SIGNAL TRANSDUCTION IN STOMATAL GUARD CELLS

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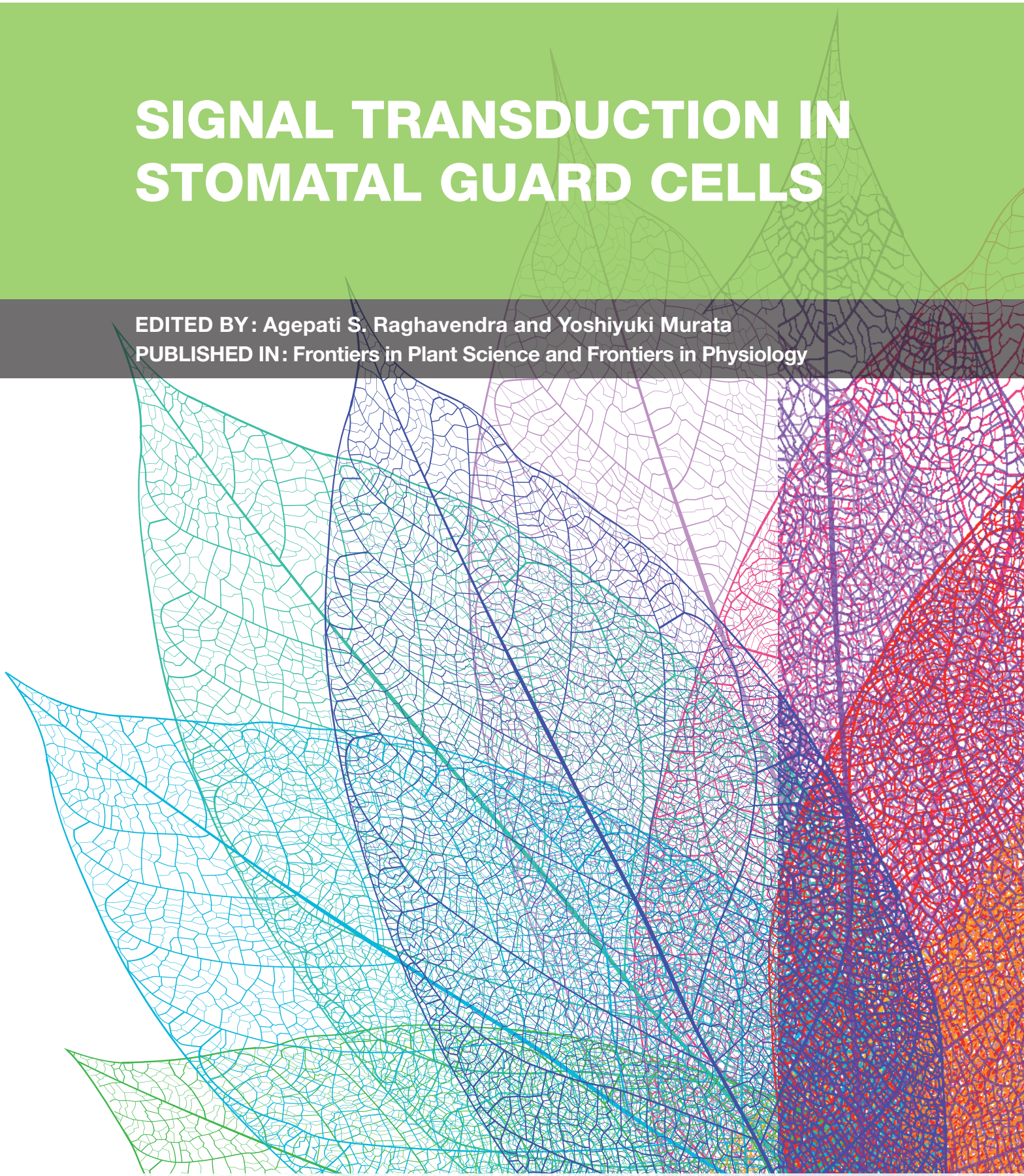

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SIGNAL TRANSDUCTION IN STOMATAL GUARD CELLS

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Stomata, the tiny pores on leaf surface, are the gateways for CO_2 uptake during photosynthesis as well as water loss in transpiration. Further, plants use stomatal closure as a defensive response, often triggered by elicitors, to prevent the entry of pathogens. The guard cells are popular model systems to study the signalling mechanism in plant cells. The messengers that mediate closure upon perception of elicitors or microbe associated molecular patterns (MAMPs) are quite similar to those during ABA effects. These components include reactive oxygen species (ROS), nitric oxide (NO), cytosolic pH and intracellular Ca^{2+}. The main components are ROS, NO and cytosolic free Ca^{2+}. The list extends to others, such as G-proteins, protein phosphatases, protein kinases, phospholipids and ion channels. The sequence of these signalling components and their interaction during stomatal signalling are complex and quite interesting.

The present e-Book provides a set of authoritative articles from 'Special Research Topic' on selected areas of stomatal guard cells. In the first set of two articles, an overview of ABA and MAMPs as signals is presented. The next set of 4 articles, emphasize the role of ROS, NO, Ca^{2+} as well as pH, as secondary messengers. The next group of 3 articles highlight the recent advances on post-translational modification of guard cell proteins, with emphasis on 14-3-3 proteins and MAPK cascades. The last article described the method to isolate epidermis of grass species and monitor stomatal responses to different signals. Our e-Book is a valuable and excellent source of information for all those interested in guard cell function as well as signal transduction in plant cells.

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EDITORIAL
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Editorial: Signal Transduction in Stomatal Guard Cells

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Keywords: abscisic acid, cytosolic calcium, cytosolic pH, ion-channels, microbial elicitors, NO, ROS, secondary messengers

Editorial on the Research Topic

Signal Transduction in Stomatal Guard Cells

INTRODUCTION

During adaptation of plants to water stress/drought, the tiny pores on the leaf surface, called "stomata," play a very important role. Stomatal movements can modulate the entry/exit of not only CO_2/water (Lawson and Blatt, 2014) but also microbial pathogens (Agurla et al., 2014; Arnaud and Hwang, 2015). The stomatal opening/closure is brought out by changes in the turgor of guard cells. The abiotic/biotic stress factors induce a series of changes in the signaling components of guard cells, such as ROS, NO, pH and calcium, leading to efflux of ions, loss of turgor and stomatal closure. Due to their dynamic responses to signals, and the ease of handling leaf epidermis, the stomatal guard cells have been popular systems to study signal transduction in plants.

