Guard cell abscisic acid signalling and engineering drought hardiness in plants

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Guard cells are located in the epidermis of plant leaves, and in pairs surround stomatal pores. These control both the influx of CO_2 as a raw material for photosynthesis and water loss from plants through transpiration to the atmosphere. Guard cells have become a highly developed system for dissecting early signal transduction mechanisms in plants. In response to drought, plants synthesize the hormone abscisic acid, which triggers closing of stomata, thus reducing water loss. Recently, central regulators of guard cell abscisic acid signalling have been discovered. The molecular understanding of the guard cell signal transduction network opens possibilities for engineering stomatal responses to control CO_2 intake and plant water loss.

Desiccation of crops and horticultural plants during periods of drought causes severe and often irreversible damage and lost yields. Fresh water scarcity has been identified as one of the principal global problems in the new century[1], and plants account for around 65% of global fresh water use. Engineering of the stomatal guard cell signal transduction network could provide a major contribution to more sustainable water use during droughts.

Plants are rooted in one place and therefore need to respond effectively to environmental stresses. Intricate signalling pathways form networks that regulate plant responses to the environment. However, many of the principal signalling components and their interactions remain largely unknown.

Guard cell signalling integrates water status, hormonal stimuli, light, CO_2 levels and other environmental conditions to regulate stomatal apertures. Guard cells are a well-developed model system for understanding how components interact within a signalling network in a single cell. They are well suited for dissecting the functions of genes and proteins in signalling cascades. Consequently, powerful techniques have been developed for guard cell signalling studies, enabling combined molecular genetic, cell biological, biophysical and proteomic and genomic analyses.

Guard cells respond at the single cell level to physiological stimulation, allowing cell biological analyses in response to diverse stimuli. Guard cells respond to most of the classical plant hormones, which illustrates that unidentified receptors and early signalling mechanisms function in these cells.

A network of ion channels in the plasma membrane and the vacuolar membrane of guard cells, which controls stomatal movements, has been characterized (see Box 1). These ion channels are targets of early signalling branches and provide probes to identify and characterize upstream transducers.

Abscisic acid (ABA)-induced stomatal closing is mediated by a reduction in the turgour pressure of guard cells, which requires an efflux of potassium and anions, sucrose removal and the conversion of malate to osmotically inactive starch[2]. Box 1 shows an extension of early models for the proposed functions of ion channels during ABA-induced stomatal closing[3,4].

Reconstitution in *Xenopus* oocytes

Cloning of an ABA receptor has remained a goal of plant cell signalling research and plant stress tolerance engineering. Heterologous expression systems, such as *Xenopus laevis* oocytes, have been used to clone animal cell receptors, and this technique has been used in attempts to identify ABA receptors and transduction mechanisms[5,6]. Simultaneous injection of guard-cell-derived poly(A^+) RNA with RNA of the *Arabidopsis* inward-rectifying K^+ channel *KAT1* reconstitutes ABA-induced downregulation of KAT1-mediated K^+_{in} channel currents (Box 1a) in oocytes[6].

ABA causes activation of endogenous Ca^{2+}-activated Cl^- currents in oocytes injected with messenger RNA from drought-stressed tobacco leaves[5]. By fractionation of the complementary DNA library, *Nt-Syr1*, a homologue of human and yeast syntaxins, was isolated. However, *Nt-Syr1* expression in oocytes constitutively activates Cl^- currents in oocytes without ABA addition[5]. Further work is required to determine whether *Nt-Syr1* is actually involved in the heterologous ABA response in oocytes.

Syntaxins and SNARE proteins have an integral function in vesicle trafficking in animal, yeast and plant cells. In tobacco guard cells, ABA regulation of slow-activating sustained (S-type) anion, K^+_{out} and K^+_{in} channels (Box 1a) is inhibited by microinjection of truncated Nt-Syr1 or injection of the neurotoxin *Clostridium botulinum C*, which inhibits syntaxin-dependent vesicle trafficking[5]. During stomatal closing, the plasma membrane surface area of guard cells decreases to accommodate volume reductions[7], which may require syntaxins. On the basis of the guard cell ion channel responses, syntaxins were proposed to function not only in membrane vesicle trafficking, but also in ABA signalling[5] (Fig. 1). These studies show functional expression of ABA perception and transduction mechanisms in oocytes, which may therefore be suitable for identifying ABA receptors.

