Chapter 22

Systems Biology of Abiotic Stress: The Elephant and the Blind Men

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Summary

The study of plant stress responses is fragmented into many separate domains by the type of stress used, the response measured, and the disciplinary perspective of the investigation. Thus, light, salt, drought, and ozone stress are commonly investigated separately. End-point measures vary from changes in stomatal aperture and cellular electrophysiology to signaling and transcriptional changes and, ultimately, cell death. Furthermore, investigations of the same system and end-point are often carried out from very different disciplinary perspectives, such as signaling, cell structure, or expression of genes and protein trafficking. In the present review, I use the old Hindu parable of the elephant and the blind men as a metaphor for the study of the stress response. Taking the well-studied environmental response of guard cells, I first assemble a "parts" list, briefly overviewing the literature on signaling, cytoskeletal changes and vesicular trafficking that mediate changes in the stomatal aperture. I then attempt to synthesize a "systems" view of stomatal responses by integrating the various perspectives. Finally, I briefly explore the relevance of guard cell responses to the more general issues of plant stress responses and comment on future areas of interest.

Keywords: cytoskeletal restructuring • reactive oxygen species • systemic acquired resistance • system biology • vericular rafficking

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I Introduction

The plant stress response literature is replete with titles asserting that a protein or a particular compound "regulates" a "signaling pathway" that activates the plant's response to a specific stress of many abiotic stressors (commonly salt, drought, cold, heat, excess light or ozone) or to a biotic stressor (in the form of a particular pathogen – bacterial, viral or fungal). The underlying concept is that there exists a specific set of interacting molecules, primarily proteins, that receives each stress signal somewhere – we often vaguely think that this must be at the cell surface – and transmits it to someplace else commonly a target gene or genes - through a series of intermediaries to give a specific response unique to the stressor. We endeavor to place a given protein "upstream" or "downstream" of another in this signaling pathway on the assumption that the pathway is somehow quite uncomplicated, linear and yes, hierarchical – else what would be the sense of asserting that some component "regulates" the "pathway"? If things don't fit neatly into a linear pathway, we postulate that a given intermediate acts both "upstream" and "downstream".

Yet it is increasingly clear that abiotic stressors, such as excess light, salt and ozone, activate the expression of many of the same genes that were identified and viewed as specific responses to infection by pathogens (Mullineaux et al. 2000; Mahalingam et al. 2005; Tosti et al. 2006). So we report the "crosstalk" between signaling pathways, as if these independent, linear pathways – wires, as it were – occasionally got crossed and the signal leaked from one to activate another – not unlike someone else's telephone conversation intruding into yours. More recently, the concept of "integrators" has entered literature, suggesting somewhat more explicit points of reconciliation amongst what we still conceptualize as independent, even parallel, linear signaling pathways.

At the cellular level, many of the same ions and small-molecules, ranging from calcium, ROS, nitric oxide and small lipids to salicylic acid, ethylene, ABA and jasmonic acid, are centrally involved in the responses of plant cells to a variety of stresses and pathogens (Howe and Schilmiller 2002; Laxalt and Munnik 2002; Wiermer et al. 2005; Gechev et al. 2006; Grun et al. 2006; Wang 2006). Calcium ions have long been called "second messengers", but reactive oxygen, nitrogen and sulfur species are also increasingly receiving this designation. Evidence is also rapidly accumulating that hormones can interact directly with multiple proteins, commonly inside the cells, to directly influence their biochemical functions, often without the intervention of second messengers (Dharmasiri et al. 2003, 2005; Razem et al. 2006; Shen et al. 2006). We still designate these targets as "hormone receptors," but the conventional separation of signal receiver from its transmitter and its target may not exist for some small molecules, traditionally regarded as hormones. Moreover, while some hormone receptors are located in the plasma membrane, others are in internal membranes, such as the endoplasmic reticulum (ER) (Wang et al. 2001b; Chen et al. 2002). Inactive conjugates and esters of hormones were identified some time ago, but evidence that these constitute physiologically important, rapidly mobilizable internal stores is rather recent (Lee et al. 2006).

Responses of plants to both abiotic stressors and pathogens are complicated in time and space. It was first reported more than a decade ago that plants respond to a variety of insults, both biotic and abiotic, themselves by producing a biphasic "oxidative burst," with a first transient increase in ROS production commencing within minutes of an insult and another some hours later (Levine et al. 1994; Lamb and Dixon 1997). Rapid transient increases in the cytoplasmic concentration of free calcium ions [$(Ca^{+2})_{cyt}$] have been known for some time (Trewavas and Gilroy 1991; MacRobbie 1993; Trewavas and Knight 1994), but recent work utilizing new kinds of cellular calcium

Abbreviations: αβγ heterotrimeric G-protein subunits; ABA abscisic acid; $(Ca^{2+})_{cyt}$ cytosolic calcium concentration; cADPR cyclic ADP ribose; DAG diacylglycerol; ER endoplasmic reticulum; GCR_2 G-protein coupled receptor-2; GFP green fluorescence protein; GPCR G-protein coupled receptor; GSH glutathione; HRhypersensitive response; IP_3 inositol-1,4,5-triphosphate; KATI-GFP K^+ channel-green fluorescent protein; PA phosphatidic acid; PIphosphoinositide; PI_3P phosphoinositide-3-phosphate; PI_4P phosphoinositide-4-phosphate; $PI_{4,5}P_2$phosphoinositide-4,5-bisphosphate; PIP_2 phosphoinositide-4,5-bisphosphate; PIP_3 phosphatidylinositol-3,4,5-triphosphate; PLCphospholipase C; PLDphospholipase D; RAFL RIKEN *Arabidopsis* full-length cDNA; ROS reactive oxygen species; SAA systemic acquired acclimation; SAR systemic acquired resistance