The guard cells are extremely efficient in their signal integration to optimize stomatal aperture. Murata et al. (2015) summarized the studies on signal transduction pathway in guard cells, with emphasis on downstream components. Extensive work has been carried out using the plant hormones, such as abscisic acid (ABA) and methyl jasmonate (Assmann and Jegla, 2016). Similarly, the elicitors, such as chitosan and flagellin, are also used to study sensing and transduction of signals (Agurla et al., 2014). Guard cells are unique in not only their ability to respond to external signals but also their structure and development. Very few groups are working on development and differentiation of guard cells (Chater et al., 2014; Keerthisinghe et al., 2015; Torii, 2015).

Besides the areas covered in the present research topic, there are additional aspects of contemporary interest. Some of these are: signaling by plant lipids in relation to guard cell function (Puli et al., 2016), molecular mechanisms of sensing CO_2 (Engineer et al., 2016), signals from underlying mesophyll cells of leaf (Lawson et al., 2014) and cross-talk of ABA with ethylene and brassinosteroids during stomatal closure (Shi et al., 2015). Another area is the systems biology to integrate and model the signaling network in guard cells (Medeiros et al., 2015).

ARTICLES IN THE RESEARCH TOPIC

There have been several reviews on signaling components during stomatal closure, which are in different journals. The present research topic has been planned to provide a set of articles as a compendium and a ready source of information for all those interested in guard cell function.

Most of the work on signal transduction in guard cells has been with ABA and MJ, while such studies with microbial elicitors are limited. The guard cells perceive the presence of microbes though the microbe associated molecular patterns (MAMPs). The signaling events initiated by MAMPs overlap with the effects of ABA, particularly with reference to the rise in ROS, NO,

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cytosolic Ca^{2+} and activation of ion channels (Ye and Murata). Agurla and Raghavendra assessed the multiple signaling components induced by plant hormones or microbial elicitors. They proposed that reactive oxygen species (ROS), cytosolic free Ca^{2+} and ion channels are the major converging points while ROS, NO and cytosolic free Ca^{2+} are points of divergence. The end result is the ion channel modulation causing an efflux of K^+/Cl^-/malate from guard cells leading to stomatal closure. The major role of ROS and NO in guard cells during the stomatal closure is well established (Gayatri et al., 2013; Song et al., 2014). However, the role of NO is quite intriguing as NO can either amplify or limit (by scavenging) the effects of ROS (Laxalt et al.). Further, other gasotransmitters such as H_2S can also regulate stomatal aperture (Scuffi et al.).

Abscisic acid induces not only stomatal closure, but also integrates multiple physiological processes, including leaf senescence. Using mutants, Song et al., describe how ABA can regulate the components of senescence, namely gene expression, calcium channel activation in plasma membrane, loss of chlorophyll and ion leakage. Thus, ABA action through Ca^{2+} signaling appears to function during leaf senescence as well.

Protein phosphorylation is an important strategy for integrating different signals in guard cells (Zhang et al., 2014; Vilela et al., 2015). Often the signal transduction processes involve mitogen-activated protein kinases (MAPK), which and drive the cascade of events. Lee et al. highlight the advances in the MAPK-mediated guard cell signaling. These kinases mediate phosphorylation of their next target protein. Balmant et al. describe the methods to study post translational modification (PTM) and redox modification of guard cell proteins. With improved technology, further studies on PTM are bound to intensify and reveal interesting insights. For example, reactive carbonyl species function downstream of ROS production in abscisic acid signaling in guard cells (Islam et al., 2016). Similarly, the 14-3-3 proteins could target and modify different proteins in guard cells (Cotelle and Leonhardt).

The role of guard cell sugars in the stomatal movement is acknowledged, but detailed studies are lacking. Using citrus plants with over-expressed hexokinase I in the guard cells, Lugassi et al. provide a convincing study that hexokinase regulates photosynthesis and promotes stomatal closure in not only annual species, but also in perennials. The description of an optimized procedure for the isolation of abaxial epidermal peels from grasses, including barley, wheat and *Brachypodium,* to study their responses to ABA and CO_2 (Shen et al.), would open up an exciting range of possibilities.

CONCLUDING REMARKS

The articles in our research topic provide interesting leads for future work. The stomatal guard cells are excellent models to study PTM of proteins by ROS as well as NO during signal transduction. Such PTM studies could explain the interactions of 14-3-3 proteins with MAP kinases in guard cells. Hexoses can contribute to the guard cell osmoticum, but their origin from within guard cells or mesophyll cells needs to be investigated. A rise in ROS, NO and cytosolic pH of guard cells is essential for stomatal closure, but their exact sequence and their interactions are quite interesting for further studies. The signaling events initiated by MAMPs are fairly understood, but the identity of MAMP-receptors is to be established.

AUTHOR CONTRIBUTIONS

AR and YM assessed the information in the Frontiers articles, as well as the available literature. Both AR and YM drafted and finalized the manuscript together.

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ORIGINAL RESEARCH
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Abscisic Acid as an Internal Integrator of Multiple Physiological Processes Modulates Leaf Senescence Onset in *Arabidopsis thaliana*

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Many studies have shown that exogenous abscisic acid (ABA) promotes leaf abscission and senescence. However, owing to a lack of genetic evidence, ABA function in plant senescence has not been clearly defined. Here, two-leaf early-senescence mutants (*eas*) that were screened by chlorophyll fluorescence imaging and named *eas1-1* and *eas1-2* showed high photosynthetic capacity in the early stage of plant growth compared with the wild type. Gene mapping showed that *eas1-1* and *eas1-2* are two novel *ABA2* allelic mutants. Under unstressed conditions, the *eas1* mutations caused plant dwarf, early germination, larger stomatal apertures, and early leaf senescence compared with those of the wild type. Flow cytometry assays showed that the cell apoptosis rate in *eas1* mutant leaves was higher than that of the wild type after day 30. A significant increase in the transcript levels of several senescence-associated genes, especially *SAG12*, was observed in *eas1* mutant plants in the early stage of plant growth. More importantly, ABA-activated calcium channel activity in plasma membrane and induced the increase of cytoplasmic calcium concentration in guard cells are suppressed due to the mutation of *EAS1*. In contrast, the *eas1* mutants lost chlorophyll and ion leakage significant faster than in the wild type under treatment with calcium channel blocker. Hence, our results indicate that endogenous ABA level is an important factor controlling the onset of leaf senescence through Ca^{2+} signaling.