Plasma membrane Ca^{2+} channels

Calcium influx and cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) increases are important for guard cell ABA signal transduction (Box 1)[4,8,9]. Impairment of ABA-induced $[Ca^{2+}]_{cyt}$ increases in the ABA-insensitive *Arabidopsis* mutants *abi1-1* and *abi2-1* provides genetic evidence for the importance of $[Ca^{2+}]_{cyt}$ elevations in ABA signalling[10] (Fig. 1). In *Vicia* guard cells, Ca^{2+} influx is enhanced when the membrane potential is hyperpolarized below about −120 mV[11,12], but ABA shifts the threshold for influx to potentials above −80 mV, which suggests that voltage-dependent Ca^{2+} influx and ABA signalling are closely linked[12]. Moreover, the probability of ABA-induced $[Ca^{2+}]_{cyt}$ elevations is increased if guard cells are maintained in hyperpolarizing conditions[12–14].

The upstream mechanisms by which ABA activates guard cell plasma membrane Ca^{2+} channels had remained unknown. Reactive oxygen species (ROS) were recently identified as an activator of hyperpolarization-activated Ca^{2+}-permeable channels (I_{Ca}) in *Arabidopsis* guard cells[15]. ROS and I_{Ca} were established as com-

ponents contributing to early ABA signalling because ABA causes a rapid increase in ROS and I_{Ca} activation in guard cells[15], whereas ROS-induced stomatal closure and I_{Ca} activation are abolished in the ABA-insensitive recessive mutant *gca2* (refs 15, 16) (Fig. 1).

A hyperpolarization-activated channel similar to I_{Ca} was identified in *Vicia* guard cells[17]. This channel shows bursts of increased activity in response to cytoplasmic ABA in isolated membrane patches[17]. ABA activation of these Ca^{2+} channels shows repetitive transients and rapidly desensitizes, suggesting that a second messenger might be generated locally at the plasma membrane in response to ABA. The Ca^{2+} influx channel is itself downregulated by $[Ca^{2+}]_{cyt}$ elevation[17], which may be part of a regulating system that generates complex ABA-induced Ca^{2+}oscillations (Fig. 2).

I_{Ca} channels are activated by ROS[15] and so could act as a focal point for integrating other, non-ABA, stress signals and the oxidative state of guard cells. For example, pathogenic elicitors cause ROS production in guard cells and stomatal pore closure[18]. Furthermore, ozone, which causes oxidative stress, downregulates K^+_{in} channels and inhibits stomatal opening[19], raising the possibility that ozone regulates I_{Ca}-type channels.

Ca^{2+} release and oscillations

In *Commelina* guard cells, plasma membrane Ca^{2+} influx is more strongly activated at high ABA concentrations, whereas at low ABA concentrations the InsP3 and cADPR pathways (see Box 1) predominate[20,21]. Combined application of the phospholipase C (PLC) inhibitor U-73122 and the cADPR inhibitor nicotinamide inhibit ABA-induced K^+ efflux and stomatal closing[21,22], whereas each inhibitor alone produces only weak inhibition[13,21,22], suggesting that these two Ca^{2+}-release signalling pathways operate in parallel during ABA signalling.

Cytosolic Ca^{2+} oscillations (Fig. 2) have been proposed as necessary for stomatal closure[9]. The four stomatal closing stimuli ABA, ROS, low temperature and external Ca^{2+} treatment produce Ca^{2+} oscillations in wild-type *Arabidopsis* guard cells[13,23] (Fig. 2). In contrast, in the *Arabidopsis* V-type H^+-ATPase mutant *det3*, ROS and external Ca^{2+} generate prolonged $[Ca^{2+}]_{cyt}$ elevations (plateaux) in guard cells, and long-term stomatal closure by these stimuli is inhibited[23]. Experimentally re-establishing external Ca^{2+}-induced Ca^{2+} oscillations in *det3* guard cells by repetitive hyperpolarizations rescues stomatal closure in *det3*. Furthermore, stomatal closure is abolished in wild-type guard cells when non-oscillating Ca^{2+} plateaux are experimentally imposed[23]. Overall, these results suggest that Ca^{2+} oscillations encode information required for stomatal closure. New techniques that combine Ca^{2+} indicators (Fig. 2) and molecular genetics in *Arabidopsis* guard cells[10,14,15,23] provide a powerful combination for future analyses of $[Ca^{2+}]_{cyt}$ oscillation generation and downstream processing in plants.