sensors has revealed that calcium signaling has a previously unappreciated temporal and spatial complexity (Allen et al., 1999, 2001; Wood et al., 2000, 2001; Lecourieux et al. 2006; Xiong et al. 2006). The transcriptional response to stress stimuli is also temporally complex, with a regular progression of changes in the expression levels of genes extending from minutes to many hours (Mahalingam et al. 2005). Moreover, signals originating within cells are communicated to neighboring cells both to propagate and to limit the propagation of localized cell death, termed the HR, triggered by pathogens and abiotic stressors. Cell-to-cell and longer-range signals initiate the development of SAR and SAA responses (Mullineaux et al. 2000; Casimiro et al. 2001; Lam et al. 2001; Grant and Lamb 2006). Hence, the emerging picture is somewhat inside-out from the conventional view, with signal perception at multiple sites both at the cell surface and within cells and signals arising from affected cells propagating both to neighboring cells and throughout the plant.

In taking on the assignment of describing the "system biology" of the stress response, I have adopted the metaphor of the elephant and the blind men, an old Hindu parable. Each of us most clearly sees the small set of interactions on which we choose to focus, rarely exploring the relationships among them. Although I cannot promise to deliver the whole elephant, I can suggest some ways to begin connecting the parts. The stress-response literature has focused historically on gene expression changes, which are somewhat removed both spatially and temporally from the initial insults. I will focus on the immediate cellular responses to stimuli because the transcriptional changes must ultimately be grounded in them. The importance of struggling towards a conceptual integration among the various aspects of stress responses lies in how it might influence our future approach to their analysis. It is time to begin viewing the system both at a new level of multidimensional detail and as a whole system to understand what is both common to all stress profiles and what uniquely crafts the plant's response and adaptation to a specific stressor. This is not just an intellectual exercise, however absorbing, but a task of immense practical importance because it is well known that exposing a plant to stress increases its ability to withstand subsequent stress of the same or of a different kind (Abel et al. 1986; Delledonne et al. 1998; Sandermann 2000; Logemann and Hahlbrock 2002). These phenomena, termed "priming" and "cross-protection," have been explored as a means of increasing the ability of crop plants to maintain yield in the face of environmental stress (Iriti et al. 2003; Engelberth et al. 2004). But even priming is not a unitary response and some priming responses appear to have a higher cost in terms of plant productivity than others, underscoring the importance of understanding the operation of the system as a whole (van Hulten et al. 2006).

II First Responders: Stomatal Guard Cells

The response of stomatal guard cells to abiotic stress is undoubtedly the best-studied plant cellular stress response. Guard cells are also the best-studied biotic stress response system, of course, as well as the best-studied cellular light-, gas- and chemical-sensing system in plants (MacRobbie 1998; Schroeder et al. 2001; Roelfsema and Hedrich 2005). The output of this system is relatively simple and easily measured**:** stomata open or close by virtue of the swelling or shrinkage of the guard cells. There are dozens of review articles on guard cell function and they can be lumped in three groups by their primary focus on signaling, vesicular trafficking or cytoskeletal re-structuring. These categories overlap to some extent, are vastly disproportionate in the size of the relevant literature, and do not include the contribution of cellular metabolism and sugar uptake (Vavasseur and Raghavendra 2005). Indeed, reviews addressing guard cell signaling outnumber those focused on either of the other areas by more than an order of magnitude. This does not necessarily reflect their eventual importance, but rather reflects both the history of discovery and the existence of appropriate tools and techniques. The availability of genome sequence information, genome-wide gene expression data and large libraries of insertion mutations, together with improving markers and microscopy, are vastly accelerating integration of cellular and molecular studies. But it seems that the tools have, if anything, gotten ahead of the ideas and it is an opportune time to derive a new "systems biology" perspective.

A Signaling

Stomatal closure can be induced by application of the stress hormone ABA, which is classically regarded as a signaling molecule or first messenger in this system (Schroeder et al. 2001). For a long time, it was assumed that ABA signaling could be represented by a linear signaling pathway, commencing with the binding of ABA to a surface receptor located in the plasma membrane. The general idea is that a signaling molecule (the first messenger) binds to a transmembrane receptor (such as a GPCR), which then activates a transducer (such as a heterotrimeric G-protein), which further activates the production or release of a diffusible second messenger, such as Ca^{2+} or ROS, which in turn activates a range of downstream effectors. This concept captures some elements of ABA signaling, but not its totality. There is a plasma-membrane GPCR, encoded by the *Arabidopsis GCR2* gene, that interacts with the α-subunit of the single heterotrimeric G protein (Liu et al. 2007).

However, it was reported more than a dozen years ago that ABA can act at both the cell surface and inside of the cell (Allan et al. 1994; Anderson et al. 1994; Schwartz et al. 1994). So there appears to be at least two different routes for ABA action, one from the outside and one from the inside of the cell. But at least two other proteins have been identified that might as well directly interact with ABA in guard cells. These are the H-subunit of the chloroplastic Mg-cheletase, reported to be the most abundant ABA-binding protein in cells, and a plasma membrane receptor-like kinase designated RLK1 (Osakabe et al. 2005; Shen et al. 2006). Moreover, stomatal aperture is also influenced by light, humidity and CO_2, inputs that impact stomatal dynamics by different molecular routes (Schroeder et al. 2001).