Keywords: abscisic acid, leaf senescence, chlorophyll fluorescence, guard cell, cytosolic calcium

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INTRODUCTION

Leaf senescence, involving photosynthesis cessation, degradation of macromolecules, and increase of reactive oxygen species (ROS), as well as contributing to the mobilization of nutrients from old leaves to growing or storage tissues, is regulated by various external and internal factors. In line with this, leaf senescence initiation is affected by many such factors, such as the age of the plant, plant hormones, ROS, transcription factors, protein kinases, nutrient limitation, and drought (Fischer, 2012; Koyama, 2014).

Earlier studies have documented the important role of the phytohormone abscisic acid (ABA) in the regulation of leaf senescence. It has long been considered that ABA accelerates leaf senescence because exogenously applied ABA was shown to promote leaf senescence (Gepstein and Thimann, 1980; Pourtau et al., 2004; Raab et al., 2009; Lee et al., 2011) and endogenous ABA levels have been found to be increased during leaf senescence in many plants (Gepstein and Thimann, 1980; Leon-Kloosterziel et al., 1996; Cheng et al., 2002; He et al., 2005; Breeze et al., 2011; Yang et al., 2014). More importantly, both the upregulation of genes associated with ABA signaling and a dramatic increase in endogenous ABA levels can be observed in many plants during leaf senescence (Tan et al., 2003). Exogenous ABA can induce the expression of many senescence-associated genes (Parkash et al., 2014). In addition, the molecular mechanistic evidence for a positive regulatory role of ABA in senescence comes from functional analyses of receptor-like kinase 1 (RPK1; Lee et al., 2011). RPK1 is a membrane-bound leucine-rich repeat receptor-like kinase that acts as an upstream component of ABA signaling, whose expression was found to increase in an ABA-dependent manner throughout the progression of leaf senescence. Moreover, leaf senescence was accelerated in transgenic plants overexpressing RPK1 and ABA-induced senescence was delayed in *rpk1* mutant plants, suggesting that RPK1 has a role in promoting leaf senescence.

Some studies have shown that ABA inhibits the senescence of cucumber plants grown under low-nitrogen conditions (Oka et al., 2012) and ABA-deficient mutants showed accelerated senescence on glucose-containing medium (Pourtau et al., 2004). In tomato, maize, and Arabidopsis, ABA could maintain shoot growth by inhibiting ethylene production (Sharp, 2002). SAG113 is a PP2C protein phosphatase that acts as a negative regulator of stomatal movement and water loss during leaf senescence (Zhang and Gan, 2012; Zhang et al., 2012). *SAG113* is expressed in senescencing leaves and induced by application of ABA. Leaf senescence was found to be delayed in a *sag113* knockout mutant line (Zhang and Gan, 2012; Zhang et al., 2012). Therefore, the role of ABA in the onset of leaf senescence remains unclear.

It has been reported that several *abscisic acid-dificient* 2 (*aba2*) alleles, as well as other ABA biosynthesis mutants including *aba1*, *aba3*, *abscisic aldehyde oxidase 3* (*aao3*), *9- cis-epoxycarotenoid dioxygenase 3* (*nced3*) have already been isolated and identified by screening *Arabidopsis* mutants (Leon-Kloosterziel et al., 1996; Leung and Giraudat, 1998; Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002; González-Guzmán et al., 2002; Finkelstein, 2013). These studies are mainly focused on stomatal regulation, developmental processes, and stress responses. However, little is known whether ABA specifically modulates leaf senescence. Recent studies showed that an *Arabidopsis* NAC-LIKE, ACTIVATED BY AP3/PI (NAP) transcription factor promotes chlorophyll degradation by enhancing transcription of ABSCISIC ALDEHYDE OXIDASE3 (AAO3), which leads to increased levels of the senescence-inducing hormone ABA (Yang et al., 2014).

In this work, we used chlorophyll fluorescence imaging to isolate two early-senescence *Arabidopsis* mutants (*eas1-1* and *eas1-2*) and performed further studies that showed that they are novel *aba2* alleles. Compared with the wild type, the *eas1* mutants display multiple phenotypes including early germination, larger stomatal aperture, insensitivity to stresses, more chloroplasts in mesophyll cells, higher chlorophyll fluorescence during the early stage of plant growth, and early leaf senescence. Meanwhile, many senescence-associated genes were found to be strongly up-regulated in the *eas1* mutants during the early stage of plant growth. Furthermore, $[Ca^{2+}]_{cyt}$ levels and calcium channel activity of *eas1* mutant guard cells were significantly lower than those of the wild type. These results revealed that the internal ABA level is involved in the control of senescence onset.

MATERIALS AND METHODS

Plant Growth Conditions and Isolation of Mutants

Arabidopsis thaliana plants used were in the C24 and the Columbia 0 background. Approximately 50,000 M1 seeds of the C24 ecotype were mutagenized by treatment with 0.4% EMS solution for 8 h. M2 seeds were obtained by self-fertilization of the M1 plants. Surface-sterilized seeds were plated in MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar and, after 5–7 days, seedlings were transplanted into pots containing a mixture of forest soil:vermiculite (3:1). The potted plants were kept under a cycle of 16 h light/8 h dark and a relative humidity of about 50–70% in a growth room at 20 ± 2°C. The seedlings were used for mapping the *EAS1* gene. The mutant plants were back-crossed twice to C24. The descendants of single progeny derived from each backcross were used for all experiments.

Chlorophyll Measurements and Stress Treatment

Leaves 4 and 5 were detached from plants under normal or stressed conditions. Total chlorophyll was extracted in ethanol and measured spectrophotometrically (He and Gan, 2002). To determine leaf senescence phenotype of *eas1* and wild-type plants under osmotic and oxidative stresses, 20-days-old leaves were floated on water or water containing 10 mM H_2O_2 or 500 mM mannitol in petri dishes under normal condition as described in the figure legends.

Dark Treatment

Seedlings grown 20 days after sowing in soil were placed in a closed opaque box in a growth room at 20 ± 2°C. To ensure that the box is not translucent, box was wrapped with aluminum foil. Pictures were taken after 2, 4, 6, 8, and 10 days as indicated in the figure legends.

