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$_2$O$_2$) 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$_2$O$_2$ 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$_2$O$_2$, whilst nitrate reductase has been identified as a source of NO in Arabidopsis guard cells. Some signalling components positioned downstream of H$_2$O$_2$ and NO are calcium, protein kinases and cyclic GMP. However, the exact interaction between the various signalling components in response to H$_2$O$_2$ 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$_2$O$_2$) and the reactive nitrogen species nitric oxide (NO) (Hetherington, 2001; Neill et al., 2003a, b). In this article, the role of H$_2$O$_2$ 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$_2$O$_2$ and NO as signalling molecules in plants

H$_2$O$_2$ and NO are forms of reactive oxygen and reactive nitrogen species, respectively, having wide-ranging effects...
et al. (2002). H₂O₂ and NO also activate common cellular responses such as elevation of cytosolic calcium levels, protein kinases and phosphatases (see below). The chemical nature of H₂O₂ 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 H₂O₂ and NO acting in parallel, together or synergistically, in various plant cells, particularly in guard cells, is clearly an important research priority.

### H₂O₂ as a signal in guard cells

The effects of H₂O₂ on guard cells were first reported by McAinsh et al. (1996). Exposure of *Vicia faba* guard cells to exogenous H₂O₂ induced elevations of cytosolic calcium and stomatal closure. At low levels (<10⁻⁵ M) H₂O₂ had no effect on membrane damage, whereas at higher concentrations this was apparent. The ability of guard cells to generate H₂O₂ has been demonstrated in tobacco, tomato and *Commelina* sp. (Allan and Fluhr, 1997; Lee et al., 1999). H₂O₂ synthesis in plant cells can easily be visualized using the cell-permeable fluorescent dye, dichlorofluorescein diacetate (H₂DCF-DA). Treatment of tobacco epidermal cells with the fungal elicitor cryptogein, induced H₂O₂ generation in guard cells and surrounding epidermal cells (Allan and Fluhr, 1997). In tomato and *Commelina*, Lee et al. (1999) observed H₂O₂ generation in response to an oligogalacturonide elicitor or chitosan, leading to stomatal closure. Treatment with catalase, a H₂O₂ scavenger, reduced both stomatal closure and H₂O₂ synthesis, confirming that the H₂O₂ was required to initiate stomatal closure. The discovery that ABA induces H₂O₂ 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 H₂O₂ production that resulted in stomatal closure. These findings have subsequently been reported in guard cells of *Vicia faba* (Zhang et al., 2001c), and observed in pea (R Desikan et al., unpublished data). Therefore, it appears that synthesis of H₂O₂ is essential for ABA-induced stomatal closure in various species.

H₂O₂ can be synthesized via several routes in plant cells (Neill et al., 2002b). Electron transport processes such as photosynthesis and respiration generate basal levels of H₂O₂, which increase in response to stress. Enzymatic sources of H₂O₂ include NADPH oxidase, cell wall peroxidases, amine oxidases, and other flavin containing enzymes (Neill et al., 2002b, c; Desikan et al., 2003). More than one source of H₂O₂ 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, H₂O₂ 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$_2$O$_2$-induced calcium channel activation, this implies a role for a NADPH oxidase-like enzyme mediating H$_2$O$_2$ synthesis in response to ABA in Arabidopsis guard cells (Pei et al., 2000; Murata et al., 2001). Zhang et al. (2001) proposed two different sources of H$_2$O$_2$ 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$_2$O$_2$ production and stomatal closure (R Desikan et al., unpublished data), also suggesting NADPH oxidase as a potential source of H$_2$O$_2$.

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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ in guard cells were soon followed by the discovery that NO is also an essential signal mediating ABA-induced stomatal closure (Neill et al., 2002a). Exogenous NO (applied as NO donors) induced stomatal closure and reduced transpiration in Vicia faba, Salpichroa argofolia 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., 2002a; SJ Neill, unpublished data), a process that is time- and dose-dependent, and fully reversible (Neill et al., 2002a).

The discovery that NO is a key signal mediating ABA responses in pea guard cells (Neill et al., 2002a), 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., 2002a). 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., 2002a, 2003a; 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., 2003b). 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-NNAME (L-nitro-L-arginine methyl ester), have provided some evidence for the presence of NOS in plants (Wendehenne et al., 2001; Neill et al., 2003b). In pea guard cells it has been observed that ABA-induced NO synthesis and stomatal closure can be partially inhibited by L-NNAME (Neill et al., 2002a), 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, H2O2 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, H2O2 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 H2O2 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 H2O2 (R Desikan et al., unpublished data). Treatment of pea epidermal peels with exogenous H2O2 alone also inhibits stomatal opening. Preliminary pharmacological data indicate that a MAPK pathway is involved in H2O2-induction of stomatal closure, but not in H2O2 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., 2003a). 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.

H2O2 and NO signalling and cross-talk in guard cells

It is clear that both H2O2 and NO regulate stomatal movements. However, there is little information on the cellular processes by which H2O2 and NO act in guard cells. An understanding of the signalling events that occur downstream of H2O2 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 H2O2-induced stomatal closure (R Desikan et al., unpublished data; Burnett et al., 2000). In previous work it was shown...
that H$_2$O$_2$ induces the activation of a MAPK in cell cultures, leaves and protoplasts of Arabidopsis (Neill et al., 2002b). In pea epidermis, H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ production in guard cells (Mustilli et al., 2002). It remains to be determined whether the ABA-activated kinase in pea also regulates H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ (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$_2$O$_2$ (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$_2$O$_2$ and NO actions in guard cells require calcium. H$_2$O$_2$ 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$_2$O$_2$ 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 shown that ABA and NO-induced stomatal closure can be inhibited with nicotinamide, an antagonist of cADPR synthesis (Neill et al., 2002a), 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$_2$O$_2$ also inhibits K$^+$ channel activity and induces cytosolic alkalisation in guard cells (Zhang et al., 2001a, b; Kohler et al., 2003). However, it is not known whether H$_2$O$_2$ 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., 2003b). 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., 2002a). Interestingly, H$_2$O$_2$-induced closure is not inhibited by ODQ (R Desikan, unpublished data), suggesting a branch in the ABA-H$_2$O$_2$/NO signalling pathway (Fig. 2).

From the findings described above, it is not yet clear whether signalling pathways unique to either H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ and NO play a central role in the guard cell ABA signalling network. Both H$_2$O$_2$ 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$_2$O$_2$ 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 wilt phenotype under normal growth conditions. Clearly, further evaluation of the mechanisms of synthesis of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ (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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ and NO in guard cells. Identification of downstream targets of H$_2$O$_2$ 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$_2$O$_2$ and NO in guard cells, for example, thiol-modified proteins, or newly synthesized proteins, and whether they are common or unique to H$_2$O$_2$ or NO. It has already been established that MAPKs, cGMP, cADPR, and protein phosphatases are likely targets downstream of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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.

References