The earliest detectable ABA-induced chemical changes in plant cells are the increases in intracellular calcium [$(Ca^{2+})_{cyt}$] and ROS (Orozco-Cardenas et al. 2001; Lecourieux et al. 2006). These have been designated "second messengers" (Lecourieux et al. 2006) according to the criterion that interfering with their production or destroying them enzymatically or with reducing agents abolishes both the increase in $(Ca^{2+})_{cyt}$ and prevents ABA-induced stomatal closure (Zhang et al. 2001). Also, there is evidence that the observed increase in $(Ca^{2+})_{cyt}$ is necessary for stomata to close, suggesting again that calcium ions act as second messengers (Blatt 2000a; Schroeder et al. 2001). However, there is also evidence that stomata can close without an increase in $(Ca^{2+})_{cyt}$ (Allan et al. 1994; Levchenko et al. 2005; Marten et al. 2007), implying that at the very least, that a calcium transient is not an obligatory feature of the closure mechanism. Efforts to place calcium and ROS in a linear chain of causation as second messengers in ABA signaling are stubbornly resisted by the fact that some studies place ROS firmly upstream of calcium signaling, while others place it downstream (Mori and Schroeder 2004). Also, both elevated external calcium level and external exposure to ROS can induce stomatal closure (McAinsh et al. 1995; Zhang et al. 2001). The changes in $(Ca^{2+})_{cyt}$ observed in guard cells have a temporal pattern, with single peaks (transients) of a characteristic shape and size or with oscillations in a few minutes following a stimulus. These have been called the "calcium signature" and there is a not insignificant cottage industry in interpreting its information content (McAinsh and Hetherington 1998; Clayton et al. 1999; Knight and Knight 2000; Allen et al. 2001; Assmann and Wang 2001; Schroeder et al. 2001). Guard cells also rapidly produce ROS from different sources in a temporal sequences that can itself be regarded as an "ROS signature" (Schroeder et al. 2001; Mahalingam and Fedoroff 2003; Gechev et al. 2006). Strikingly, chloroplasts are the initial site of ROS production in ABA-treated guard cells, followed by cytoplasmic sources (Allan and Fluhr 1997; Zhang et al. 2001). But it has been reported that the ROS produced by plasma membrane-bound NADPH oxidases, which transport electrons across the plasma membrane to generate ROS outside of cells, are required for activation of calcium channels and stomatal closure (Pei et al. 2000; Kwak et al. 2003).

Many other small molecules and signaling proteins are involved in stomatal movements. Phospholipases C and D and the small molecules derived from membrane lipids, such as IP_3, PA, DAG, and sphingosine-1-phosphate have been implicated in ABA signaling (Gilroy et al. 1990; Jacob et al. 1999; Coursol et al. 2003; Zalejski et al. 2005). Protein phosphorylation and dephosphorylation are known, as judged by both the ability

of protein kinase and phosphatase inhibitors, as well as mutations in two type 2C protein phosphatase genes (*ABI1* and *ABI2*), to interfere with stomatal movements (Schroeder et al. 2001). cADPR also participates in ABA signaling and stomatal closure (Wu et al. 1997; Leckie et al. 1998). Two GPCRs, GCR1 and GCR2, the α subunit of the heterotrimeric G protein and the small GTPase AtRac1 (Rop6) have been implicated in ABA signaling and guard cell function (Lemichez et al. 2001; Wang et al. 2001a; Pandey and Assmann 2004; Liu et al. 2007).

B Vesicular Trafficking

Guard cells swell and shrink to alter the size of the stomatal aperture. The substantial increase in guard cell volume during stomatal opening necessitates an increase in surface area that can be as much as 40%, significantly exceeding the membrane elasticity limit of about 3% (Homann 1998; Blatt 2000b; Morris and Homann 2001; Shope et al. 2003; Meckel et al. 2005). Experiments employing fluorescent membrane dyes reveal a continuous slow internalization of membrane material in unstimulated guard cell protoplasts (Kubitscheck et al. 2000). Changes in osmotic pressure trigger rapid variation in membrane area, which has been reported to change by more than 10% in 10 min (Homann and Thiel 1999). The changes in surface area occur by incorporation of membranous material by exocytosis and its retrieval by endocytosis (Homann and Thiel 1999; Kubitscheck et al. 2000; Shope et al. 2003). Guard cells expand both in diameter and in length during stomatal opening (Meckel et al. 2007). Most of the increase in volume can be attributed to cell expansion; however, extension of the tips of guard cells appears to be primarily responsible for the mechanical deformation that increases the size of the stomatal opening (Meckel et al. 2007). Moreover, changes in guard cell volume are accompanied by re-organization of vacuoles. Closed guard cells contain many small vacuoles (Gao et al. 2005). These undergo rapid fusion upon guard cell opening, with a simultaneous increase in the area of the vacuolar membrane (tonoplast) (Gao et al. 2005).