More lipid-derived second messengers

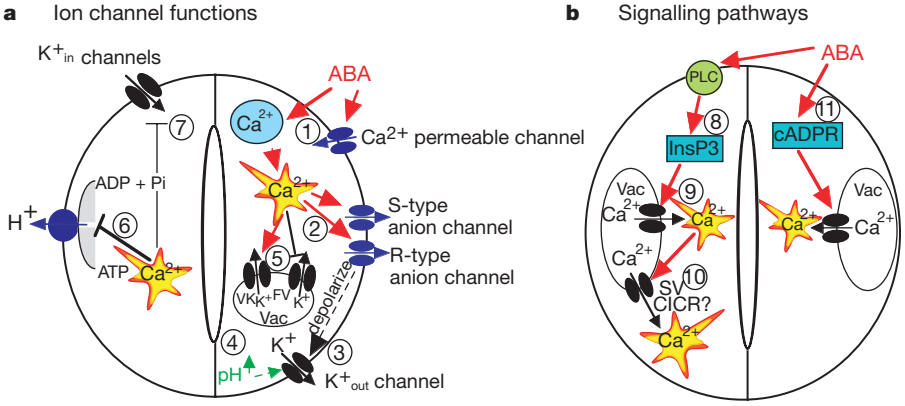
ABA stimulates production of another inositol-phosphate second messenger, myo-inositol-hexakisphosphate (InsP6), to a greater extent than InsP3 in guard cells[24] (Fig. 1). InsP6 inhibits K^+_{in} channels in a Ca^{2+}-dependent manner, and more efficiently than InsP3 (ref. 24). It was hypothesized that microinjection of InsP3 functions by affecting the InsP6 pathway[24]. Whether InsP6 causes $[Ca^{2+}]_{cyt}$ elevations and whether these two messengers function in the same or separate signalling branches is unknown.

Another phospholipase acts in ABA signalling. The level of phosphatidic acid (PtdOH) generated by phospholipase D (PLD) transiently increases after ABA treatment of aleurone cells and guard cells[22]. PtdOH promotes partial (50%) stomatal closure, inhibits stomatal opening and partially inactivates K^+_{in} channel currents. PtdOH does not elicit a $[Ca^{2+}]_{cyt}$ increase in guard cells[22] and PLD is therefore likely to act downstream of or parallel to ABA-induced $[Ca^{2+}]_{cyt}$ elevations (Fig. 1).

Box 1
Components and pathways of guard cell ABA signalling

a, ABA is detected by as yet unidentified receptors (right guard cell) and induces cytosolic Ca^{2+} elevations (1) through extracellular Ca^{2+} influx and release from intracellular stores (for reviews see refs 2, 8, 9). (2) $[Ca^{2+}]_{cyt}$ elevations activate two types of anion channel that mediate anion release from guard cells, slow-activating sustained (S-type) or rapid transient (R-type) anion channels[3,8]. (3) Anion efflux causes depolarization, which activates outward-rectifying K^+ (K^+_{out}) channels and results in K^+ efflux from guard cells[2,3]. (4) ABA causes an alkalization of the guard cell cytosol, which enhances K^+_{out} channel activity[44]. Overall, the long-term efflux of both anions and K^+ from guard cells contributes to the loss of guard cell turgor, leading to stomatal closing[3]. Over 90% of the ions released from the cell during stomatal closing must be first released from vacuoles into the cytosol. (5) At the vacuole, $[Ca^{2+}]_{cyt}$ elevation activates vacuolar K^+ (VK) channels, which are thought to mediate Ca^{2+}-induced K^+ release from the vacuole[8]. In addition, fast vacuolar (FV) channels can mediate K^+ efflux from guard cell vacuoles at resting $[Ca^{2+}]_{cyt}$[45]. ABA also inhibits ion uptake, which is required for stomatal opening (left guard cell). (6) $[Ca^{2+}]_{cyt}$ elevations inhibit the electrogenic plasma membrane proton-extruding H^+-ATPases[46] and K^+ uptake (K^+_{in}) channels[2,3,44] (7). Initiation of ion efflux (1–5, right guard cell) and inhibition of stomatal opening processes (6, 7, left guard cell) provide a mechanistic basis for ABA-induced stomatal closing[8].

b, Ca^{2+}-releasing second messengers that mediate ABA-induced stomatal closing. Second messengers have been implicated in ABA signalling in guard cells, including InsP3 and cADPR. (8) Biochemical evidence indicates that ABA stimulates InsP3 production in guard cells[2]. (9) InsP3 can release Ca^{2+} from intracellular stores, inhibit K^+_{in} channels and cause stomatal closure[9,13,22,44]. (10) $[Ca^{2+}]_{cyt}$ elevations may be amplified by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism from the vacuole through activation of the Ca^{2+}-dependent slow vacuolar (SV) channel[8,45]. Data questioning this SV model for CICR[47] and recent other data supporting it[48] indicate the potential for molecular genetic analyses. (11) ABA stimulates the production of cADPR in plant cells[49], which also increases $[Ca^{2+}]_{cyt}$ in guard cells[20,45] and promotes stomatal closing.



