Behind the scenes: the roles of reactive oxygen species in guard cells

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Summary
Guard cells regulate stomatal pore size through integration of both endogenous and environmental signals; they are widely recognized as providing a key switching mechanism that maximizes both the efficient use of water and rates of CO2 exchange for photosynthesis; this is essential for the adaptation of plants to water stress. Reactive oxygen species (ROS) are widely considered to be an important player in guard cell signalling. In this review, we focus on recent progress concerning the role of ROS as signal molecules in controlling stomatal movement, the interaction between ROS and intrinsic and environmental response pathways, the specificity of ROS signalling, and how ROS signals are sensed and relayed. However, the picture of ROS-mediated signalling is still fragmented and the issues of ROS sensing and the specificity of ROS signalling remain unclear. Here, we review some recent advances in our understanding of ROS signalling in guard cells, with an emphasis on the main players known to interact with abscisic acid signalling.

I. Introduction
Reactive oxygen species (ROS) are chemical species that are formed upon the incomplete reduction of oxygen; they include the superoxide anion (O2−), hydrogen peroxide (H2O2), singlet oxygen (1O2) and the hydroxyl radical (HO•). The enzymatic activity of superoxide dismutase (SOD) was discovered 45 yr ago (McCord & Fridovich, 1969). This was a turning point that opened up the field of ROS biology, leading to more attention being directed to cellular oxidative stress. Many reviews have covered the basic principles of new mechanisms of ROS regulation and their coordination with large molecules (Mittler, 2002; Mittler et al., 2004; Wang & Song, 2008). Along with aerobic respiration, ROS were long regarded as unwanted and toxic by-products of physiological metabolism. However, ROS are now recognized as central players in the complex signalling network of cells. Modulation by ROS is now understood to occur in virtually every type of organism. Significant progress has been achieved specifically in plant cells. Several timely reviews have outlined progress in understanding the regulatory role of ROS (Moller & Sweetlove, 2010; Mittler et al., 2011; Tripathy & Oelmuller, 2012). This includes advances in our understanding of plant defence responses.
(Scandalios, 1997; Oliver & Solomon, 2004; Yang et al., 2004; Seong et al., 2007; Cechin et al., 2008; Martin et al., 2009; Noriega et al., 2012; Ravet & Pilon, 2013), growth and morphogenesis (Wang et al., 2010), cell death (Kachroo et al., 2003; Zhou et al., 2004; Montillet et al., 2005; Steffens & Sauter, 2009; Samuilov et al., 2010; Wang et al., 2013b,c) and guard cell signalling (Hetherington, 2001; Schroeder et al., 2001; Wang & Song, 2008; Acharya & Assmann, 2009; Kim et al., 2010).

Stomatal opening and closing control water transpiration and the diffusion of gases into and out of air spaces in plants. Guard cells, which form stoma, can sense and integrate both extra- and intracellular signals and rapidly respond to exogenous signals, such as light, humidity, carbon dioxide, pollutants, pathogens, as well as various hormones, including abscisic acid (ABA), auxin (IAA), cytokinin (CTK) and ethylene (ET) (Schroeder et al., 2001; Melotto et al., 2006; Shimazaki et al., 2007; Shope et al., 2008). In 1996, it was reported for the first time that ROS cause stomatal closure and inhibit stomatal opening through increases in concentrations of cytosolic free calcium ([Ca2+]cyt) in Commelina communis (McAinsh et al., 1996). The field of ROS signalling in guard cells has expanded since then. Recently, many important advances have been made in unravelling the roles of ROS and the ROS-regulated genetic pathways that control stomatal movement. However, rather than providing a review of the mechanisms that regulate stomatal closing and opening, this paper focuses on ROS as signal molecules that control stomatal movement in response to exogenous and endogenous conditions. These conditions include abiotic and biotic stresses that trigger ROS generation in guard cells, as well as ABA and other phytohormones that modulate stomatal closure and opening. We attempt to outline the signal transduction pathways of ROS in guard cells and their targets. Finally, we discuss the specificity and dynamics of ROS signalling in guard cells. Rather than providing an all-inclusive survey, we highlight key advances in an attempt to provide a comprehensive overview of the roles of ROS in the regulation of stomatal responses.

II. Multiple signals for production and signalling integration of ROS in guard cells

1. Multiple pathways for ROS production

The accumulation of ROS that occurs under conditions of biotic and abiotic stress causes oxidative stress (Xia et al., 2010; Mittler et al., 2011; Pucciariello et al., 2012a; Tripathy & Oelmuller, 2012). Owing to their exposure to diverse environmental conditions, plants have evolved an elaborate system to control cellular ROS concentrations (Mittler et al., 2011). There are multiple mechanisms by which an increase of ROS is generated in guard cells. Under different environmental conditions, ROS are generated in the chloroplasts and mitochondria; they are essential for the maintenance of normal energy and metabolic fluxes, optimization of different cell functions, activation of acclimation responses through retrograde signalling and control of whole-plant systemic signalling pathways (Moller, 2001; Jaspers & Kangasjarvi, 2010). For example, under conditions of water deficiency, electron flow continues and excitation energy can be passed from photoexcited Chl (chlorophyll) pigments to ground-state oxygen (O2), forming ‘O2. In addition, O2−, H2O2 and OH− can be produced by photosystem II (Suzuki et al., 2012a; Tripathy & Oelmuller, 2012).

Compared with other epidermal cells, guard cells have fewer, smaller chloroplasts (Allaway & Setterfield, 1972) and their Chl content is between 1 and 4% of that in mesophyll cells (Zemel & Gepstein, 1985; Birkenhead & Willmer, 1986; Reckmann et al., 1990). Although guard cell chloroplasts contain the principal enzymes of the Calvin cycle (Zemel & Gepstein, 1985), such as Rubisco, their activity is negligible (Reckmann et al., 1990). In transgenic tobacco plants that have reduced amounts of Rubisco and impaired photosynthesis as a result of antisense-mediated suppression of Rubisco, there is no relationship between photosynthetic capacity and stomatal conductance under conditions of high light intensity (von Caemmerer et al., 2004). However, studies have also shown that photophosphorylation in guard cell chloroplasts is as high as 80% of that in mesophyll cells (Shimazaki & Zeigler, 1985). Hence, it is proposed that ROS generated from the photosynthetic electron transport chain in guard cell plastids may play an important role in plant responses to various stimuli (Pfannschmidt, 2003). Meanwhile, chloroplasts in guard cells and those in mesophyll cells exhibit different characteristics in response to stressors, with the former being less stable and more vulnerable to breakdown. For example, following UV treatment, indications of damage to the chloroplasts of guard cells developed more rapidly than for the chloroplasts of mesophyll cells and, within 24 h, most guard cells had no chloroplasts (Blakey & Chessin, 1959). This rapid disappearance of guard cell plastids obviously cannot be completely attributed to guard cell metabolism. Instead, an attractive, but still speculative way to explain these phenomena is that more ROS may accumulate in guard cell plastids.

Given that ABA plays a key role in controlling stomatal closure during drought stress, much work has focused on ABA signalling in guard cells (Cutler et al., 2010; Kim et al., 2010). Working with broad bean (Vicia faba), Miao et al. (2000) demonstrated that ABA induced H2O2 production in guard cells, which resulted in stomatal closure. A detailed follow-up study demonstrated the generation of H2O2 in Vicia guard cells in response to ABA, suggesting two distinct sources of H2O2: one chloroplastic and the other via a possible plasma membrane NADPH oxidase (Zhang et al., 2001b,c). At the same time, an independent study reported that ABA-stimulated ROS accumulation induced stomatal closure via activation of plasma membrane calcium channels in Arabidopsis (Pei et al., 2000). Kwak et al. (2003) further showed that the two NADPH oxidase subunit genes AtRbohD and AtRbohF are both involved in ABA-mediated stomatal closure. Although the AtRbohD single mutant was not affected, the AtRbohDF double mutant was more impaired in terms of ABA-mediated stomatal closure than the AtRbohF single mutant, suggesting partial redundancy in function. The AtRbohDF double mutant showed reduced ABA-induced stomatal closure and ROS production compared with wildtype plants. The demonstration that exogenous application of H2O2 restored stomatal closure revealed the connection between ROS production by NADPH oxidase and ABA-mediated stomatal closure. Consistent with chloroplast functions in guard cells.
described earlier, ROS production during ABA and methyljasmonate (MeJA) stimulation in Arabidopsis guard cell chloroplasts was more than tripled (Lesher & Levine, 2013). Moreover, ROS were detected on the suborganelle level in compartments that are typically occupied by starch grains. This observation suggests that these ROS are involved in redox control, which leads to the inactivation of starch degradation that takes place in these compartments, thus contributing to the stoma closure in an additional way.