Stomatal opening occurs by changes in turgor pressure driven by the influx of K^+ ions, sugar and anions (Cl^- and malate) resulting from the development of an electrical potential by proton pumps (H^+-ATPases) in the plasma and vacuolar membranes (Schroeder et al. 2001; Roelfsema and Hedrich 2005). The H^+-ATPases are activated by a decrease in pH and contain a C-terminal autoinhibitory domain (Roelfsema and Hedrich 2005). Stomatal opening is stimulated by both red and blue light (Schroeder et al. 2001; Roelfsema and Hedrich 2005). The plasma-membrane and vacuolar H^+-ATPases are activated by phosphorylation and binding of 14-3-3 proteins in response to blue light, which is perceived by phototropins (Hentzen et al. 1996; Kinoshita and Shimazaki 1999; Kinoshita et al. 2001; Sakamoto and Briggs 2002; Takemiya et al. 2006). Although phototropins are themselves protein kinases and might be responsible for the direct phosphorylation of the proton pumps, additional regulatory components are suggested by the report that inhibition of type1 protein phosphatases by tautomycin interferes with phosphorylation of the H^+-ATPase, but not that of the phototropin (Takemiya et al. 2006). Stomatal aperture is also regulated by CO_2, although the underlying mechanisms are not well understood (Roelfsema and Hedrich 2005). There is evidence that ABA, light and CO_2 responses are linked to some extent and also that they are independent (Vavasseur and Raghavendra 2005).

While little is yet known about the range of mechanisms by which ion channels are regulated, results of studies employing a K^+ channel-green fluorescent protein (KAT1-GFP) fusion have established the existence of membrane patches carrying a higher density of K^+ channel clusters than is characteristic of the plasma membrane as a whole (Hurst et al. 2004; Meckel et al., 2004). The KAT1-GFP fluorescence exhibits a punctate distribution in guard cells, with an intensity greater by two-fold at the tips of the cells than elsewhere, which is consistent with greater membrane turnover at the tips (Meckel et al. 2007). Rapid and reversible changes in guard cell volume are also induced by exposure to hypo- and hyperosmotic solutions (Shope et al. 2003). Such changes occur by water uptake and extrusion. These processes also entail changes in surface area of up to 30% by membrane endo- and exocytosis (Shope et al. 2003; Shope and Mott 2006). Agents that promote depolymerization of microtubules, actin filaments and the PI_3 kinase inhibitor wortmannin, all of which affect vesicular

trafficking, were found to interfere either with the expansion or the contraction of the membrane (Shope and Mott 2006).

Evidence that vesicular trafficking is critical to guard cell expansion and contraction is provided by the discovery that disrupting the function of a syntaxin encoded by the tobacco *Nt-SYR1* (*SYP121*) gene blocks ion channel responses to ABA (Leyman et al. 1999). Syntaxins comprise a family of proteins that participate in protein-protein interactions which mediate the vesicle-membrane fusions that are required for the vesicle trafficking of secretory processes and endocytosis (Sutter et al. 2006a). Over-expression of a fragment of this protein disperses plasma membrane KAT1-GFP clusters and results in the apparent retention of the channel in the endoplasmic reticulum (ER) (Sutter et al. 2006b). Similarly, SYP22 is involved in vacuolar fusion (Gao et al. 2005).

C Cytoskeletal Restructuring

The precise organization of the cytoskeleton, a complex of microtubules and actin filaments, is central both to the development of guard cells and to their function of controlling the stomatal aperture (Galatis and Apostolakos 2004). Many investigations have documented the rapid rearrangement of the cytoskeleton from a form in which the microtubules and actin filaments are in a radial array and extend from the ventral to the dorsal wall of each guard cell in open stomates to a form in which they are shorter and occur in many different orientations in closed stomates (Staiger 2000). The ordered, radially arrayed microtubules and actin filaments rearrange to the more disordered form during ABA-induced stomatal closure, as well as stomatal closure during the light-dark transition (Kim et al. 1995).

Stabilization of actin filaments with phalloidin promotes formation of densely packed radial actin filament arrays and inhibits both the inward K^+ current and ABA-induced stomatal closure (Kim et al. 1995; Hwang et al. 1997). Destabilization of actin filaments with cytochalasin B or D causes the partial opening of closed stomata, promotes light-induced stomatal opening, and increases the inward K^+ current (Kim et al. 1995; Hwang et al. 1997). Similar observations have been made on guard cell microtubules (Fukuda et al. 1998; Huang et al. 2000; Marcus et al. 2001; Lahav et al. 2004). Thus, reorganization of microtubules and actin filaments is closely correlated with changes in stomatal aperture. Although it was originally thought that the fungal toxin fusicoccin, which promotes stomatal opening by activating plasma membrane H^+-ATPase, uncouples microtubule and actin filament reorganization from stomatal opening (Assmann and Schwartz 1992; Marcus et al. 2001), cytoskeletal changes have been reported in fusicoccin-treated cells (Eun and Lee 2000).

III A Systems View of the Stress Response: The Elephant

A The Stomate as a System

The variety of stimuli to which guard cells respond and the multiplicity of molecular components participating in the response seriously defy efforts to construct a satisfying array of conventional linear signaling pathways by incorporating concepts such as “crosstalk” and “integrators.” It is the stomate itself, comprising the guard cell pair that is the “system”, integrating input signals and adjusting cell size and shape. While this may seem like a retreat from molecular rigor to animism, it is actually a first tentative attempt to synthesize the various presently disparate observations on the chemical and structural dynamics of guard cells.

1 Integrating Signal, Structure and Function

ROS and calcium “signals” are both ubiquitous intertwined everywhere in plants and animals. This is a phenomenon that some authors refer to as “cross talk” (Knight and Knight 2001; Mori and Schroeder 2004; Hidalgo 2005; Camello-Almaraz et al. 2006), while others dismiss with the argument that ROS and calcium aren’t really signals, just regulators or switches in the chemical machinery of life (Scrase-Field and Knight 2003; Plieth 2005). But signal and switch may be one and the same. Calcium ions and ROS share several important characteristics**:** both are extremely toxic to cells, both influence protein structure and activity, both act rather indiscriminately over short distances (albeit for different reasons) and there are multiple sources of each,

both inside of plant cells and in the apoplastic space (Gechev et al. 2006; Lecourieux et al. 2006).

