothesis presented here, are:

- Comparison of NO production by ABI1 and ABI2 mutants and their wild parents.
- Study of stomatal movement and Ca^{2+} channel activity in ABA-induced epidermal strips treated with NR inhibitors.
- Using affinity chromatography to test whether NR and 14-3-3 proteins co-purify in ABA-treated protoplast of ABI1 and wild-type guard cells.
- Localization of phosphorylated and dephosphorylated NR forms in guard cells using immunoassays.
- Transformation of *Arabidopsis* with NR–GFP fusion proteins and visualization of the subcellular localization pattern in both guard and mesophyll cells.

These and other biochemical, molecular and genetic approaches should confirm or refute the involvement of NR in stomatal movement.

Acknowledgements

This work was supported by Grants to L.L. by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 1–6496–99), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) (PIP 0898/98), Fundación Antorchas (14156-43) and Universidad Nacional de Mar del Plata (UNMdP) (EXA 177/00), Argentina.

References

1 Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.* 2, 48–54
2 Blatt, M.R. (2000) Ca^{2+} signalling and control of guard-cell volume in stomatal movements. *Curr. Opin. Plant Biol.* 3, 196–204
3 Neill, S.J. *et al.* (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. *Plant Phsysiol.* 128, 13–16
4 García-Mata, C. and Lamattina, L. (2002) Nitric oxide and abscisic acid cross talk in guard cells. *Plant Physiol.* 128, 790–792
5 Wendehenne, D. *et al.* (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.* 6, 177–183
6 Griffith, O.W. and Stuehr, J.F. (1995) Nitric oxide synthase: properties and catalytic mechanisms. *Annu. Rev. Physiol.* 57, 707–736
7 Barroso, J. *et al.* (1999) Localization of nitric-oxide synthase in plant peroxisomes. *J. Biol. Chem.* 274, 36729–36733
8 Ribeiro, E.A. Jr *et al.* (1998) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Lett.* 445, 283–286
9 The Arabidopsis Genome Initiative, (2000) Analysis of the genome sequence of the flowering plant. *Nature* 408, 796–815
10 Klepper, L.A. (1987) Nitric oxide emissions from soybean leaves during *in vivo* nitrate reductase assay. *Plant Physiol.* 85, 96–99
11 Campbell, W.H. (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 277–303
12 Correll, C.C. *et al.* (1993) Structural prototypes for an extended family of flavoproteins reductases: comparison of phthalate dioxygenase reductase with ferredoxin reductase and ferredoxin. *Protein Sci.* 2, 2112–2133
13 Yamasaki, H. *et al.* (1999) An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant Sci.* 4, 128–129
14 Rockel, P. *et al.* (2002) Regulation of nitric oxide (NO) by plant nitrate reductase *in vivo* and *in vitro*. *J. Exp. Bot.* 53, 103–110
15 Kaiser, W.M. *et al.* (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. *J. Exp. Bot.* 53, 875–882
16 Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* 468, 89–92
17 Huber, S.C. *et al.* (1996) Post-translational regulation of nitrate reductase activity: a role for Ca^{2+} and 14-3-3 proteins. *Trends Plant Sci.* 1, 432–437
18 García Mata, C. and Lamattina, L. (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiol.* 126, 1196–1204
19 Grabov, A. and Blatt, M.R. (1998) Membrane voltage initiates Ca^{2+} waves and potentiates Ca^{2+} increases with abscisic acid in stomatal guard cells. *Proc. Natl Acad. Sci. U. S. A.* 95, 4778–4783
20 Hamilton, D.W.A. *et al.* (2000) Ca^{2+} channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proc. Natl Acad. Sci. U. S. A.* 97, 4967–4972
21 Schroeder, J.I. *et al.* (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 627–658
22 Murata, Y. *et al.* (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatases. *Plant Cell* 13, 2513–2523
23 Stohr, C. *et al.* (2001) A plasma membrane bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* 212, 835–841
24 Bolotina, V.M. *et al.* (1994) Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368, 850–853
25 Navazio, L. *et al.* (2000) Calcium release from the endoplasmic reticulum of higher plants elicited by the NADP metabolite nicotinic acid adenine dinucleotide phosphate. *Proc. Natl Acad. Sci. U. S. A.* 97, 8693–8698
26 Li, J. *et al.* (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* 287, 300–303
27 Blatt, M.R. (2000) Cellular signaling and volume control in stomatal movements in plants. *Annu. Rev. Cell Dev. Biol.* 16, 221–241
28 Armstrong, F. *et al.* (1995) Sensitivity to abscisic acid of guard cell-K^+ channels is suppressed by *abi-1*, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. *Proc. Natl Acad. Sci. U. S. A.* 92, 9220–9224
29 Pei, Z.M. *et al.* (1997) Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and *abi1* and *abi2* mutants. *Plant Cell* 9, 409–423
30 Luan, S. *et al.* (1993) Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. *Proc. Natl Acad. Sci. U. S. A.* 90, 2202–2206
31 Thiel, G. and Blatt, M.R. (1994) Phosphatase antagonist okadaic acid steady-state K^+ current in guard cell of *Vicia faba*. *Plant J.* 5, 727–733
32 Provan, F. *et al.* (2000) Deletion of the nitrate reductase N-terminal domain still allows binding of 14-3-3 proteins but affects their inhibitory properties. *Plant Physiol.* 123, 757–764
33 Clint, G.M. and Blatt, M.R. (1989) Mechanism of fusicoccin action: evidence for concerted modulations of secondary K^+ transport in higher plant cell. *Planta* 178, 495–508
34 De Boer, B. (1997) Fusicoccin -a key to multiple 14-3-3 locks? *Trends Plant Sci.* 2, 60–65
35 Assmann, S.M. and Shwartz, A. (1992) Synergistic effect of light and fusicoccin on stomatal opening. *Plant Physiol.* 98, 1340–1355
36 Kaiser, W.M. *et al.* (1999) Nitrate reductase in higher plants: a case study for transduction of environmental stimuli into control of catalytic activity. *Physiol. Plant.* 105, 385–390
37 Kojima, M. *et al.* (1995) Phosphorylation/dephosphorylation of Komatsuna (*Brassica campestris*) leaf nitrate reductase *in vivo* and *in vitro* in response to environmental light conditions: effects of protein kinase and protein phosphatase inhibitors. *Physiol. Plant.* 93, 139–145
38 Booij, P.P. *et al.* (1999) 14-3-3 proteins double the number of outward-rectifying K^+ channels available for activation in tomato cells. *Plant J.* 20, 673–683
39 Wilkinson, S. and Davies, W.J. (1997) Xylem sap pH increases: a drought signal received at the apoplastic face of the guard cell that involves the suppression of saturable abscisic acid uptake by the epidermal symplast. *Plant Physiol.* 113, 559–573
40 Schwartz, A. *et al.* (1994) Inhibition of inward K^+ channels