Measurements of Ion Leakage, Total DNA Content, and Protein Extraction

Ion leakage and total DNA content in the sixth rosette leaves grown for 25 days under osmotic and oxidative stresses. For measuring ion leakage, leaf samples were immersed into deionised water, shaken in a 25°C water bath for 30 min, and the conductivity was measured using an electrical conductivity meter (B-173, Horiba, Kyoto, Japan). Samples were boiled for 10 min before total conductivity was determined. The conductivity was expressed as the percentage of the initial conductivity versus

the total conductivity (Jing et al., 2002). Total DNA content was measured by densitometry method. Leaf total proteins were extracted from 250 mg FW of frozen leaf tissue at 4°C with 2 ml of 100 mM potassium phosphate buffer, pH 7.5. The homogenate was centrifuged (2000 g, 4°C, 5 min) and supernatant was collected.

ABA Quantification

Fresh leaf samples (usually 1 g) was used for ABA content determination assay. Fully expanded leaflets immediately immersed in liquid N_2 and then stored at −20°C before being used for ABA content determination. ABA was extracted and measured using enzyme-linked immunosorbent assay (ELISA).ELISA kits were purchased from China Agriculture University (China). The assays were performed according to the instructions given by the manufacturer.

Chlorophyll Fluorescence Imaging and Photosynthetic Parameters

Images of chlorophyll fluorescence were obtained as described by Barbagallo et al. (2003) using a CF Imager (Technologica Ltd.,Colchester, UK). Seedlings were adapted to the dark for 30 min before minimal fluorescence (Fo) was measured using a weak measuring pulse. Then, maximal fluorescence (Fm) was measured during 800-ms exposure to a saturating pulse having a photon flux density (PFD) of 4800 μmol $m^{-2}s^{-1}$. Plants were then exposed to an actinic PFD of 300 μmol $m^{-2}s^{-1}$ for 15 min and steady-state F′ was continuously monitored, while Fm′ (maximum fluorescence in the light) was measured at 5-min intervals by applying saturating light pulses. This was repeated at a PFD of 500 μmol $m^{-2}s^{-1}$. Fv/Fm, maximum quantum efficiency of PSII photochemistry.

Genetic Analysis and Map-Based Cloning of the *EAS1* Gene

Backcrosses of *eas1* mutants to the wild type and intercrosses among *eas1* mutants, as well as those of *eas1* with *aba* mutants, were performed by transferring pollen to the stigmas of emasculated flowers. The mapping population was generated by crossing *eas1* (C24) to the Col-0 wild type. From the F_2 generation, 800 homozygous *eas1* individuals were isolated. Genomic cDNA of the young seedling was extracted individually to perform PCR using simple sequence length polymorphism (SSLP) markers to identify recombinants, as described previously (Cheng et al., 2002). Fine mapping was performed by designing new indel markers. The primers were synthesized based on bacterial artificial chromosome (BAC) DNA sequences and tested by PCR using DNA isolated from three ecotypes. *eas1* was found to be linked to the SSLP marker nga280 on the long arm of chromosome I. Thus, SSLP markers were developed based on the sequences of the BAC clones F5F19, F6D8, F12M16, F15I1, T15A14, F16N3, and F7F22.

Real-Time RT-PCR

Total RNA was extracted with TRIzol reagent (Ambion) from leaves 6 and 7 under different conditions and digested with RNase-free DNase I; it was then used for real-time RT-PCR, employing oligo (dT) primers with M-MLV (Promega) in a 30-μL reaction, in accordance with the manufacturer's instructions. The cDNA was used for quantitative real-time PCR amplification. One microliter of the RT reaction was used as a template to determine the levels of transcripts of the tested genes using a PTC-200 DNA Engine Cycler with a Chromo 4 Detector in 25-μL reactions. The levels of actin is described as the control, and the values given are expressed as the ratios to the values in the wild type. Three biological replications were performed for each experiment. The values shown represent averages of triplicate assays for each RT sample. PCR conditions were as follows: 5 min at 95°C (one cycle), and 30 s at 95°C, 30 s at 55–60°C, and 60 s at 72°C (40 cycles). The primers for real-time PCR are shown in **Table S1**.

Thermal Imaging

A ThermaCAMSC3000-equipped quantum-well infrared photodetector was used as it provides image resolution of 320 × 240 pixels and is responsive to a broad dynamic range, with extraordinary long-wave (8–9 μm) imaging performance. The specified temperature resolution was below 0.03°C at room temperature. The camera was mounted vertically at ∼35–45 cm above the leaf canopy for observations, and was connected to a color monitor to facilitate visualization of individual plants. Digitally stored 14-bit images, live IR video, or real-time high-speed dynamic events were analyzed.

Electrophysiological Assays and Data Acquisition

Arabidopsis guard cell protoplasts of leave 5 were isolated as described previously (Tallman, 2006; Zhang et al., 2008). The whole-cell voltage-clamp or single-channel currents of *Arabidopsis* guard cells were recorded with an EPC-9 amplifier (Heka Instruments), as described previously (Bai et al., 2009). Pipettes were pulled with a vertical puller (Narishige, Japan) modified for two-stage pulls. Data were analyzed using PULSEFIT 8.7 software. Standard solutions for Ca^{2+} measurements were used, including 10 mM $BaCl_2$, 0.1 mM DTT, 10 mM MES-Tris (pH 5.6) in a bath, and 100 mM $BaCl_2$, 0.1 mM DTT, 4 mM EGTA, and 10 mM HEPES-Tris (pH 7.1) in a pipette. ABA was freshly added to bath solutions at the indicated concentrations. For ABA-activated Ca^{2+} current measurements, 50 μM ABA was added to the standard pipette solution. Osmolalities of pipette and bath solutions were adjusted to 510 and 490 mM kg^{-1}, respectively, using *D*-sorbitol (Sangon, China).

Flow Cytometric Analysis

Analyses were performed on three Cytomics FC500 flow cytometers (Beckman-Coulter, Villepinte, France). To limit background noise from dust and crystals, all three instruments were operated using 0.22-μm filtered sheath fluid (Isoflow™; Beckman-Coulter). CXP ACQUISITION and CXP ANALYSIS software packages (Beckman-Coulter) were used for data acquisition and analysis, respectively. *Arabidopsis* protoplasts of leave 5 were immersed in 5 μM FDA (Sigma; in MES buffer, pH

6.1) for 20 min at room temperature in the dark, and then washed three times with MES buffer (pH 6.1). Cells were stained with Annexin V using the Annexin V-FITC fluorescence detection kit (BD Biosciences, San Jose, CA, USA), in accordance with the manufacturer's instructions. Briefly, cells cultured on cover slips, and then washed twice with PBS. The slides were examined and photographed with a Nikon Eclipse TE 2000 U motorized inverted microscope (Nikon Corp., Tokyo, Japan). The apoptotic index was calculated as the percentage of cells stained positive for Annexin V. A total of 100 cells were counted in each experimental group in three independent experiments and results arethe mean proportion of apoptotic cells in sixscanning electron micrographs.