Life evolved in a reducing atmosphere and adaptation to an increasingly oxidizing environment did not alter its basic biochemistry. Rather, it led to the evolution of multiple mechanisms to maintain a reducing intracellular environment (Sitia and Molteni 2004). Life is powered by the conversion of solar energy to chemical energy, its storage through photosynthesis and extraction through oxidative phosphorylation. In all organisms, and especially plants, this entails the dangers of unbalanced electron flow and the possibility of generating ROS, which are partially reduced oxygen molecules. The cellular mechanisms that protect the cell from oxidation of proteins and nucleic acids by these strong oxidants include the maintenance of high intracellular concentrations of low molecular weight redox couples, enzymes that rapidly reduce ROS to water, and protective mechanisms such as glutathionylation of proteins, activation of chaperones and protein disulfide isomerases, as well as nucleic acid repair mechanisms (Sitia and Molteni 2004). The existence of rapid chemical and enzymatic mechanisms for quenching ROS, in turn, makes it possible to make use of them as short-range signals. Moreover, the oxidation and reduction of disulfide bonds within and between proteins is not only essential for their correct folding, but is also as fundamental a regulatory mechanism as protein phosphorylation (Paget and Buttner 2003; Fedoroff 2006).

It has been suggested that efficient calcium ion sequestration is essential in view of the central role of phosphate in both energy metabolism and regulating protein structure, simply because of the insolubility of calcium phosphate (Sanders et al. 1999). Plant cells maintain an internal free calcium concentration about 1–2 orders of magnitude lower than the extracellular calcium concentration, both by the extrusion of calcium and its rapid sequestration by the many calcium-binding proteins in cells (Gilroy et al. 1990; Sanders et al. 1999). As in the case of ROS, these mechanisms make it possible for rapid, transient spikes in the concentration of free calcium to serve as protein and enzyme activators and inactivators, both directly and through interaction with calcium-binding proteins. Oscillations that bring the $(Ca^{2+})_{cyt}$ in guard cells above 0.5 mM trigger guard cell closure (Gilroy et al. 1990; McAinsh et al. 1992, 1995).

The prevailing view is that calcium is actively sequestered by calcium pumps in "internal stores" comprising vacuoles and the endoplasmic reticulum and that calcium transients are generated by the release of calcium from these internal stores, which in turn, triggers its uptake through calcium channels in the plasma membrane (Penner and Fleig 2004; Putney 2007). A radically different view holds that the main calcium-sequestering protein in the cell is filamentous actin (F-actin) and that actin depolymerization transiently releases free calcium, which is then rapidly bound by ATP-G-actin (which has a high calcium affinity), and reincorporated into F-actin (Lange and Brandt 1996; Lange and Gartzke 2006). The notion that F-actin is the major calcium store is a particularly attractive hypothesis for plants cells whose cortical ER has an actin backbone (Boevink et al. 1998). However, it is increasingly recognized that ER resident proteins, such as calreticulin and calsequestrin, with an extremely high calcium-binding capacity, also participate actively in the sequestration and release of calcium (Beard et al. 2004; Persson et al. 2004).

A cytoskeletal network of both actin filaments and microtubules comprises the heart of the highly dynamic cortical ER of plant cells, including stomata (Hepler et al. 1990; Boevink et al. 1998). Actin depolymerization and reorganization underlie all the shape changes that guard cells undergo in response to prevailing external conditions (Fig. 1). Virtually every type of stimulus that alters the stomatal aperture triggers actin depolymerization and cytoskeletal restructuring (Kim et al. 1995; Hwang et al. 1997; Eun and Lee 2000; Hwang and Lee 2001; Marcus et al. 2001; Lahav et al. 2004; Xiao et al. 2004). Assuming that F-actin and other calcium-binding proteins of the cortical ER are the calcium stores, then calcium spikes reflect the transient release of Ca^{2+} from ER proteins and the actin depolymerization that initiate the changes in stomatal aperture (Fig. 1). The release of calcium, as noted earlier, transiently increases the $[Ca^{2+}]_{cyt}$, activating a variety of enzymes, including the calcium-dependent membrane-bound NADPH oxidase, as well as the further influx of calcium (Schroeder et al. 2001).