Extensive and detailed studies on ABA receptors and early signalling components have provided new insights that helped to unravel the regulation of ABA signalling. Progress in understanding ABA sensing and signal transduction has been the subject of several recent reviews (Kim et al., 2010; Raghavendra et al., 2010; Joshi-Saha et al., 2011; Nakashima & Yamaguchi-Shinozaki, 2013; Xu et al., 2013). The perception of ABA by PYR/PYL/RCAR (PYRABACTIN RESISTANCE/PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) proteins induces protein complex formation between these proteins and the PP2Cs, which subsequently inactivates the negative regulatory function of the PP2Cs and activates SnRK2 protein kinase open stomata 1 (OST1) to transmit an ABA signal to the downstream signalling components (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Umezawa et al., 2009). ABA-activated SnRK2 protein kinase acts upstream of ROS in guard cell ABA signalling. Recent studies have indicated that OST1 physically interacts with and phosphorylates Ser13 and Ser174 on AtrbohF (Sirichandra et al., 2009) and that Ca2+ binding and phosphorylation synergistically activate the ROS-producing enzyme activity of AtrbohD (Ogasawara et al., 2008). It was shown that ABA was unable to generate ROS in the abi1 (ABA insensitive 1) mutant plants, but that ABA could still induce ROS production in the abi2 (ABA insensitive 2) mutant plants (Murata et al., 2001). These data suggest that the abi2-1 mutation impairs ABA signalling downstream of ROS production (Murata et al., 2001).

Constitutive ROS accumulation caused by mutation of the CATALASE 1 (CAT1), CAT2 and CAT3 genes or application of an inhibitor of CAT, 3-amino-1,2,4-triazole (3-AT), was shown not to affect the stomatal aperture in the absence of ABA or MeJA (Jannat et al., 2011a,b, 2012). Meanwhile, ROS production and [Ca2+]cyt oscillation were enhanced during ABA-induced stomatal closure (Jannat et al., 2011a). This suggests a tight link between ABA-induced stomatal closure and inducible ROS production, rather than constitutive ROS accumulation. By contrast, the knockout mutants lacking APX1 (KO-APX1) showed high sensitivity to wounding or MeJA treatment (Maruta et al., 2012). Recently, a nuclear-encoded cytosol-located yellow fluorescent protein-based sensor for H2O2, called HyPer, was used to examine spatial and temporal changes in H2O2 in high light-exposed plants (Esposito-Rodriguez et al., 2013). It could be helpful in revealing the functions of constitutive ROS accumulation in guard cells, other tissues, or even in other hormone signalling pathways.

Unlike ABA, salicylic acid (SA) mediates ROS production, not via NADPH oxidases, but rather via a peroxidase-catalysed reaction (Mori et al., 2001). In fact, the reduced stomatal apertures in the SA-accumulating mutant siz1 were inhibited by the application of salicylhydroxamic acid and azide, peroxidase inhibitors, which inhibit SA-dependent ROS production, but not by diphenyl iodonium chloride (DPI), an NADPH oxidase inhibitor that inhibits ABA-dependent ROS production (Miura et al., 2012). Moreover, SA induces stomatal closure accompanied by extracellular ROS production mediated by peroxidase, intracellular ROS accumulation, and inactivation of K+ channels (Khokon et al., 2011). By contrast, there is also evidence that SA induces a rapid increase in NADPH oxidase activity. SA-induced stomatal closure is inhibited by DPI treatment, and NADPH-oxidase RbohD-deficient plants exhibited impaired stomatal reaction upon exposure to exogenous SA (Kalachova et al., 2013). Thus, it has been postulated that NADPH oxidase plays a key role in the process of stomatal closure in response to SA, and that NADPH oxidase is involved in SA signalling. Plants with internally reduced SA concentrations (eds5-1, eds16/sid2 or nabG lines) or plants affected in SA signalling (npr1) were compromised in their ability to close stomata in response to either bacteria or pathogen-associated molecular patterns (PAMPs) and displayed lower stomatal defence (Melotto et al., 2006; Zeng & He, 2010). An underlying link between SA and ABA signalling has been further suggested based on the observation that the ABA-deficient mutant line aba2-1 no longer closed stomata in response to exogenously applied SA, whereas guard cells of SA-deficient sid2 and nabG plants responded normally to ABA (Zeng & He, 2010; Montillet & Hirt, 2013). Therefore, these data suggested that SA signalling acts upstream of ABA signalling in bacterium-triggered stomatal closure. It also clearly indicates that interaction between ROS and SA can vary under different contexts, concentrations and conditions.

As mentioned earlier, it has been shown that MeJA evokes ROS production in guard cells. Exogenous application of DPL results in the suppression of MeJA-induced stomatal closure. Simultaneously, MeJA-induced stomatal closing is suppressed in the NADPH oxidase double mutant atrbohD/F (Suhita et al., 2004). These findings indicate that the major ROS sources are NADPH oxidases AtrbohD/F in guard cell MeJA signalling. Besides NADPH oxidases, other ROS-producing enzymes also play important roles in various plant responses, and some of them (e.g. cell wall-bound peroxidase and copper amine oxidase) have been shown to regulate stomatal movement (Munemasa et al., 2011). MeJA fails to induce production of ROS and NO in the Arabidopsis rcn1 mutant, which has a mutation in a gene encoding a regulatory subunit of protein phosphatase type 2A (PP2A) (Saito et al., 2008). This finding suggests that RCNI-regulating PP2As function upstream of ROS and NO production in guard cell MeJA signalling.

Of course, there are numerous other sources of ROS that are produced by plant cells in response to other environmental conditions and phytohormones. These are discussed in Section IV on the specificity of ROS signalling. For a list of ROS-related findings in Arabidopsis vs other plants discussed in this review, see Table 1. In addition, a ROS-dependent systemic response, an autopropagating ROS systemic signal that travels from the local site to the entire plant, can enhance plant resistance such as the wounding response (Suzuki and Mittler, 2012b). The autonomous nature of guard cells may enable the ROS systemic signal to reach
Table 1 Overview of reactive oxygen species (ROS) involved in the regulation of stomatal movement

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Mediated components</th>
<th>Stomatal response</th>
<th>Species</th>
<th>References</th>
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<tr>
<td>H₂O₂, OGA, MV, chitosan</td>
<td>H₂O₂</td>
<td>Stomatal closure</td>
<td>Commelina communis</td>
<td>McAinsh et al. (1996); Lee et al. (1999)</td>
</tr>
<tr>
<td>ABA, SA, H₂O₂</td>
<td>H₂O₂, O₂⁻</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
<td>Miao et al. (2000); Pei et al. (2000); Dong et al. (2001); Mori et al. (2001); Zhao et al. (2003)</td>
</tr>
<tr>
<td>ExtCaM</td>
<td>GPA1, PTPase, H₂O₂</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
<td>Chen et al. (2004); Shi et al. (2004); Li et al. (2009)</td>
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<tr>
<td>ABA</td>
<td>CuAO, H₂O₂</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
<td>An et al. (2008)</td>
</tr>
<tr>
<td>Darkness</td>
<td>S1P, H₂O₂</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
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</tr>
<tr>
<td>UV-B</td>
<td>ET, peroxidase</td>
<td>Stomatal closure</td>
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</tr>
<tr>
<td>ABA, H₂O₂</td>
<td>MEK1/2, p38-like kinase</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
<td>Jiang et al. (2008)</td>
</tr>
<tr>
<td>ABA, H₂O₂, MeJA</td>
<td>H₂O₂, pH₀</td>
<td>Stomatal closure</td>
<td>Vicia faba</td>
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<tr>
<td>CTK, IAA</td>
<td>H₂O₂, ET</td>
<td>Stomatal closure</td>
<td>Vicia faba, Arabidopsis thaliana</td>
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<tr>
<td>ABA</td>
<td>PI3P, H₂O₂</td>
<td>Stomatal closure</td>
<td>Nicotiana tabacum</td>
<td>Park et al. (2003)</td>
</tr>
<tr>
<td>MeJA, ABA, O₃, Wounding</td>
<td>H₂O₂, NtMPK4</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
<td>Gomi et al. (2005); Hettenhausen et al. (2012)</td>
</tr>
<tr>
<td>Chitosan, pyrabactin</td>
<td>H₂O₂</td>
<td>Stomatal closure</td>
<td>Pisum sativum</td>
<td>Srivastava et al. (2009); Puli &amp; Raghavendra (2012)</td>
</tr>
<tr>
<td>Drought</td>
<td>H₂O₂</td>
<td>H₂O₂ accumulation in subsidiary cells</td>
<td>Zea mays</td>
<td>Yao et al. (2013)</td>
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<tr>
<td>Drought</td>
<td>SNAC1, OsSRO1c</td>
<td>H₂O₂ accumulation and stomatal closure</td>
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<tr>
<td>ABA, H₂O₂</td>
<td>OST1, ABI1, ABI2, H₂O₂</td>
<td>Stomatal closure</td>
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<td>Meinhard &amp; Grill (2001); Murata et al. (2001); Meinhard et al. (2002); Mustilli et al. (2002)</td>
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<tr>
<td>ABA, bicarbonate</td>
<td>H₂O₂, AtrohβD, AtrohβF</td>
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<td>Kwak et al. (2003); Bright et al. (2006); Kolla et al. (2007)</td>
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<td>H₂O₂</td>
<td>ETR1</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
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<td>ET</td>
<td>AtrohβD, H₂O₂</td>
<td>Stomatal closure</td>
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<td>Desikan et al. (2006)</td>
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<td>Drought, ABA</td>
<td>H₂O₂, ALGPX3</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
<td>Miao et al. (2006)</td>
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<td>ABA, H₂O₂, yeast elicitor</td>
<td>MPK1, MPK9, MPK12</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
<td>Gudesblat et al. (2007); Jammes et al. (2009); Salam et al. (2013)</td>
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<td>MeJA</td>
<td>CO11, H₂O₂</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
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<td>Pathogen, ABA</td>
<td>GPA1, H₂O₂</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
<td>Zhang et al. (2008); Zhang et al. (2011)</td>
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<td>ABA, MeJA, H₂O₂</td>
<td>Myrosinase</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
<td>Islam et al. (2009)</td>
</tr>
<tr>
<td>Wounding, heat, H₂O₂, etc.</td>
<td>RBOHD</td>
<td>System ROS signals</td>
<td>Arabidopsis thaliana</td>
<td>Miller et al. (2009)</td>
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<td>ABA, SA, MeJA</td>
<td>PLD, PA, H₂O₂</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
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<td>O₃</td>
<td>SLAC1, OST, H₂O₂</td>
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<td>Arabidopsis thaliana</td>
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<td>Chitosan, SA, MG yeast elicitor</td>
<td>Peroxidase</td>
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<td>ABA, UV-B</td>
<td>GPD1, H₂O₂</td>
<td>Stomatal closure</td>
<td>Arabidopsis thaliana</td>
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<td>ABA, eATP</td>
<td>GPD1, H₂O₂</td>
<td>Stomatal opening</td>
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<td>ABA, H₂O₂</td>
<td>GHR1</td>
<td>Stomatal closure</td>
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<td>Blue light</td>
<td>CYCH;1</td>
<td>Stomatal opening</td>
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<td>Pathogen</td>
<td>CPK5</td>
<td>ROS-mediated cell-to-cell communication</td>
<td>Arabidopsis thaliana</td>
<td>Dubiella et al. (2013)</td>
</tr>
</tbody>
</table>