and stomatal response by abscisic acid: An intracellular locus of phytohormone action. *Proc. Natl Acad. Sci. U. S. A.* 91, 4019–4023
41 Hedrich, R. *et al.* (2001) Changes in apoplastic PH and membrane potential in leaves in relation to stomatal responses to CO_2, malate, abscisic acid or interruption of water supply. *Planta* 213, 594–601
42 Blatt, M.R. and Armstrong, F. (1993) K^+ channels of stomatal guard cells: abscisic acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* 191, 330–341
43 Kandlbinder, A. *et al.* (2000) Nitrate reductases from leaves of Ricinus (*Ricinus communis* L.) and spinach (*Spinacea oleracea* L.) have different regulatory properties. *J. Exp. Bot.* 51, 1099–1105
44 Dean, J.V. and Harper, J.E. (1988) The conversion of nitrite to nitrogen oxide(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean. *Plant Physiol.* 88, 389–395
45 Doyle, M.P. *et al.* (1984) Structural effects in alkyl nitrite oxidation of human hemoglobin. *J. Biol. Chem.* 259, 80–87
46 Sanders, D. *et al.* (1999) Communicating with calcium. *Plant Cell* 11, 691–706
47 Campbell, W.H. (2001) Structure and function of eukaryotic NAD(P)H: nitrate reductase. *CMLS. Cell Mol. Plant Sci.* 58, 194–204