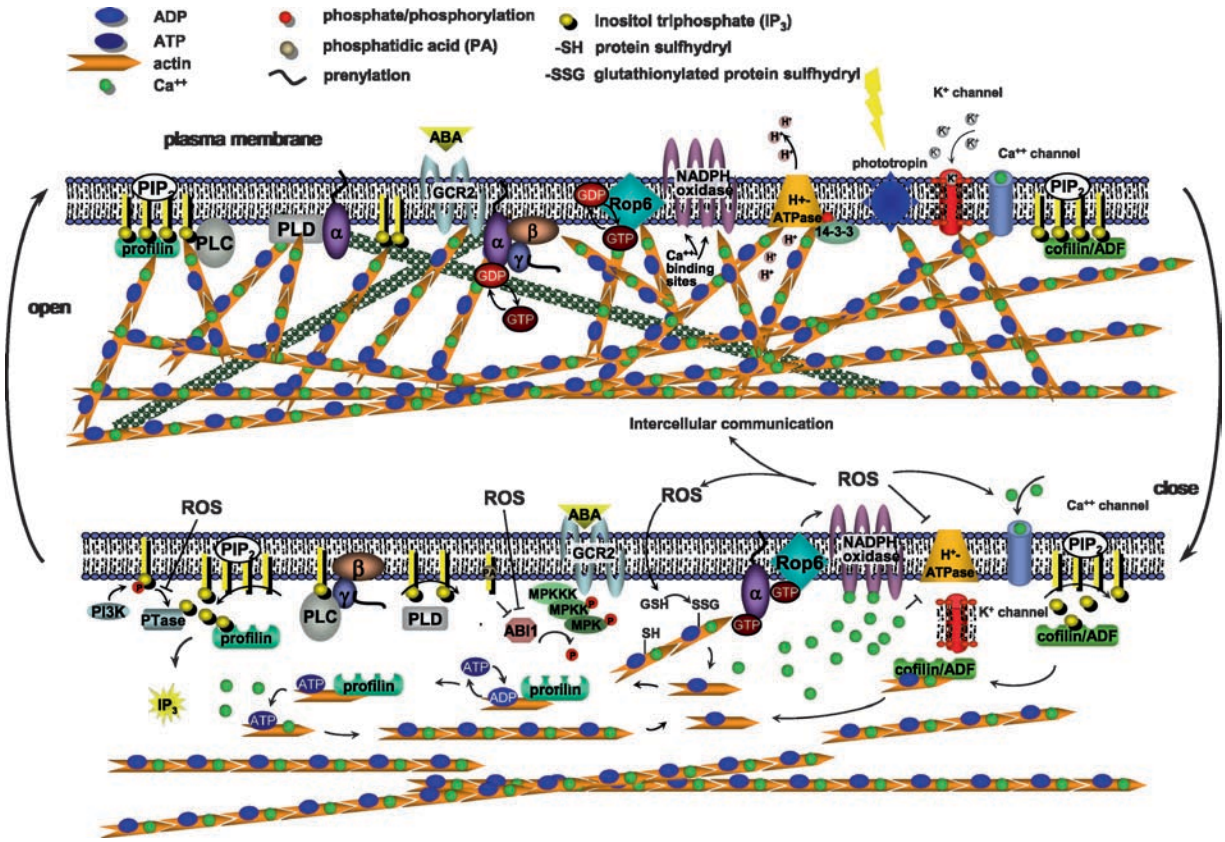

Fig. 1. A diagrammatic representation of some of the changes known to occur in the structural and signaling component during stomatal movements, as described in the text. Abbreviations (used in the figure): ABA, abscisic acid; αβγ, heterotrimeric G-protein subunits; GCR2, G-protein coupled receptor 2; PIP_2, phosphatidylinositol 4,5 bisphosphate; GSH, glutathione; MPK, MAP kinase; MPKK, MAPK kinase; MPKKK, MAPKK kinase; PLC and PLD, phospholipases C and D; ROS, reactive oxygen species [See Color Plate 13, Fig. 20].

Similarly, ROS are likely to affect both cytoskeletal organization and enzymatic activity. Only one route of ROS production is represented in Fig. 1, which is initiated by the perception of ABA at the cell membrane, stimulating dissociation of the heterotrimeric G protein. Activated Gα, in turn, is believed to activate the small GTPase Rop6 which further activates the membrane-bound NADPH oxidases, primarily AtrbohF and AtrbohD in guard cells (Kwak et al. 2003). But it is also known that ROS are initially and rapidly elicited from guard cell chloroplasts upon ABA stimulation, as well as ozone exposure (Zhang et al. 2001; Joo et al. 2005). There are additional intra- and extra-cellular sources of ROS that may well be triggered by other environmental stimuli and the most stable ROS, H_2O_2, is membrane permeable (Bolwell et al. 2002). Thus, ROS arising from either external or internal sources are likely to exert an effect in the vicinity of the cell surface.

ROS stimulate the glutathionylation of many proteins, particularly actin, in both animal and plant cells (Giustarini et al. 2004; Dixon et al. 2005). Actin glutathionylation has been shown to be essential for actin polymerization and reorganization in response to growth and chemotactic factors in animal cells (Wang et al. 2003; Fiaschi et al. 2006). Specifically, glutathionylation of sulfhydryl group of the single superficial cysteine residue (Cys^{374}) of actin interferes with the disassembly of the actinomyosin complex that initiates cytoskeletal reorganization (Fiaschi et al. 2006). The catalytic subunit of the mammalian NADPH oxidase, designated Nox2, is associated with the ER and is transported to the membrane at the leading edge of migrating epithelial cells, where it interacts with actin and an actin-binding

scaffold protein designated IQGAP1 (Ikeda et al. 2005). While such studies have not yet been done in plants, plant cells contain myosins, at least some of which are thought to play an F-actin stabilizing role and participate in organelle movement (Volkmann et al. 2003; Lee and Liu 2004; Abu-Abied et al. 2006; Holweg 2007). There are likely to be additional F-actin-stabilizing proteins and in view of the fact that ROS production and cytoskeletal restructuring are required for stomatal closure, it appears a quite reasonable conjecture that ROS promote actin reorganization in guard cells of plants, as they do in animal cells (Fig. 1).