OGA, oligogalacturonic acid; MV, methyl viologen; P13P, phosphatidylinositol 3-phosphate; ABA, abscisic acid; SA, salicylic acid; ExtCaM, extracellular calmodulin; MeJA, methyl jasmonate; CTK, cytokinin; IAA, auxin; ET, ethylene; OST1, open stomata 1; PTPase, protein tyrosine phosphatase; CO11, coronatine-insensitive 1; CuAO, copper amine oxidase; PLD, phospholipase d alpha 1; PA, phosphatidic acid; GSH, intracellular glutathione; eATP, extracellular ATP; MG, methylglyoxal; S1P, sphingosine-1-phosphate; SNAC1, stress-responsive NAC1; O₂⁻, superoxide anion; pH, intracellular pH; pyrabactin, an ABA agonist; CYCH;1, core cell cycle protein.
optimum regulation between a pair of guard cells and even neighbouring cells in response to a variety of environmental stresses (Bauer et al., 2013). In short, guard cells can finely regulate ROS generation to control stomatal behaviour by integrating chloroplast metabolic pathways with plant hormone signalling pathways. It makes sense that guard cells need to perceive and respond to multiple stimuli if they are to adjust stomatal aperture to balance water loss via transpiration with the need to optimize CO₂ uptake to support photosynthesis.

2. Integration of ROS signalling with multiple signals in guard cells

What are the advantages of the use of ROS signalling in guard cells? ROS ‘signals’ are ubiquitous in plants and animals. Given that ROS production increases after exposure to many stresses, it is not surprising that ROS act as ‘cellular indicators of stress’ and function as integral signalling components in response to both abiotic and biotic stresses (Mittler, 2002). Several important environmental factors, including light, atmospheric CO₂ concentration, air humidity and soil moisture, have major impacts on stomatal movement (Shimazaki et al., 2007; Kim et al., 2010). Meanwhile, plant hormones, including IAA, CTK, ET, brassinosteroids (BRs), MeJAs and SA, also affect stomatal movement (Acharya & Assmann, 2009). Therefore, ROS might be the hub of a signalling network involved in the response to different environmental conditions; guard cells thus provide a helpful single-cell system to elucidate how individual signalling mechanisms function as parts of this cellular signalling network, in which ROS could be integrated with several different interacting signalling pathways.

It is the stoma itself, which comprises a pair of guard cells, that integrates input signals and adjusts cell size and shape accordingly. An advantage of ROS signalling is that ROS can be used as rapid long-distance autopropagating signals that are transferred throughout the plant. Each individual cell along the path of the signal can activate its own ROS-producing mechanisms in an autonomous manner, carrying a ROS signal over long distances. The NADPH oxidase homologue RbohD transmits a rapid systemic signal that travels at a rate of 8.4 cm min⁻¹ and is dependent on the accumulation of ROS in Arabidopsis (Miller et al., 2009). Intra-cellularly generated ROS can move apoplastically as H₂O₂ into neighbouring cells (Allan & Fluhr, 1997). We also found that an ROS-related signal of salt stress at the roots moves rapidly to the stomata in V. faba (G. An & C-P. Song, unpublished). Therefore, ROS display the advantage of being versatile signal molecules with regard to their properties and mobility within a pair of guard cells to integrate various environmental stimuli.

How is specificity achieved in ROS signalling? Different ROS have distinct biological and molecular properties, and differ in terms of their chemical reactivity, half-life and lipid solubility (D’Autreaux & Toledano, 2007; Tripathy & Oelmuller, 2012). For example, H₂O₂ is a small, diffusible and ubiquitous molecule that can be synthesized and destroyed rapidly in response to external stimuli; it thus meets all of the important criteria for an intracellular messenger (Bienert et al., 2006). O₂⁻ is a charged molecule under most physiological conditions and cannot move across a membrane by passive transfer. By contrast, O₂⁻ can be easily converted into H₂O₂, which readily crosses across membranes passively or through water channels (Bienert et al., 2006; Hooijmaijers et al., 2012). O₂⁻ and H₂O₂ can also mediate the formation of lipid peroxides that are membrane-soluble. However, O₂⁻ and H₂O₂ have different preferred biological targets. The observation of a preference for the use of the redox-sensitive transcription factors SoxR and OxyR in Eicheria coli is the best example of how specificity of ROS signalling is achieved. These enable bacteria to discriminate and regulate distinct responses towards ROS. In Arabidopsis, among seven potential ROS-responsive cis-acting elements (ROSE), ROSE4 and ROSE6 were found to be strongly activated by methyl viologen (MV), whereas expression under the control of ROSE5 was increased by the application of H₂O₂. However, the promoter that contained ROSE5 was only activated effectively by 3-AT, and the same promoter was not induced by H₂O₂ (Wang et al., 2013a). Thus, the oxidative responsiveness of individual target genes might be determined by distinct DNA-binding selectivity of the ROSE binding factors in the presence of different ROS. Further experiments should be carried out on guard cells to clarify the roles of the identified ROSE motifs in oxidative signalling, including the characterization of ROSE-binding factors by yeast one-hybrid assays and analysis of the phenotypes of homozygous Arabidopsis plants in which ROSE-binding factors have been knocked out. An important follow-up issue is how guard cells ‘balance’ generic ROS-specific signalling pathways with stimulus-specific systems such as ROS receptors or sensor-mediated responses.