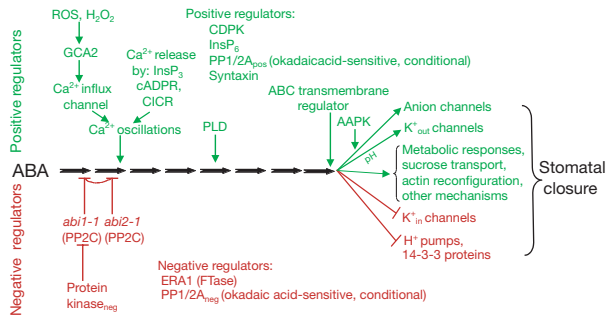


Figure 1 Simplified model for proposed functions of positive and negative regulators mediating guard cell ABA signal transduction. Positive ABA-activated regulators (top, in green) and negative ABA-signalling regulators (bottom, in red) in guard cells are shown. The sequence of events among regulators remains largely unknown and will require in-depth analyses. Positive and negative regulators for which the position relative to cytosolic Ca^{2+} elevations remains to be determined are listed alphabetically. Some parallel signalling branches are excluded for simplicity.

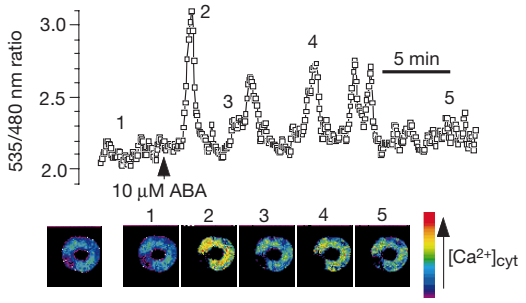


Figure 2 ABA-induced $[Ca^{2+}]_{cyt}$ oscillations in *Arabidopsis* guard cells expressing yellow cameleon 2.1. $[Ca^{2+}]_{cyt}$ oscillations elicited by ABA are indicated by an increase in the fluorescence emission ratio 535/480 nm[14]. Bottom, pseudo-coloured ratio images of cameleon fluorescence in a stomate after ABA application. The trace is from one of the imaged guard cells; images are shown at the points marked.

Protein kinases and phosphatases

Phosphorylation events are central mediators of ABA signalling in guard cells[25–31]. Some of the underlying protein kinase[31] and phosphatase genes[16,32] have been cloned. Both positively and negatively regulating protein kinases, and positively and negatively regulating PP2C- and PP1/2A-type protein phosphatases (Fig. 1, PP2Cs, $PP1/2A_{pos}$ and $PP1/PP2A_{neg}$) have been proposed to function in ABA signalling (for details see refs 10, 16, 29, 32). ABA signalling can be transduced by several types of positively regulating protein kinases[31,33,34]. For example, biochemical analyses have led to the hypothesis that Ca^{2+}-dependent protein kinases (CDPKs) may function upstream of ABA-activated Ca^{2+}-independent kinases in maize protoplasts and *Vicia* guard cells[28,34].

In-gel phosphorylation assays showed that in guard cells ABA rapidly activates a Ca^{2+}-independent protein kinase[27,28] with a relative molecular mass of 48,000. A corresponding guard-cell-specific protein kinase, AAPK, was isolated by two-dimensional gel separation and mass spectrometry[31]. Transient transformation of guard cells with a dominant mutant form of AAPK with reduced activity impairs ABA activation of S-type anion channels and disrupts ABA-induced stomatal closing[31] (Fig. 1).

With one exception[30], dominant ABA-insensitive mutations in the known protein kinase and phosphatase genes *ABI1*, *ABI2* and *AAPK*, have been analysed in guard-cell signalling[25,29,31]. It will be important to isolate recessive loss-of-function mutations in these enzymes[16,30] to test the proposed functions of the wild-type proteins (Fig. 1). Scaffolding mechanisms may hold the key for target specificity of the many proposed protein kinases and phosphatases (Fig. 1).

Scaffolding mechanisms and signalling complexes

In yeast and animal cells proteins are clustered into signalling complexes or 'transducisomes', which provide a structural basis for specificity in signalling[35]. Little is known about signal transduction complexes in plants, although more than ten genes in the *Arabidopsis* genome encode for 14-3-3 proteins, which function as scaffolding proteins by linking protein kinases to their targets or by mediating protein–protein interactions[36]. 14-3-3 proteins have a direct function in blue-light-induced protein kinase activation of guard cell plasma membrane H^+-ATPases, by controlling phosphorylation of serine and threonine residues in the carboxy-terminus of the ATPases[37].