ROS also impact enzymatic activity directly through oxidation of sulfhydryl residues and promotion of protein disulfide bond formation (Finkel 2001; Dietz 2003; Fedoroff 2006). Protein phosphatases contain readily oxidizable cysteine residues in their active sites and are therefore likely to be immediate targets of ROS regulation (Xu et al. 2002). It is well established that ABA and ROS activate stress MAP kinases (Mishra et al. 2006b). Likely targets include the protein phosphatases encoded by the *ABI1* and *ABI2* genes, as well as the MAP kinase phosphatases (Meinhard and Grill 2001; Ulm et al., 2001, 2002; Meinhard et al. 2002; Meskiene et al. 2003). Thus activation of ROS production and changes in $[Ca^{2+}]_{cyt}$ are intimately interconnected with cytoskeletal reorganization. Moreover, as illustrated in the Fig. 22.1, they may well feed-back on each other, since the NADPH oxidase is activated by calcium. It has also been suggested that Ca^{2+} channels are activated by ROS (Pei et al. 2000), although the effect of ROS on cytoskeletal structure was not addressed in these experiments.

Moreover, vesicular trafficking in plant cells is inseparable from the cytoskeletal network, since F-actin filaments and microtubules are integral to the structure and function of the ER (Boevink et al. 1998; Brandizzi et al. 2003). The exocytotic and endocytotic membrane trafficking that are required for the constant adjustments in membrane surface area are sensitive to agents that interfere with microtubule and actin filament dynamics (Shope and Mott 2006). It has been reported in mammalian cells that both actin depolymerization and polymerization are necessary for excocytosis (Malacombe et al. 2006). It is thought that actin depolymerization enhances the ability of secretory granules to reach the plasma membrane, while actin polymerization provides a motive force that accelerates the membrane fusion process (Malacombe et al. 2006).

The recognition that K^+ channels are regulated by altering their numbers through the insertion of membranous packets containing multiple copies of them, as well as by Ca^{2+}-dependent activation of their phosphorylation, identifies the link between cytoskeleton and K^+ currents (Hurst et al. 2004; Sutter et al. 2006b). In a variety of animal cells, most or all membrane proteins are clustered in cholesterol-rich domains, designated islands, separated by protein-free, cholesterol-poor membrane domains (Lillemeier et al. 2006). The protein-rich islands include previously characterized lipid rafts, and different proteins form sub-clusters within them. The protein islands are connected to the actin cytoskeleton and can be dispersed by latrunculin, an actin-depolymerizing agent (Lillemeier et al. 2006). Recent observations on KAT1-GFP fusions are consistent with a similar organization of plant membranes, revealing the existence of KAT1-rich membrane clusters (Hurst et al. 2004; Meckel et al., 2005; Sutter et al. 2006b) firmly anchored in the plasma membrane (M. Blatt, personal communication).

Although it is known that Golgi bodies travel rapidly along the cytoskeletal network (Boevink et al. 1998; daSilva et al. 2004), there may be other protein-lipid complexes that travel the same cytoskeletal highway from ER to cell surface. A possibility yet to be explored is that Ca^{2+} currents are regulated by altering Ca^{2+} channel densities in the plasma membrane by a similar secretory mechanism. In addition to their role in supporting the secretory trafficking required to expand membranes and to add ion channels, it is also possible that the cytoskeletal restructuring observed during stomatal opening serves a structural role. The microtubules and actin filaments form highly distinctive radial arrays when stomates open (Kim et al. 1995; Fukuda et al. 1998; Lahav et al. 2004). Also, guard cells are encircled by radial arrays of cellulose microfibrils (Lahav et al. 2004). There is growing evidence that cellulose synthase complexes are mobile and critically oriented by microtubules (Smith and Oppenheimer 2005; Somerville 2006; Chu et al. 2007; DeBolt et al. 2007). As turgor pressure increases with the influx of ions and water during guard

cell opening, it is likely that the arrays limit the radial expansion of the cells by guiding cellulose deposition. The observation that blue light specifically promotes the reconfiguration of guard cell microtubules suggests a connection between phototropins and microtubule-associated proteins (Lahav et al. 2004).

It is increasingly recognized that membrane phospholipids are essential elements of plant cellular signaling (Laxalt and Munnik 2002). PI_3P, and PI_4P and PIP_2 have all been implicated in stomatal movements, as have PLC, PLD and PI3 kinase (MacRobbie 1998; Jacob et al. 1999; Staxen et al. 1999; Jung et al. 2002; Park et al. 2003; G. Mishra et al. 2006a). As shown in Fig. 1, recent work has revealed that PLDα1-derived PA binds to, inhibits and membrane-sequesters the ABI1 protein phosphatase 2C to promote stomatal closure (Mishra et al. 2006a). An interaction between PLDα1 and the Gα subunit of the heterotrimeric G protein inhibits opening of closed stomata by inhibiting PLD activity and stimulating the GTPase activiy of Gα. (Mishra et al. 2006a). It has also been reported that PI3 kinase inhibitors prevent stomatal closing and the production of ROS (Park et al. 2003).