III. Signal transduction pathway of ROS in guard cells

1. ROS sensing and targeting

Over the past decade, a large number of ROS sensors and targets have been uncovered and identified by genetics, biochemistry and genomics technologies (Fig. 1). Nonetheless, the exact mechanisms through which their sensors or receptors remain largely unknown. Unlike ABA, H₂O₂ is too simple structurally to be recognized specifically by a protein. It is thus unlikely that the modulation of protein phosphorylation by H₂O₂ is mediated by the reversible binding of this molecule to protein kinases or phosphatases. On the other hand, H₂O₂ is a mild oxidant that can oxidize cysteine residues in proteins to form cysteine sulphenic acid or disulphide bonds, both of which are readily reduced back to cysteine by various cellular reductants. Several possible ROS sensors have recently been shown to be specifically localized in guard cells. One is ATGPX3, the mutation of which impairs ABA and drought stress responses. Not only do atgpx3 mutant plants produce more H₂O₂ in their guard cells under stress conditions than their wildtype counterparts, but the intense expression of ATGPX3 in guard cells suggests that it functions in the stomata to control water loss (Miao et al., 2006). Moreover, ATGPX3 interacts strongly with ABI2 and controls stomatal aperture in an ABA-dependent manner. Interestingly, the redox states of both ATGPX3 and ABI2 were found to be altered after exposure to H₂O₂. These results suggest that ABI2...
probably represents a sensor for redox regulation by the oxidized form of ATGPX3 in the ABA signalling pathway (Miao et al., 2006). Many documents have established that thioredoxin and not glutathione (GSH) is the physiological electron donor system for the enzymes of the GPX family in Arabidopsis (Comtois et al., 2003; Iqbal et al., 2006; Miao et al., 2006), suggesting that GSH does not contribute to the scavenging of ROS via GPX. Moreover, a GSH-deficient mutant, chlorinal-1 (ch1-1), accumulated less GSH in guard cells than wildtypes, which resulted in ABA-induced stomatal opening was not impaired (Jahan et al., 2003; Iqbal et al., 2006). These results suggest that GSH modulates signalling factors downstream of ROS production in the ABA signalling cascade in guard cells.

In terms of oxidative stress signalling, major roles are played by the transcription factor OxyR in E. coli (Zheng et al., 1998) and by Yap1 in budding yeast (Delaunay et al., 2000). OxyR is activated through the formation of a disulphide bond and is deactivated by enzymatic reduction with glutaredoxin 1 (Grx1); the gene encoding Grx1 is regulated by OxyR (Zheng et al., 1998). The transcription factor Yap1 is activated by oxidation and deactivated by enzymatic reduction with Yap1-controlled thioredoxins. The two cysteines essential for Yap1 oxidation are also essential for its activation by H$_2$O$_2$ (Delaunay et al., 2000). Heat shock transcription factors (HSFs) are also potential ROS sensors. In mammals, redox regulation of heat shock factor 1 is essential for Hsp gene activation and protection from stress, and redox-dependent thiol-disulphide exchange can provide a mechanism that regulates the conformation and activity of the human heat shock transcription factor 1 (HSF1) (Nishizawa et al., 1999; Manalo et al., 2002; Ahn & Thiele, 2003). In plants, HSFs are essential for protection against high-temperature stress, but also participate in the modulation of other abiotic and disease stress responses (Akerfelt et al., 2007; Anckar & Sistonen, 2011). Plant HSFs are involved in signalling crosstalk with several key components of ROS signalling and play a central role in the early sensing of H$_2$O$_2$ stress (Davletova et al., 2005a; Pucciarriello et al., 2012b). It is believed that HSFs function as molecular peroxide sensors that respond to alterations in ROS concentrations during stress by conformational change and multimer formation, leading to subsequent transcriptional activation of their target genes (Miller & Mittler, 2006). Other types of transcription factor that are activated by ROS include members of the WRKY, Zat, RAV, GRAS and Myb families (Tripathy & Oelmuller, 2012). For example, expression of Zat12 (a zinc finger protein) is activated at the transcriptional level during different abiotic stresses and in response to wound-induced systemic signalling, as determined by fusion between the Zat12 promoter and the reporter gene.

Fig. 1 Reactive oxygen species (ROS) sensors and targets in guard cells. Many ROS sensors and targets have been proposed in plants. These components not only monitor intra- and extracellular ROS concentrations, but also respond to ROS signals. Black and red lines indicate activation and suppression, respectively. A dashed line indicates that the exact details of the signal pathway are still unknown. Abbreviations: PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; ABI1, ABA insensitive 1; ABI2, ABA insensitive 2; ATGPX3, Arabidopsis glutathione peroxidase 3; OST1, open stomata 1; CPK, calcium-dependent protein kinase; MPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase kinase; GHR1, plasma membrane receptor kinase 1; HSFs, heat shock transcription factors; Zats, zinc finger proteins; WRKYs, WRKY transcription factors; SLAC1, S-type anion channel; SLAH3, SLAC1 homolog 3; QUAC1, R-type anion channel; GPA1, Arabidopsis $\alpha$-subunit of the trimeric G protein; EBFs, ethylene-responsive factors; cGMP, cyclic guanosine monophosphate; 8-nitro-cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate; GPCR, G protein-coupled receptor; PYL/PYL/RCA, ABA receptor; PP2C, type 2C protein phosphatase.

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In chloroplasts, ROS signalling is coupled to the redox state of the plastoquinone (PQ) pool and plays an important role in the response of plants to changes in environmental conditions (Muhlenbock et al., 2008; Li et al., 2009; Pfannschmidt et al., 2009). Plants optimize their photosynthetic activity by regulating the association of light-harvesting complexes with thylakoid membranes and by adjusting photosystem stoichiometry to rearrange the balance of excitation energy. Putative ROS sensors in plants include peroxiredoxins, NADPH kinases and hitherto unidentified proteins that can detect ROS homeostasis (Moon et al., 2003; Dietz et al., 2010; Moller & Sweetlove, 2010; Rouhier et al., 2010). These results demonstrate a complex interaction among ROS signalling, environmental stresses and plant development. Many downstream targets associated with ROS sensing have been identified. These include Ca2+-, Ca2+-binding proteins such as calmodulin, G-proteins, phospholipids, many protein kinases such as the OXI serine/threonine protein kinase, and the guard cell SLAC1 protein (Wang & Song, 2008; Mittler et al., 2011; Tripathy & Oelmuller, 2012).

Interestingly, the functional genomic-based approaches used to identify potential ROS receptors or sensors described earlier failed to detect ROS receptors or ROS-sensing transcription factors in plant extracts. Assuming that they do exist, this failure could be the result either of the relatively low abundance of these factors or of the fact that ROS are too small and too reactive to function as ligands.

2. Channels and transporters

It is well documented that the concentration of ions determines guard cell turgor and the size of the stomatal aperture. It appears that ROS play a role in the regulation of ABA- and non-ABA-induced ion changes in guard cells. Regarding K+ channels, Zhang et al. (2001b) reported that \( I_{\text{in}} \) (the current of inwardly rectifying K+ channel) in Vicia guard cells is suppressed by exogenous H2O2. However, Köhler et al. (2003) found that \( K^+\)-permeable channels of Vicia guard cells responded differently to ABA and H2O2, whereas ABA depressed the activity of \( K^+\) channels in a reversible manner, H2O2 irreversibly depressed the activities of both \( K^+\)in and \( K^+\)out channels in guard cells immersed in H2O2 in the concentration range 1–50 \( \mu \)M. This suggests that H2O2 induced by different stimuli could act on \( K^+\)out channels. The identity of the \( K^+\) channels regulated by H2O2 in response to ABA is not yet known, although it is known that jasmonic acid (JA)-induced stomatal closure via ROS is affected in the \( K^+\)out channel mutant gork1 (the gated outwardly rectifying K+ channel 1) (Suhita et al., 2004). With regard to Ca2+-channels, \( H_2O_2 \) has been shown to mediate ABA signalling by activating Ca2+-permeable (\( I_{\text{Ca}} \)) channels, through which Ca2+ is released into the cytoplasm, triggering stomatal closure (Pei et al., 2000). A more recent study indicated that PYR/PYL/RCAR ABA receptors regulate \( K^+\) and Cl− channels through ROS-mediated activation of Ca2+ channels at the plasma membrane of intact Arabidopsis guard cells. Although basal activity of Ca2+-channels was not affected in pyr1/pyl1/pyl2/pyl4 quadruple mutant, ABA-induced ROS increases were impaired and loss of ABA-evoked Ca2+ channel activity in the mutant (Wang et al., 2013b). By investigating activation of the plasma membrane, Kwak et al.
(2003) found that a significantly smaller proportion of atrbohD/F guard cells displayed ABA-induced increases in [Ca\(^{2+}\)]\(_{\text{c}}\), than wild-type guard cells (Kwang et al., 2003). Moreover, the observation that the activation of \(I_{\text{Ca}}\) by exogenous \(H_2O_2\) was not affected in atrbohD/F guard cells suggests that ATRBOH functions as the \(H_2O_2\)-activating calcium channel in ABA signalling (Kwang et al., 2003). However, the possibility that exogenously applied \(H_2O_2\) may not act in the same way as \(H_2O_2\) produced and/or released within the guard cells cannot be ruled out. As previously mentioned, \(H_2O_2\) may not act in the same way as ABA during stomatal closure. This suggests that, rather than acting as a critical component of the ABA pathway, \(H_2O_2\) signalling converges with that of ABA at the point of Ca\(^{2+}\) activation.