Ca^{2+} Measurements of the Seedlings by Aq Bioluminescence and Calibration of Calcium Measurements

Ca^{2+} measurements of wild-type and *eas1-1* mutant seedlings by Aq luminescence were carried out according to the method of Bai et al. (2009). Seven-days-old seedlings were incubated in distilled water containing 2.5 μM coelenterazine (Promega) overnight in the dark at room temperature. A seedling was put into a cuvette with 100 μL of distilled water for 1–2 h in the dark, and then the cuvette was placed inside a TD20/20n digital luminometer (Turner Biosystems). Luminescence was recorded after counting for 20 s, the different reagents were added to the cuvette and the luminescence was measured. At the end of each experiment, the remaining Aq was discharged by the addition of an equal volume of 2 M $CaCl_2$ and 20 % ethanol. Luminescence values were converted to the corresponding calcium concentrations. Ten seedlings were used in each experiment.

Statistical Analyses

All experiments were repeated at least three times. To determine significant differences among different lines or different treatments, all the data were analyzed by Dunnett's test using SPSS16.0 software.

RESULTS

Leaves of *Eas1* Mutant Plants Display Early-Senescence Phenotypes

Chlorophyll content and photochemical efficiency are well-established senescence parameters and convenient markers, which can be used for assaying leaf senescence (Oh et al., 1997; Woo et al., 2001). To obtain further insights into the role of photosynthesis in leaf senescence, we developed a novel genetic screen for *Arabidopsis* mutants with altered photochemical efficiency during leaf development. This approach uses the ratio of variable (Fv) to maximal (Fm) fluorescence, which represents the quantum efficiency of PSII reaction centers. Fv/Fm can be measured continuously and nondestructively using chlorophyll fluorescence imaging, which provides a convenient indicator of the photosynthesis during leaf development and senescence (Barbagallo et al., 2003; Rolfe and Scholes, 2010; Harbinson et al., 2012). We used chlorophyll fluorescence imaging to screen for *Arabidopsis* mutants that displayed an increased or reduced Fv to Fm ratio during leaf development, in which photosynthesis efficiency thus appeared to be altered (Harbinson et al., 2012). One group of mutants that exhibited a clear increase in Fv/Fm at day 20 after planting was isolated. Two allelic mutants, designated *eas1-1* and *eas1-2* (*early senescence 1-1 and -2*), showing increased photochemical efficiency and early-aging syndrome throughout the whole of their development, were chosen for detailed characterization.

Fv/Fm of *eas1-1* plants was significantly higher than that of the wild type before approximately day 30, whilst it declined from day 30 in *eas1-1* plants (**Figures 1A,B**). The dark-green leaf phenotype and high chlorophyll content in *eas1-1* mutant plants are also consistent with their high photosynthetic efficiency in the early growth stage (**Figures 1C,D**). Moreover, the *eas1* mutant appears to have smaller or no trichomes (**Figure 1C**). Twenty-days-old leaf cross-section anatomy showed more chloroplasts in mesophyll cells in *eas1-1* mutant plants than in wild-type plants (**Figure 1E**). The pattern of change of Fv/Fm was consistent with the change of chlorophyll content throughout the whole of leaf development (**Figure 1C**).

To determine whether *EAS1* is involved in the regulation of senescence, we observed the aging syndrome in *eas1* and wild-type plants throughout the whole of leaf development. Wild-type plants exhibited a consistent increase in Fv/Fm before day 45, while *eas1* plants displayed enhanced quantum efficiency of PSII at day 30, which rapidly decreased thereafter (**Figure 1B**). Consistent with this, the aging syndrome of *eas1* mutants, including leaf yellowing and rosette bolting, appeared early compared with that of the wild type plants (**Figure 1F**; **Figure S1**). Furthermore, under unstressed conditions, the *eas1* mutant plants displayed accelerated leaf senescence in soil and Murashige and Skoog (MS) medium (**Figure 1F**; **Figures S1A–C**).

Map-based cloning and sequencing showed that the *eas1-1* and *eas1-2* missense mutations were generated in the second exon of the At1g52340 gene and the 190 glutamic acid and the 265 glycine were replaced by lysine and arginine in *eas1-1* and *eas1-2,* respectively (**Figure S2A**). Surprisingly, this gene is allelic to *ABA2/GIN1/SRE1*, which encodes a short-chain dehydrogenase/reductase (SDR1) that catalyze the final oxidation steps in the conversion of xanthoxin to ABA aldehyde (ABAld; Cheng et al., 2002; González-Guzmán et al., 2002). To further confirm the results of positional cloning, *EAS1* complementation was performed by transforming the missense *eas1-1* mutant with a 7.7-kb *ABA2* genomic clone that includes the 3-kb promoter through *Agrobacterium tumefaciens* (Cheng et al., 2002). Six independent- homozygous T3 lines with clear complementation were isolated. These complementary transgenic plants were restored to wild-type phenotype, such as the premature leaf, the growth defects in cotyledons, and rosettes, the wilting and lack of seed dormancy phenotypes of *eas1*. These results are consistent with the previous obervations (Cheng et al., 2002; González-Guzmán et al., 2002). Moreover, genetic crosses also showed that *eas1* is ABA2 allelic mutants (**Table S2**).

We have detected ABA content from 20-days-old rosette leaves in *eas1-1* and *eas1-2* plants under unstressed conditions.