There must also be additional mechanisms for forming signalling complexes in guard cells. Pharmacological studies have suggested that an ATP-binding-cassette transmembrane regulator (ABC protein) may form a junction point in guard cell ABA signalling, thus simultaneously regulating S-type anion channels and K^+_{out} channels[38] (Fig. 1). Inhibitors of sulphonylurea receptor (SUR) ABC proteins, such as glibenclamide, inhibit both S-type anion channels and K^+_{out} channels, and abolish stomatal closure triggered by ABA or external Ca^{2+}. The SUR activator cromakalin reverses the glibenclamide inhibition of these channels[38,39].

Several soluble signalling proteins have been proposed to regulate guard cell ion channels (Fig. 1). Farnesyltransferases mediate membrane targeting of specific soluble signalling proteins by attachment of a hydrophobic farnesyl group to C-terminal target sequences. The ERA1 farnesyltransferase β-subunit is a negative regulator of ABA signal transduction in seeds[40] and guard cells[41], which suggests farnesylation-dependent membrane targeting of soluble, negative regulators of ABA signalling (Fig. 1). Future proteomic-based research[31,42] should provide a more complete molecular characterization of transducisomes in guard cells.

Molecular engineering

It would be beneficial to agriculture for crop plants to show wide stomatal opening for CO_2 intake when water is available, but to close stomata during drought periods, thereby slowing desiccation and damage. However, conventional high-yield breeding approaches may have contributed to selection of crop plants with reduced stomatal ABA responsiveness, because genes controlling guard cell signalling are also expressed in other tissues and control other yield parameters (for example, refs 15, 16, 29, 32, 40, 41).

Studies on stomatal ion channel regulation have identified mechanisms that enhance ABA-induced stomatal closing. Deletion of the *ERA1* gene causes ABA hypersensitivity of both anion channel activation and stomatal closing, and during drought treatment *era1* plants show reduced wilting and enhanced stomatal closure[41]. Notably, when *era1* plants are watered the stomatal pores open, although to a lesser extent than in the wild type, allowing CO_2 influx and growth. Intragenic suppressor mutants of *abi1-1* (*abi1-1R*) also show reduced water loss during drought[30]; however, *era1* and *abi1-1R* alleles have additional growth and developmental phenotypes.

Based on the model in Fig. 1, downregulation of negative regulators (such as ERA1) or upregulation of positive regulators specifically in guard cells using guard-cell-specific[43] and stress-responsive promoters, or isolation of guard cell specific mutants, will allow engineering of plants that lose less water during drought. Some mutations may constitutively reduce stomatal opening, which could reduce the water requirements of horticultural plants and turf grass, or in agricultural regions of marginal fresh water availability[1].

For plants growing in humid regions, weakening of ABA signal transduction components (Fig. 1) may enhance stomatal opening and CO_2 intake for carbon fixation and growth[31].

Targeted mutations that only affect stomatal movements will also provide powerful tools to quantitatively analyse photosynthetic carbon fixation, gas exchange and plant growth as a direct function of specific alteration of stomatal apertures. Such work, together with engineering of leaf waxiness and osmoprotectant production, may also contribute to future molecular engineering of improved plant survival during droughts.

Recent efforts have led to major advances in understanding the molecular mechanisms of guard cell ABA signalling. A model for the functions of ion channels in regulating stomatal movements (Box 1) has provided a framework for the characterization of new, early ABA transduction mechanisms (Fig. 1). However, many components within the nonlinear and branched pathways of this network remain to be identified. Moreover, the relative positioning of the mechanisms identified and the exact functions of pharmacologically predicted transducers remain to be determined using combined genetic, cell biological and biophysical approaches. Given the large gene families, gene duplications and redundancies in the *Arabidopsis* genome, it will be important to identify guard-cell-expressed isoforms of these transducers using DNA microarrays, and to use guard cell promoters[43] to cell-specifically manipulate redundant, and often widely expressed, genes. Guard cell and other single cell analyses of signal transduction are moving towards achieving a time-resolved understanding of an entire plant cell signalling network. Postgenomic and proteomic approaches will be essential for future advances. The new insights into guard cell and ABA signalling, combined with interdisciplinary approaches, will lead to future revelations in this field that will be relevant to plant signal transduction, environmental ecology and agriculture. □

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