It has been proposed that increased intracellular CO\(_2\) enhances anion channel activity to mediate the efflux of osmoregulatory anions (Cl\(^{-}\) and malate\(^{3-}\)) from guard cells; these anions function as important regulators of stomatal closure and are essential for mediating stomatal responses to physiological and stress stimuli (MacRobbie, 1998; Hetherington & Woodward, 2003). The SLAC1 protein was the first component of the guard cell S-type anion channel to be identified (Negi et al., 2008; Vahisalu et al., 2008). Mutation of the SLAC1 gene causes defects in S-type channel currents and in stomatal responses to \(O_3\), CO\(_2\), light–dark transitions, \(H_2O_2\), NO, Ca\(^{2+}\) and ABA (Vahisalu et al., 2008). However, whether \(H_2O_2\) acts directly on these ion channels or regulates their function via other signalling cascades is not known.

Geiger et al. (2011) identified another anion channel, the SLAC1 homologue 3 protein (SLAH3), as being involved in the guard cell pathway downstream of ABA and defined its mode of regulation through an ABA receptor–phosphatase RCAR1-ABI complex and a calcium-dependent kinase, CPK21. Unlike previously characterized anion channels that are regulated by ABA and contribute to stomatal closure, the activation of SLAH3 is promoted by nitrate and is 20 times as permeable to nitrate ions as it is to chloride ions. Thus, SLAH3 may integrate nitrate signalling and metabolism with signals initiated by drought conditions to control respiration and water loss. An interesting model illustrated the putative pathway of NO biosynthesis. Mediated by \(H_2O_2\)-activated mitogen-activated protein kinase 6 (MPK6) in Arabidopsis, this pathway proceeds through phosphorylation of the NIA2 isoform of nitrate reductase (NR) at Ser-627 (Wang et al., 2010). Mutants affected in nitrate assimilation or nitrate transport show altered responses to ABA, and crosstalk between nitrate and ABA signals has been discussed (Matakiadis et al., 2009). ABA-IMPORTING TRANSPORTER (AIT) 1, which had been characterized as the low-affinity nitrate transporter NRT1.2, mediates cellular ABA uptake. Because AIT1/NRT1.2 imports nitrate in addition to ABA, it is possible that the ABA-related phenotypes in stomatal aperture observed in ait1 might be caused indirectly by disruption of nitrate signalling (Kanno et al., 2012). In addition, the effects of SA promotion of stomatal closure and NO synthesis are significantly suppressed in NR single mutants of nia1 and nia2 or the double mutant nia1/nia2, compared with the wildtype plants (Hao et al., 2010). However, it remains unknown whether there is a link between SLAH3 activity and the NO produced by NR in guard cells. It would be possible that SLAH3 is connected to NO signalling in guard cells through NR involved in nitrate signalling and this would also be a promising challenge.

3. Phosphorylation of the main players in guard cell signalling

As key messengers of the cellular redox state, ROS also modulate protein phosphorylation through redox and the promotion of the formation of protein disulphide bonds (Salmeen & Barford, 2005; Fedoroff, 2006). Likely targets include the protein phosphatases, the mitogen-activated protein kinases (MAPKs), MAPK phosphatases and the calcium-dependent protein kinase (CPK) (Meinhard & Grill, 2001; Ulm et al., 2001, 2002; Meinhard et al., 2002; Meskiene et al., 2003; Kimura et al., 2012). For example, ABI1 and ABI2 phosphatase activity is very sensitive to \(H_2O_2\) and both the enzyme activities are significantly inactivated by \(H_2O_2\) (Meinhard & Grill, 2001; Meinhard et al., 2002). Arabidopsis isofrom CPK5 of the plant calcium-dependent protein kinase family becomes rapidly biochemically activated in response to PAMPs stimulation. CPK5-dependent in vivo phosphorylation of RBOHD occurs on both PAMP and ROS stimulation (Dubiella et al., 2013).

The ABA-binding receptor (PYR/PYL/RCAR) inhibits PP2C (ABI1) activity and releases OST1 to phosphorylate and activate NADPH oxidase, and then causes \(H_2O_2\) production (Leung et al., 1997; Merlot et al., 2001; Santiago et al., 2009; Kepka et al., 2011). \(H_2O_2\) is an important signalling molecule located downstream of OST1 in guard cells because ABA-induced ROS production does not occur in ost1 mutant plants (Mustilli et al., 2002; Assmann, 2003). This signalling model has also been verified in another study (Yoshida et al., 2006). In French bean, both forskolin (an adenylylcyclase activator) and analogues of cAMP, such as dibutyryl cAMP, potentiate the oxidative burst that increases \(H_2O_2\) production (Kurosaki et al., 1994). Secondly, \(H_2O_2\) is also an important second messenger, which results in activation or inactivation of downstream signalling components and ultimately regulates stomatal movement (Desikan et al., 2004; Wang & Song, 2008). \(H_2O_2\) activates MAPK and promotes stomatal closure (Lu et al., 2002; Desikan et al., 2004; Jiang et al., 2008). Time-course analysis of \(H_2O_2\) production and MAPK activation showed that the accumulation of \(H_2O_2\) preceded the activation of MAPK (Jiang et al., 2008). Given that AtMPK3 antisense plants are less sensitive to exogenous \(H_2O_2\)-induced stomatal closure than wildtype plants, MPK3 probably acts downstream in the cellular signalling pathway activated by \(H_2O_2\) (Gudesblat et al., 2007).

On the basis of a cell type-specific functional genomics approach, two MAPK genes, MPK9 and MPK12, were shown to be preferentially and highly expressed in guard cells. Moreover, mpk9/12 double mutants showed enhanced transpirational water loss and ABA- and \(H_2O_2\)-insensitive stomatal response relative to the mpk9 or 12 single mutant. Although ABA and calcium failed to activate anion channels in guard cells of mpk9/12, ABA and \(H_2O_2\) treatments enhanced the protein kinase activity of MPK12. These results provide genetic evidence that these two MAPKs act upstream of anion channels and downstream of ROS to promote ABA signalling in guard cells (Jammes et al., 2009).
4. Transcriptional regulation

Previous studies indicated that ROS can regulate gene expression in Arabidopsis. Although individual studies revealed different expression profiles, probably as a result of variation in experimental approaches and types of stress applied, certain general trends emerged. Specifically, the genes induced or inhibited by ROS were classified into the functional groups of signal transduction, antioxidative effects, transcriptional regulation and chloroplast function (Desikan et al., 2001; Wang et al., 2006).

For example, the steady-state abundances of transcripts encoding RbohD and the transcription factor HSFA4a were found to be rapidly elevated in apx1 null mutant plants (Davletova et al., 2005a). The zinc finger protein Zat12 is thought to be involved in cold and oxidative stress signalling in Arabidopsis. Zat12 expression is activated by different abiotic stresses and in response to a wound-induced systemic signal, as shown by fusion between the Zat12 promoter and the luciferase reporter gene (Davletova et al., 2005b). Treatment of wildtype plants with \text{H}_2\text{O}_2 resulted in a significant increase in the abundances of 564 transcripts, including those that encode HSFs, NADPH oxidase, mitochondrial alternative oxidase, MAPKs, WRKY transcription factors, several zinc finger proteins and a number of transcripts associated with calcium signalling (Davletova et al., 2005b). Another microarray analysis showed that the transcripts induced by wounding are similar to those responsive to \text{H}_2\text{O}_2 and that 84% of the transcripts that were systemically elevated after wounding were previously reported to be \text{H}_2\text{O}_2-responsive (Davletova et al., 2005a,b). These included at least three proteins implicated in calcium signalling, a number of ROS-responsive transcription factors and several proteins with putative kinase activity, including two receptor-like kinases. Although ROS are the signal molecules capable of effecting large changes in the transcriptome, it is not known whether it is actually the signal per se or whether the time-frame of ROS effects on the gene expression is required to generate an intracellular signal in guard cells. Certainly, increased ABA and sugar signal in guard cells results in new transcriptional profiles (Leonhardt et al., 2004; Bates et al., 2012), so guard cells must import ROS signalling to the nucleus.

Redox signalling represents a special form of redox regulation linked to transcription, translation and associated changes that are induced by ROS. In yeast and bacteria, transcription factors such as AP-1, Yap1 and OxyR have been described to form disulphides either directly or through glutaredoxin in response to \text{H}_2\text{O}_2. The link between transcription factor and guard cell signalling is still unclear. One example is nonexpressor of pathogenesis-related genes 1 (NPR1), which is a redox-sensitive protein that confers immunity to a broad spectrum of pathogens regulated by disulphide bonds. In an uninduced state, NPR1 exists as an oligomer formed through intermolecular disulphide bonds in the cytoplasm. Upon induction of systemic acquired resistance (SAR), a bihasic change in cellular reduction potential occurs, resulting in reduction of NPR1 to a monomeric form. Monomeric NPR1 accumulates in the nucleus and activates gene expression (Mou et al., 2003). The activities of NPR1 and of the TGA family of transcription factors TGA1 and TGA4 have been shown to be modulated by SA-induced redox modifications of key cysteine residues (Fobert & Despres, 2005).