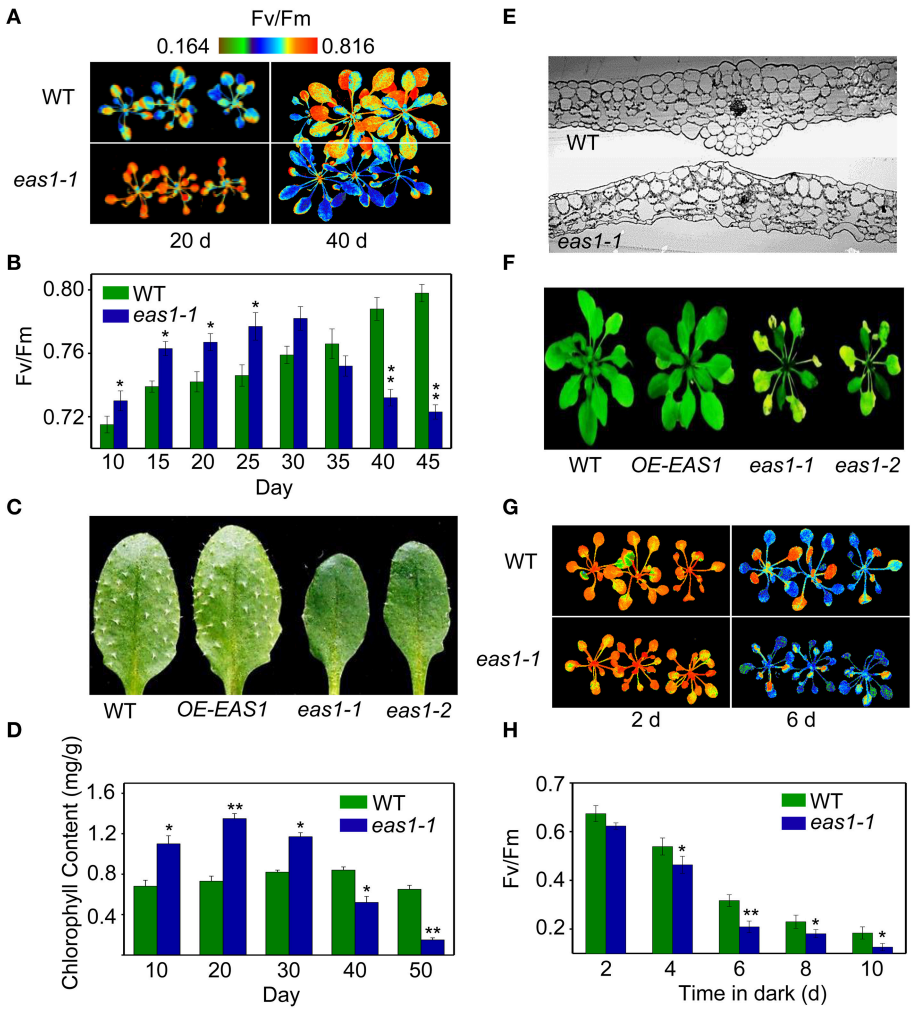

FIGURE 1 | The *eas1* mutation accelerates leaf senescence in *Arabidopsis thaliana*. **(A)** Images of Fv/Fm of wild-type and *eas1-1* mutant leaves on the 20th and 40th day under unstressed conditions in soil. **(B)** Fv/Fm values depend on different growth stages in wild-type and *eas1-1* mutant plants. **(C)** Twenty-five-days-old leaves of wild-type and *eas1-1* mutant plants. The sixth rosette leaves are shown. **(D)** Determination of chlorophyll contents of wild-type and *eas1-1* mutant leaves at different stages of plant growth. **(E)** Twenty-five-days-old leaf cross-section anatomy of wild-type and *eas1-1* mutant plants. **(F)** Thirty-five-days-old natural aging of the leaf rosettes of wild-type, overexpression of *EAS1* (OE-EAS1), and *eas1* mutant plants grown under unstressed conditions in soil. The flowers and stems of *eas1* plants were removed. **(G)** Images of Fv/Fm of 20-days-old wild-type and *eas1-1* mutant leaves under dark treatment for 2 and 6 days. **(H)** Time-dependence of Fv/Fm values in wild-type and *eas1-1* mutant plants in dark treatment. Three experiments were performed with similar results. Error bars indicate standard deviations, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$).

Indeed, compared with the wild type, ABA content decreased by about 20.8 and 23.5% in *eas1-1* and *eas1-2* plants, respectively (**Figure S2B**). Seed germination of *eas1* occurred significantly more rapidly than that of the wild type under stress conditions (e.g., mannitol and NaCl) or unstressed conditions (**Figures S2C,D**). The detached leaves of the *eas1* mutants exhibited excessive transpiration under unstressed conditions (**Figures S2E,F**).

To further demonstrate whether overexpression of *EAS1* may delay leaf senescence or other unexpected phenotypes, EAS1 overexpression transgenic plants were generated. More than 12 independent transgenic lines were isolated and 8 homozygous transgenic lines presents a consistent phenotype during the whole development. One line (OE-EAS1) were chosen for further study. Confusingly, OE1-EAS1 exhibited no apparent phenotypic differences in aerial structures from the wild type in the early stage (**Figures 1C,F**; **Figure S1C**). The young leaves of OE-EAS1 have a slightly larger but not significantly differences in chlorophyll content than that of wild type plants. However, OE-EAS1 could delay leaf senescence and plant flowering (**Figure S1A**). Leave 5 or 6 of wild-type plants show earlier senescent phenotypes (yellow tip and margin) under unstressed conditions in soil than that of OE-EAS1 plants. The average number of OE-EAS1 rosette leaves before flowering is more 2 leaves than that of wild-type plants.

We also examined the senescence syndrome of detached leaves under dark treatment. Upon exposure to dark conditions, the 20-days-old *eas1* leaves displayed more dramatic decreases in

Fv/Fm than that of the wild type (**Figures 1G,H**). Because Fv/Fm of *eas1* plants was significantly higher than that of the wild type (**Figures 1A,B**) before dark treatment, day 0 control picture is not be shown in **Figure 1G**. In order to confirm the leaf senescence phenotypes of *eas1* mutant plants, 25-days-old detached leaves were placed under osmotic and oxidative stresses. The results showed that 500 mM mannitol and 10 mM H_2O_2 could accelerate leaf senescence of *eas1-1* mutant and wild-type leaves, but the aging symptoms (yellowing and necrotic spots) of *eas1-1* mutant leaves appeared earlier and were more pronounced than those of wild-type leaves (**Figure 2A**). Relative chlorophyll contents in *eas1-1* mutant leaves were significantly decreased under 10 mM H_2O_2 and 500 mM mannitol treatment from the second day of treatment (**Figures 2C,D**). By contrast, ion leakage in *eas1-1* leaves increased significantly from the third day (**Figures 2E,F**). The detected level of DNA content was significantly reduced in *eas1-1* mutant leaves (**Figure 2B**).