Although little is as yet known about the detailed mechanisms by which they exert their effects on stomatal aperture, PI_3P, PI_4P and $PI_{4,5}P_2$, appear to be located in different cell compartments (Jung et al. 2002; Vermeer et al. 2006). Membrane phospholipids target many signaling and cytoskeletal proteins to the plasma membrane and there is recent evidence that such targeting can be attributed to both polybasic clusters in the targeted proteins and lipid modifications (Heo et al. 2006). The membrane association of small GTPases and heterotrimeric G proteins is likely to be regulated by the specificity of their phospholipid interactions, as well as the activity of phospholipases. Many actin-binding proteins that participate in cytoskeletal remodeling are membrane-tethered and maintained in an inactive form by binding to membrane phospholipids, particularly PIP_2 and PIP_3 (Nebl et al. 2000; Hilpela et al. 2004; Huang et al. 2006). Both the spatial localization of the phospholipids and the localized activation of lipid kinases, phosphatases and phospholipases can trigger actin remodeling by the differential release and sequestration of actin-remodeling proteins from the membrane (Toker 1998). Plants contain both profilin and cofillin/ADF, whose mammalian counterparts bind to and are regulated by PIP_2 and PIP_3 (McCurdy et al. 2001; Skare and Karlsson 2002; Feng et al. 2006; Gorbatyuk et al. 2006). Also, there is substantial evidence that Ca^{2+} channels are reciprocally regulated by membrane phospholipids PIP_2 and PIP_3 and the calcium-binding protein calmodulin (Kwon et al. 2007), a mechanism similar to that reported for cofillin (Gorbatyuk et al. 2006). Thus, the local activation of phospholipid-modifying enzymes, such as phospholipid kinases and phosphatases, as well as phospholipases, can both activate cytoskeletal restructuring and affect ion movements across the membrane (Tall et al. 2000).

B Stress Beyond the Stomate

To what extent are the signaling and cytoskeletal/vesicular restructuring observed in stomata representative of cellular stress responses in general? Perhaps because guard cells respond to changing environmental conditions rapidly, visibly and reversibly, immediate structural and cellular changes have dominated their study, with relatively little attention devoted to transcriptional changes (Leonhardt et al. 2004). By contrast, the literature on abiotic stress responses is dominated by the analysis of gene expression changes, increasingly relying on cDNA and oligonucleotide microarray technology in recent years (Seki et al. 2004; Kilian et al. 2007). Another obvious difference is that guard cell studies are generally investigated within a range of environmental conditions under which they are reversible, while responses to pathogens and other stresses are often investigated near or beyond the limits of plants' ability to adapt, as judged by either local cell death or tissue collapse.

Nonetheless, transient Ca^{2+} spikes and the production of ROS are common early components of both biotic and abiotic stress responses in plants (Price et al. 1994; Doke et al. 1996; Knight et al. 1996, 1997; Takahashi et al. 1997; Lecourieux et al. 2002; Mahalingam and Fedoroff 2003; Torres and Dangl 2005; Lecourieux et al. 2006). Phospholipid signaling is a well-established early plant stress response and there is a direct connection between cytoskeletal reorganization and activation of enzymes that modify membrane phospholipids (Munnik 2001; Laxalt and Munnik

2002; Dhonukshe et al. 2003; Testerink et al. 2004; Wang 2005; Williams et al. 2005; Huang et al. 2006). Cytoskeletal rearrangements have been reported in response to pathogens and pathogen-derived toxins, as well as a variety of abiotic stresses, including cold, excess light, and osmotic stress (Gross et al. 1993; Aon et al. 2000; Staiger 2000; Binet et al. 2001; Lipka and Panstruga 2005; Komis et al. 2006; Shoji et al. 2006; Yuan et al. 2006; D'Angeli and Altamura 2007). Also, upregulation of secretory process and vesicular trafficking, are components of both biotic and abiotic stress responses (Schmelzer 2002; Collins et al. 2003; Wick et al. 2003; Takemoto and Hardham 2004; Lipka and Panstruga 2005; Wang et al. 2005).

C Wherein Lies the Specificity?

But why is the response of a guard cell different from that of an epidermal cell or a mesophyll cell? And how does common cellular machinery develop a response that is unique and appropriate for a given stress? The answers to such questions are not yet available at a satisfying level of granularity, but there are some generalizations. Plant cells are highly specialized, yet remarkably flexible in their developmental fates. They acquire and maintain their structural and physiological identities by virtue of constant chemical communications among cells within the plant (Dolan and Okada 1999; Golz 2006; Sieburth and Deyholos 2006; Anastasiou and Lenhard 2007). Many plant cells, including guard cells of some plants, can regenerate into complete plants (Hall et al. 1996). The mechanisms that underlie the development of a plant from a single cell, be it a vegetative cell or a zygote, are just beginning to be explicated, as are the mechanisms underlying the regeneration and maintenance of specialized cell types (Willemsen and Scheres 2004; J.Xu et al. 2006; Anastasiou and Lenhard 2007).

IV The Future

There are many challenges for future research in understanding how plants elaborate appropriate adaptations to environmental extremes. Information about sensors and how they trigger cellular responses is still relatively meager (Albrecht et al. 2003; Samaj et al. 2004; Zhu et al. 2007). The connections between signaling relays, such as stress-activated MAP kinase cascades, and transcription factors is as yet little explored (Cheong et al. 2003; Ahlfors et al. 2004). It has been reported that the stress-activated transcriptional co-factor NPR1 is maintained in a multimeric, disulfide-bonded cytoplasmic complex in unstressed cells and activated by reduction to monomeric form and translocation to the nucleus, but little is known about the requisite cellular and molecular changes that underlie its mobilization (Mou et al. 2003; Rochon et al. 2006). It is known that the small GTPases, designated ROPs, in plants are involved in both activating ROS production and in stress-mediated cytoskeletal changes (Lemichez et al. 2001; Nibau et al. 2006), but little is known about how such changes affect gene expression. Finally, very little is known about the spatial localization of stress signals and signaling molecules within plant cells, although there is growing evidence that such spatial localization is central to how plant cells are structured in development (Fu et al. 2005; Hwang et al. 2005).

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