A recent study indicated that NPR1 acts downstream of SA, but upstream of ABA, in stomatal guard cell signalling (Zeng & He, 2010). Moreover, in \textit{apx1} plants and plants expressing antisense CATALASE gene, as well as the CATALASE inhibitor 3-AT, the pathogen-triggered or SA-induced NPR1 nuclear translocation is prevented by accumulation of \text{H}_2\text{O}_2 in the cytosol. Increased accumulation of cytoplasmic ROS in \textit{apx1} mutants reduced the NPR1-dependent gene expression (Peleg-Grossman et al., 2010). These data show that \text{H}_2\text{O}_2 is a negative regulator of NPR1 translocation to the nucleus, limiting the NPR1-dependent gene expression.

A previously unknown zinc finger transcription factor, drought and salt tolerance (DST), was also characterized in rice (Huang et al., 2009). DST negatively regulates stomatal closure by the direct modulation of genes related to \text{H}_2\text{O}_2 homeostasis. Loss of DST function increases stomatal closure and reduces stomatal density, which results in enhanced drought and salt tolerance in rice. DST binds directly to the DST-binding sequence (DBS) element in the promoter of genes related to \text{H}_2\text{O}_2 homeostasis and activates their transcription, thereby inhibiting \text{H}_2\text{O}_2 accumulation. Inhibition of \text{H}_2\text{O}_2 accumulation influences stomatal closure and, ultimately, abiotic stress tolerance. However, the pathway for regulation of stomatal density remains to be elucidated in future studies. The suppression of DST expression following drought and salt stress resulted in the down-regulation of genes related to \text{H}_2\text{O}_2 homeostasis, such as the peroxidase 24 precursor, the promoter of which contains DBS; the resulting accumulation of \text{H}_2\text{O}_2 and promotion of stomatal closure ultimately enhance drought and salt tolerance. This ABA-independent pathway differs from the ABA-induced \text{H}_2\text{O}_2 accumulation pathway that controls stomatal closure. These findings provide a novel pathway for the signal transduction of DST-mediated \text{H}_2\text{O}_2-induced stomatal closure, and an important genetic engineering approach for improving abiotic stress tolerance in crops (Huang et al., 2009; Song & Matsuoka, 2009).

Is there a ROSE or specific transcription factor? Recently, bioinformatics and approaches that involve chromatin immuno-precipitation (ChiP) identified seven potential ROSEs. The APETALA2/ethylene-responsive element binding factor 6 (ERF6) binds specifically to the ROSE7/GCC box. Coexpression of ERF6 enhances luciferase activity driven by ROSE7. Moreover, ERF6 interacts physically with MPK6 and MPK6-mediated ERF6 phosphorylation that affected the dynamic alternation of the ERF6 protein, which resulted in changes in ROS-responsive gene transcription (Wang et al., 2013a). Although there is presently no solid evidence for the involvement of \textit{de novo} mRNA synthesis in response to ROS in guard cells, this could stimulate new research in this respect.

IV. Specificity of ROS signalling

Why do common cellular machinery and responses originate from multiple environmental conditions? In addition, how do common
stomatal closure and opening develop a response that is unique and appropriate for a given stress? The answers to these questions remain unclear at a satisfying level of granularity, but the connections between a pair of adjacent guard cells, and the key role played by ROS in coordinating this signalling, provide another possible example of how cells coordinate the activity of various types of stress response and optimize stomatal aperture size to adapt to environmental conditions.

1. Regulation of redox homeostasis

Changes in the redox state of proteins are a reversible post-translational alteration in the properties of a protein (typically the activity of an enzyme) as a result of a change in its oxidation state. These changes are independent of terminal oxidation: an irreversible reaction that marks proteins for degradation. There are several sources of ROS and antioxidant mechanisms within various subcellular locations in plant cells (Bray, 2000; Neill et al., 2002). Under normal physiological conditions, the appropriate redox balance in each subcellular compartment is determined by the relative rates of ROS generation and removal (Buchanan & Balmer, 2005; Foyer & Noctor, 2005). Any stimulus that increases ROS and/or decreases antioxidant activity will disturb the redox balance and therefore induce oxidative stress. In addition to damaging effects, oxidative stress may alter the cellular redox potential (also termed the ‘redox environment’; Schafer & Buettner, 2001). The intracellular environment is maintained within a range of voltages, usually lower than ~200 mV, which supports appropriate reductions of the cellular pools of NADPH and NADH. The electronegativity of the cell is maintained by millimolar concentrations of reduced GSH (Konigshofer et al., 2008). It is possible that thiol-containing signalling proteins with midpoint potentials within the physiological range might exist in either a reduced or an oxidized state under physiological conditions, with the protein adopting a conformation commensurate with the state of reduction. Altered conformations may modify protein function by, for example, activating or inactivating it. Oxidative stress will cause the intracellular redox environment to become more electropositive. This may induce a shift in the redox environment away from the physiological range at which thiol groups can exist in either an oxidized or reduced state, and thus potentially interfere with signalling pathways.

As mentioned earlier, signalling mediated by ROS involves heterotrimeric G-proteins (Joo et al., 2005) and protein phosphorylation regulated by specific MAPKs and tyrosine phosphatases (Kovtun et al., 2000; Gupta & Luan, 2003). The biochemical and structural bases of the activation of kinase pathways by ROS remain to be established in plants, but thiol oxidation probably plays a key role. The thiol-based regulation of glutamate-cysteine ligase provides a post-translational mechanism for modulating enzyme activity in response to the in vivo redox environment and suggests a role for oxidative signalling in the maintenance of GSH homeostasis in plants (Hicks et al., 2007). Thiol groups are probably important in other types of redox signal transduction, including ROS sensing by receptor kinases, such as ETHYLENE RECEPTOR1 (ETR1), which mediates stomatal closure in response to H₂O₂ (Desikan et al., 2005). Membrane-bound ETR1 is a homodimer formed by an intermolecular disulphide bridge. The formation of this disulphide bridge is necessary for ETR1 function (Schaller & Bleeker, 1995). The observations that guard cells with a higher than normal reduction of the ascorbate pool were less responsive to H₂O₂ or ABA signalling than normal guard cells, and plants with this alteration in the redox state of guard cell ascorbate exhibited enhanced water loss after the imposition of drought conditions, suggested that the redox state of ascorbate plays an important role in controlling H₂O₂-mediated stomatal closure (Chen & Gallie, 2004). Deficiency of cytosolic ascorbate peroxidase (APX1) results in the accumulation of H₂O₂ in Arabidopsis grown under optimal conditions. Knockout-Apx1 plants exhibit altered stomatal responses during light stress (Pnueli et al., 2003). Furthermore, genetic and pharmacological experiments show that GSH functions as a negative regulator of ABA signalling and may control redox status of certain signal components downstream of ROS production in guard cells (Okuma et al., 2011; Akter et al., 2012); and suppression of CAT activity enhances ABA-induced stomatal closure (Jannat et al., 2011a). Importantly, the apx1 mutant phenotype and GSH-related mutants show different phenotypes in stomatal responses. These studies expand our understanding of how redox and ROS concentrations balance some of the key metabolic pathways in guard cells.

2. Specific stimulus–response pairs

The connections between a pair of guard cells in relation to ROS provide another good example of how cells coordinate the activity of various types of stress response and optimize stomatal aperture size to adapt to environmental conditions. It has been proposed that ABA can regulate a large proportion of BR-responsive genes and crosstalk between ABA and BR after BR perception, but before their transcriptional activation (Zhang et al., 2009). The Arabidopsis sax1 mutant, which is BR-deficient, shows altered sensitivity of growth responses to ABA and enhanced stomatal closure in response to ABA (Ephritikhine et al., 1999). The observation that reductions in the sizes of stomatal apertures of BR-deficient (det2) and BR-insensitive mutants (bri1) in Arabidopsis were more severely inhibited by ABA than in normal plants suggests that BR counteracts ABA in regulating stomatal movement (Xue et al., 2009). Recently, BRs were shown to play opposing roles in the regulation of stomatal development in opposite stomatal phenotypes through two different stomatal signalling pathways (Kim et al., 2012; Ye et al., 2012).