EAS1/ABA2 is Involved in Regulation of the Expression of Senescence- Associated Genes in the Early Stage of Plant Growth

Since the role of ABA in leaf senescence has not been clearly defined, and only circumstantial evidence has been obtained, we further examined the expression of some senescence-associated genes (SAGs) in wild-type and *eas1* mutant plants at different developmental stages. Quantitative reverse transcription PCR (qRT-PCR) was performed using RNA samples from leave 6 and 7 of wild-type and *eas1* mutant plants grown for 25, 35, and 45 days (**Figure 3**). The *eas1* mutation has been shown to have dramatic effects on the expression of *SAG12* (Noh and Amasino, 1999), *SAG29* (Seo et al., 2011), *SAG113* (Zhang and Gan, 2012), and *SAG101* (He and Gan, 2002) at young and old developmental stages. Compared with the wild type, the expression levels of *SAG12*, *SAG29*, *SAG113*, and *SAG21* in leaves of 25-days-old *eas1-1* were increased by 1523-, 59-, 8.5-, and 8-fold, respectively. However, at day 45, the expression of all four genes was significantly inhibited by the mutation (**Figure 3**). The expression levels of the other *SAGs* (*SAG13*, *SAG14*, *SAG18*, *SAG101*) were also up-regulated from 1.5- to 7.8-fold in *eas1* mutants at day 25. The age-dependent induction of *SAG13*, *SAG14*, *SAG18*, and *SAG101* also substantially increased at days 35 and 45.

We also examined the mRNA levels of chloroplast-related genes (CRGs; *CAB3*, *RBCS*, *PSBA*, *PETB*) at 25-, 35-, and 45-d-old *eas1-1* plants (**Figure 3**). Compared with *eas1-1* mutant plants, the expression levels of almost all CRGs in wild-type plants gradually increased, and *RBCS*, *PSBA*, *PETB*, and *CAB3* were significantly up-regulated.

Mutation of *EAS1* Enhances Cell Apoptosis under Leaf Senescence

It is known that leaf senescence is a programmed event that can be induced by a variety of endogenous factors and environmental cues (Lim et al., 2003; Guo and Gan, 2005; Zhang and Zhou, 2013; Li et al., 2014). To confirm whether *EAS1* mutation could affect the programmed cell death (PCD) in leaf senescence, several fluorescence-based dyes for the measurement of cell death were applied. In the first set of experiments, trypan blue (TB) staining was used for the investigation of cell viability (Lee et al., 2011). When seedlings were stained with TB, a large number of blue patches were observed in leaves of *eas1-1*, but were rarely present in wild-type plants (**Figure 4A**), indicating that age-dependent cell death is increased in *eas1* mutants during leaf senescence. Consistent with these observations, total protein extracted from *eas1-1* was significantly decreased compared with that of wild-type plants (**Figure S3C**).

Membrane deterioration is one of the early events during leaf senescence (Leshem et al., 1984). Therefore, we next examined the membrane integrity of protoplasts of leave 6 in wild-type and *eas1-1* plants by using propidium iodide (PI) staining under fluorescence microscopy (Rolny et al., 2011). To test the cell membrane integrity in *eas1-1* and wild-type plants, PI staining was used to estimate cell death. When 35-days-old detached leaves were incubated for 0.5 h, there was significant staining of mesophyll and epidermal tissues in *eas1-1*. However, PI staining was hardly observed in the leaves of wild-type plants (**Figure 4B**). Furthermore, mesophyll protoplast activity was detected by fluorescein diacetate (FDA) staining (**Figure S3A**).There was a greater proportion of active mesophyll protoplasts in *eas1-1* mutant plants than in wild-type plants after 35 days (**Figure S3B**).

For the assessment of cell apoptosis in *eas1* mutant and wild-type plants at different development stages, we stained mesophyll protoplasts with Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and detected apoptotic cells by flow cytometry. After approximately 30 d, the number of normal mesophyll protoplasts in *eas1* mutant plants significantly decreased compared with that in the wild type, while the number of apoptotic cells was increased in *eas1-1* mutant plants (**Figures 4C,D**). The rates of apoptotic cells in wild-type and *eas1-1* mutant plants were 6.4 and 11.5%, respectively, at day 45, indicating that *EAS1* mutation was associated with a lower survival rate. Together, these results suggest that *EAS1* partially protects cells against senescence-induced PCD.

ABA Signaling, not Stomatal Behavior, is the Causal Factor of Senescence

The previous series of data establish a general parallel between stomatal aperture size and senescence (Thimann and Satler, 1979a,b; Gepstein and Thimann, 1980), with a strong indication that the stomatal apertures are the causal factor and the effects of stomatal apertures on senescence are actually mediated by the internal concentration of ABA.

To assess the relationship between stomatal apertures and age-dependent leaf senescence, we first measured stomata aperture size and density in several stomatal response and development mutants under unstressed conditions. The results showed that *constitutive photomorphogenic1* (*cop1*; Mao et al., 2005), *slow anion channel-associated 1* (*slac1*; Vahisalu et al., 2008), *open stomata 1* (*ost1*; Mustilli et al., 2002), and *ABA insensitive 1* (*abi1*) showed larger stomatal apertures than that of the wild type (**Figures 5A,C**). Stomatal openings of the double-mutant *epidermal patterning factor (epf)1-1epf2-1* (Hunt and Gray, 2009)