Both ABA and ET are able to induce stomatal closure independently (Desikan et al., 2006; Wang & Song, 2008). However, when applied simultaneously, they fail to achieve full closure (Tanaka et al., 2005). A recent study has shown that the effects of ROS in guard cells are almost entirely dependent on the amount and duration of ROS production through the time-course measurements of ROS and aperture over 60 min in guard cells under single stimuli (ABA or ET) and under combined stimulus (ABA plus ET). When both hormones are present and ROS are removed swiftly after an initial burst of production, the closure process was reversed. Thus, there is a distinct role for two
antioxidant mechanisms during stomatal closure: a slower, delayed response activated by a single stimulus (ABA or ET) and another more rapid mechanism that is only activated when both stimuli are present (Beguerisse-Diaz et al., 2012).

Previous studies showed that an increased JA content of plants promoted stomatal closure in response to water stress and drought stress (Creelman & Mullet, 1995). MeJA has concentration-dependent effects on guard cell K+ channels and promotes stomatal stress (Creelman & Mullet, 1995). MeJA has concentration-promoted stomatal closure in response to water stress and drought stress (Okuma et al., 2011). These results indicate that GSH negatively modulates a signal component other than ROS production, Ca2+ oscillation and cytosolic alkalization in the pathways that transduce ABA and MeJA signals in Arabidopsis guard cells.

Stomata are also active ports of bacterial entry during infection. SA induces stomatal closure to prevent bacterial attack (Melotto et al., 2006). In SA-deficient mutant plants, stomatal closure is impaired in response to bacterial pathogens, which indicates that SA is required for stomatal defence (Melotto et al., 2008). The ABA-insensitive ost1 mutant and the ABA-deficient aba3 mutant do not show stomatal closure in response to bacterial infection, and SA also markedly induces NO synthesis in guard cells and stomatal closure in Arabidopsis wildtype plants (Melotto et al., 2006). Furthermore, the MAPK phosphatase PP2C5, which modulates ABA-dependent MPK4 activation, positively regulates stomatal aperture (Brock et al., 2010). These results imply that ABA-induced stomatal closure is also involved in plant–bacteria interactions and that the synergy of SA and ABA signalling mediates stomatal closure in response to pathogen attack. However, a recent study revealed that increasing MPK4 activity compromises plant basal defense to pathogen invasion and reduces pathogen-induced SA accumulation (Berrieri et al., 2012). Therefore, Arabidopsis MPK4 may play a role in plant immunity, which is not related to stomatal closure or at least not measurable for the time points and conditions analysed.

Recent studies demonstrated that the SIZ1 mutation, which leads to impairment in the SIZ-type small ubiquitin-related modifier E3 ligase in Arabidopsis (Ezaki et al., 2001; Muraoka & Miura, 2005), conferred ABA hypersensitivity (Miura et al., 2009) and enhanced the accumulation of SA and SA-inducible gene transcripts (Lee et al., 2007). SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling SA-induced accumulation of ROS (Miura et al., 2012). However, another report showed that endogenous ABA is not involved in SA-induced stomatal closure because SA can induce stomatal closure not only in Arabidopsis wildtype plants but also in ABA-deficient aba2 mutant plants. Moreover, SA-induced stomatal closure was accompanied by the production of ROS mediated by salicylhydroxamic acid-sensitive peroxidases (Issak et al., 2013). Taken together, these lines of evidence suggest that ABA and SA coordinately regulate ROS production in response to drought and pathogen invasion; however, these signalling pathways are independent of each other. Unfortunately, many of the detailed steps of crosstalk between SA and ABA signalling in guard cells remain to be discovered.

A recent review summarized the evidence that the red-light response of stomatal movement is sensed by the redox state of the photosynthetic electron transport chain (Busch, 2013). Interestingly, blue light-induced stomatal opening has also been closely linked with ROS. An increase in ABA concentrations, which is mediated by H2O2 accumulation, inhibits blue light-dependent phosphorylation of H+-ATPase in guard cells (Zhang et al., 2004) and stimulates stomatal closure (Goh et al., 1996). Phototropins could be the target of H2O2, because phototropins have cysteine residues that are essential for their activities (Salomon et al., 2000). It has been found that ABA is accumulated in response to high light stress in wildtype leaves (Rossel et al., 2006) and 61% of the HL-responsive genes were altered in their expression in ABA-treated leaves (Bechthold et al., 2008).

O3 severely inhibits K+-stimulated ATPase activity and this inhibition is completely reversed by the addition of sulphhydryl compounds (Dominy & Heath, 1985). Subsequently, it was found that O3 can close the stomata by an alteration of Ca2+ transport (Castillo & Heath, 1990), an increase of Ca2+ concentrations in guard cells (De Silva et al., 2001) or an inhibition of guard cell K+ channels (Torsethaugen et al., 1999). Indeed, transcription profiling in response to O3 showed twofold or greater differential expression for 2385 genes after 3 and 6 h of treatment (Ludwikow et al., 2004). Interestingly, the Arabidopsis heterotrimeric G protein mediated ROS generation by the regulation of NADPH oxidase in response to O3 (Joo et al., 2005); in addition, preferential expression of tobacco MPK4 (NtMPK4) under O3 treatment and abnormal regulation of stomatal closure were observed in NtMPK4-silenced plants (Gomi et al., 2005).

3. Specificity of ROS signals

An intriguing feature that has emerged from functional genomics and biochemistry studies is the apparent specificity of ROS signals (Fig. 2). How is ROS signal specificity determined by guard cells? Despite the lack of sufficient evidence for the specificity of ROS signals in guard cells, perhaps analysis of other aspects of the research
data from different biological processes will be given the appropriate cues in the future study. The differences in gene expression and/or biological responses caused by different types of ROS may be attributed to specific signal transduction mechanisms that act on different sets of response elements in the promoters of the target genes. As discussed in Section II, different ROS signals are also associated with particular degrees of specificity, and this specificity depends on the ROS type or its subcellular site of production (Gadjev et al., 2006). An analysis of transcriptome data generated from ROS-related microarray experiments was performed to assess the specificity of ROS-driven transcript expression (Gadjev et al., 2006). In addition to several transcripts that act as general oxidative stress response markers, some marker transcripts that are specifically regulated by H$_2$O$_2$, O$_2$ or 1O$_2$ were identified (Gadjev et al., 2006). A series of cis-regulatory elements specific for different types of ROS were identified in Arabidopsis thaliana (Petrov et al., 2012).

However, there was little overlap with genes reportedly activated by different types of ROS, including H$_2$O$_2$, O$_2$ or 1O$_2$ (Miller et al., 2009).

Moreover, an analysis of 217 antioxidant and 180 ROS marker genes also showed that some of the genes were specifically regulated by either the catalase inhibitor 3-AT or PQ (Mehterov et al., 2012). Consistent with this, the same ROSE-fused luciferase reporter displayed different luciferase activity under different ROS stimuli (Wang et al., 2013a). Intriguing examples of ROS signal specificity were reported from analysis of an Arabidopsis double-mutant knockout of APX1 and CAT2, as well as single mutants in tobacco. The double mutants were surprisingly more tolerant to different environmental conditions than the wildtype and the single mutants of apx1 and cat1 (Rizhsky et al., 2002; Vanderauwera et al., 2011). The mechanism behind this was that a lack of APX1 and CAT2 in Arabidopsis caused a unique ROS signature in cells, which triggered a novel acclimation response that involved activation of the DNA damage response. A similar response was not found in the apx1 or cat2 single mutants, which demonstrated the need for a specific ROS signature for its activation, a signature that was only found in the double mutants (Vanderauwera et al., 2011). This protective response requires a coordinated balance between different ROS-removal mechanisms that are active outside the nucleus, that is, in the cytosol and peroxisomes. In a particularly interesting review, three different models of elucidating ROS specificity were proposed (Mittler et al., 2011). However, deciphering the complexity of ROS signalling within guard cells would require the development and characterization of many more mutants deficient in ROS signalling and the combined use of these mutants with advanced ROS imaging tools that are specific to particular subcellular compartments.

Furthermore, it was found that ROS production increased during the O$_3$ stress response (Overmyer et al., 2008), and rapid accumulation of NO induced by O$_3$ was initiated from guard cells, spread to adjacent epidermal cells and eventually moved to mesophyll cells (Ahlfors et al., 2009). Subsequently, novel molecular mechanisms of O$_3$-triggered rapid stomatal response were proposed, as follows: O$_3$-induced ROS production leads to the activation of OST1, which phosphorylates SLAC, followed by the activation of S-type anion channels and anion efflux out of guard cells; this, in turn, causes plasma membrane depolarization and activation of GORK1, and K$^+$ efflux from guard cells; finally, the overall efflux of anions and K$^+$ contributes to the loss of guard cell turgor, leading to stomatal closure. Owing to the fact that protein phosphatases ABI1 and ABI2 can regulate OST1 activity, it is suggested that this process is an ABA-dependent pathway (Vahisalu et al., 2010). Consistent with these observations, guard cells of the Arabidopsis de-etiolated 3 (det3) mutant, a V-ATPase mutant, were shown to be disrupted in terms of stomatal closure and [Ca$^{2+}$]$^{\text{cyt}}$ oscillation in response to H$_2$O$_2$, but not to ABA, which again suggests the existence of stimulus-specific signalling pathways (Allen et al., 2000).

Under intense-light stress, guard cell chloroplasts can generate a large number of ROS (${}^1$O$_2$, O$_2$ and H$_2$O$_2$), which are implicated as triggers of signalling pathways that influence the expression of nuclear-encoded genes that might initiate acclimation processes or

**Fig. 2** Specificity of reactive oxygen species (ROS) in plants. ROS signal specificity has been shown at several different levels. First, plants can produce different types of ROS (O$_2$-, H$_2$O$_2$ and 1O$_2$) in response to different stresses, such as moderate light, strong light, UV-B, cold and drought. Secondly, different compartments (plastid, mitochondria, peroxisome, cytosol) can generate different types of ROS under multiple stresses. Given that no ROS signalling network has been identified in vacuoles, it might be informative to study ROS signalling and metabolic mechanisms in vacuoles. Thirdly, ROS marker transcripts were specifically regulated by O$_2$-, H$_2$O$_2$ and 1O$_2$. However, these were general markers of a response to oxidative stress, because their expression levels were at a steady state under different ROS stresses. In other words, there was crosstalk between different types of ROS activating distinct signalling pathways. Finally, the same kind of ROS in response to different environmental stimuli may generate different ROS signalling pathways (see Mittler et al., 2004, 2011; Gadjev et al., 2006; Laloi et al., 2007; Petrov et al., 2012).
trigger cell death responses, depending on the degree of photooxidative stress suffered (Danon et al., 2005; Rossel et al., 2006). O2 has been proposed to be the major ROS produced in plant cells exposed to intense light (Gonzalez-Perez et al., 2011) and to be responsible for photooxidative damage to plant leaves (Triantaphylides et al., 2008). In addition to its cytotoxicity, O2 is known to be a key signal molecule involved in cellular responses (Suzuki et al., 2012a) and O2 signalling can communicate with other ROS signals, as shown by the antagonising effects of O2- on hydrogen peroxide (H2O2) on O2-induced gene expression (Laloi et al., 2007). Recent research indicated that Arabidopsis b-cyclocitrinal, a volatile derivative of b-carotene that accumulates in Arabidopsis leaves under intense-light stress, induced changes in the expression of a large set of O2-specific genes. Treatments of Arabidopsis plants with this volatile molecule were also associated with increased tolerance to photooxidative stress, which suggests that b-cyclocitrinal is an intermediate in the O2 signalling pathway that results in acclimation (Ramel et al., 2012). Moreover, it was found that ABA accumulates in response to intense-light stress in wildtype leaves (Rossel et al., 2006) and 61% of high light-responsive genes exhibited altered expression in ABA-treated leaves (Bechtold et al., 2008). These findings emphasize the strong linkage between leaf water potential and the capacity to dissipate excitation energy.

4. Dynamic ROS signalling and systematic stomatal responses

How are local stress signals transmitted systemically to distant parts of a plant for cell-to-cell communication and long-distance signalling in the response of plants to extreme environmental conditions? Miller et al. have shown that the gene RhodD encodes a plant NADPH oxidase that generates ROS as a rapid form of systemic signalling travelling at a rate of 8.4 cm min-1 (Miller et al., 2009). Signal propagation was accompanied by the accumulation of ROS in the extracellular spaces between cells and was inhibited by the suppression of ROS accumulation at locations distant from the initiation site. Their data reveal the profound role that ROS play in mediating rapid, long-distance, cell-to-cell propagating signals in plants.

There is a feedback control mechanism for modulating dynamic changes of ERF6 activity, which operates via MAPK cascade-mediated phosphorylation and leads to increased transcription of ROS-responsive genes by both sorting of the nucleus and degradation of transcription factors (Wang et al., 2013a). These dynamic changes of ERF6 activity are probably important for photoprotection because timely and efficient modulation of ROS homeostasis benefits plants that are faced with a fluctuating light environment, in terms of either hourly variation of light emission over the course of a day or sudden exposure to a different intensity of light.

Interestingly, many environmental stimuli can induce dynamic changes in stomatal behaviour. Such oscillation usually occurs after a sudden change in one environmental factor in a relatively constant environment (Wang et al., 2005). As a special rhythmic stomatal movement that usually occurs at smaller stomatal apertures, stomatal oscillation can maintain CO2 absorption at a sufficient rate and reduce water loss at the same time, which may improve water-use efficiency. The oscillation of ROS signals in root hairs was also clearly demonstrated (Monshausen et al., 2007; Takeda et al., 2008). However, further research is required to establish whether there is a link between stomatal oscillation and the temporal-spatial concept of an ROS wave or ROS oscillation.

V. Conclusions and prospects

Unquestionably, ROS signalling is a central hub for information flow in guard cells and much progress has been made in recent years to reveal important components in this signalling network; however, the current picture remains very fragmented. Future efforts should uncover many of the details related to the region upstream of guard cell signalling and the interaction of ROS with other signals, which would advance our understanding of ROS sensing. A very attractive focus of research is the identification of guard cell-specific ROS sensors/receptors using this excellent single-cell model system and building genetic screening systems for mutants with impaired ROS signalling. Future questions that need to be answered include the following. How do guard cells sense ROS or redox homeostasis? Do thiol-based redox sensors exist in guard cells? If so, how are they integrated into ROS signalling pathways and how do they interact with guard cell signalling networks?

Conventionally, H2O2 has been believed to cross membranes freely. However, although many studies have now indicated that H2O2 diffusion across membranes is limited and its transport is dependent on channel proteins such as aquaporins (Bienert et al., 2006), the specific genes involved still need to be identified. Recently, an exciting finding was made which suggests that ROS are a rapid systemic signal dependent on the respiratory burst associated with the oxidase homologue D (RhodD) gene. These results confirm that ROS play a role in mediating rapid, long-distance, cell-to-cell propagating signals in plants. However, this leads to further questions. How are ROS signals transmitted in a pair of guard cells for the orchestration of stomatal function as a systemic response? Apart from the water channels, is there another possibility that ROS accumulation leads to membrane depolarization, then resulting in the channel activation/deactivation that enables the ROS signalling transduction? Can isolated single guard cells generate ROS waves, and, if so, how are these waves propagated? Finally, what is the difference in ROS waves between guard cells and other cells, and how do ROS signals travel within or between guard cells?

Recent work has resulted in significant advances in our understanding of the underlying pathway controlling stomatal development (Pillitteri & Torii, 2012). Considering that ABA and ROS are tightly connected in regulating stomatal movement, it will be interesting to explore the possibility that ROS might be involved in the development of guard cells and stomata. A MAPK signalling cascade is predicted to act downstream of the receptors to negatively regulate stomatal development, headed by the MAPK kinase kinase YODA (Bergmann et al., 2004) and including the MAPK kinases MKK4 and MKK5 and the MAPKs MPK3 and MPK6. In this respect, it is interesting to note that H2O2 activates MAPK modules in guard cells (Gudesblat et al., 2007; Jiang et al., 2008; Wang et al.,
2010). How does environmental and developmental signalling, through the ROS pathway, impinge on the core MAPK pathway to regulate stomatal development? Can ROS be regarded as a potential integration point of environmental and developmental signals that regulate stomatal development? It is also worth investigating whether ROS might be partially responsible for the increase of stomatal density and index in some species owing to water stress and altered ABA content, and for the decrease of the same indices in other species (Beerling et al., 1993; Luomala et al., 2005).

Reactive oxygen species-specific probes and cellular imaging have long been used and developed for real-time detection. These range from ROS probes based on chemical synthetic dyes to genetic reporters and the monitoring of features ranging from ROS generation to dynamic changes of ROS (Meyer & Dick, 2010). For example, the Zat12-luc reporter systems, which were successfully used to monitor ROS dynamics (Davletova et al., 2005b; Miller et al., 2009), and a series of redox probes derived from green fluorescent protein (roGFP) were reported to have been successfully applied for the detection of ROS specificity, quantitative analysis, long-distance transmission and determination of temporal and spatial distribution (Belousov et al., 2006; Meyer & Fricker, 2008; Mullineaux & Lawson, 2008; Meyer & Brach, 2009; Niethammer et al., 2009; Maughan et al., 2010; Rosenwasser et al., 2010). Recently, the properties of roGFP have been constantly updated to ensure increased suitability for in vivo studies of dynamic samples (Oku & Sakai, 2012; Wierer et al., 2012). However, the development of ROS-specific probes for the detection of different ROS in different subcellular compartments is a major challenge.

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