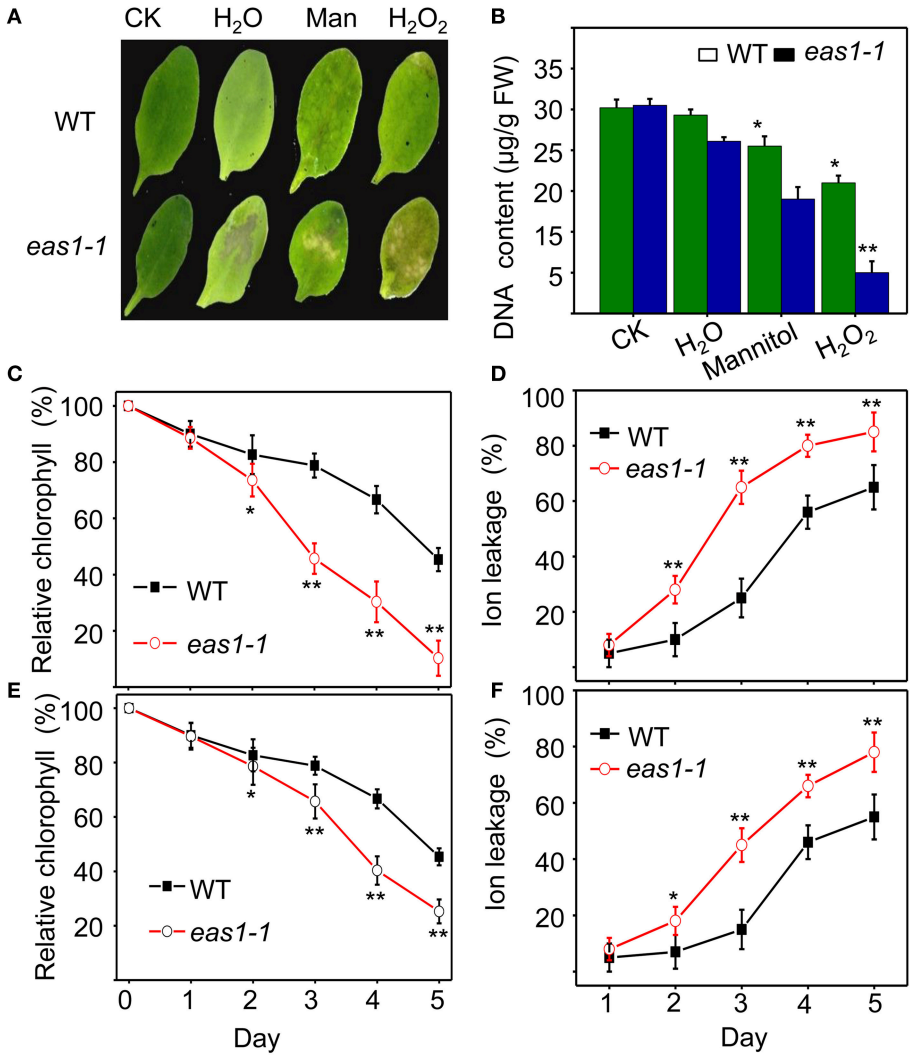

FIGURE 2 | *EAS1* mutations accelerate leaf senescence under osmotic and oxidative stresses. (A) Aging symptoms of detached leaves from wild-type and *eas1-1* mutant plants under different stresses (deionized water, 500 mM mannitol, and 10 mM H_2O_2,for 3 days).The sixth rosette leaves grown for 25 days under unstressed conditions in soil are treated. **(B)** DNA content in leaves of different treatments corresponding to panel **(A)**. **(C,D)** Relative chlorophyll contents of wild-type and *eas1-1* leaves under 10 mM H_2O_2 **(C)** and 500 mM mannitol **(D)** treatments at different stress times. **(E,F)** Ion leakage of wild-type and *eas1-1* plants at different times under 10 mM H_2O_2 **(C)** and 500 mM mannitol **(D)** treatments. Three experiments were performed with similar results. Error bars indicate standard deviations, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$).

and *too many mouths* (*tmm*; Yang and Sack, 1995) were smaller, but their stomata were present at a higher density than in the wild type (**Figures 5A,D**). The level of water loss of detached leaves of these mutant plants was higher than that of wild-type plants (**Figure 5B**). Similar to *aba2-1* mutants, *ost1-4,* and *abi1* mutants displayed earlier leaf senescence than wild-type plants. However, leaf senescence phenotypes of *cop1*, *tmm*, and *epf11-1 epf2-1* mutants did not differ from those of wild-type plants (**Figures 6A,B**). The changes of Fv/Fm at different developmental stages displayed similar patterns to those in the observations on leaf senescence (**Figure 6C**).

To confirm that ABA rather than stomatal behavior modulates the onset of leaf senescence, we determined the ABA content of the above-mentioned stomatal mutants. Except for *aba2-1*, the ABA content of the mutants was similar to that of the wild type (**Figure 6D**). These results demonstrated that the degree of opening and density of stomata are not necessarily linked to leaf senescence, and ABA signaling is involved in regulation of the onset of leaf senescence.

Calcium Ions are Involved in ABA Signaling in the Regulation of Leaf Development and Senescence

The transduction of hormonal signals and other environmental stimuli in plant systems is in many instances mediated through the secondary messenger action of Ca^{2+} (Poovaiah and Reddy, 1987), which is involved in the regulation of leaf

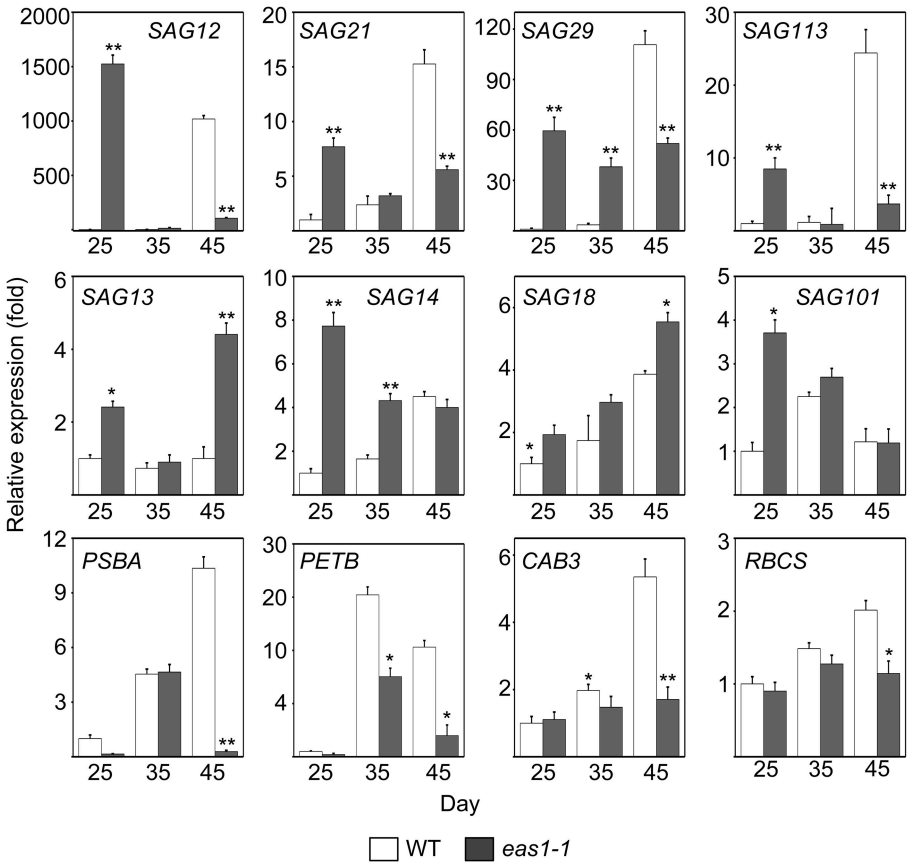

FIGURE 3 | Expression analysis of SAGs and CRGs in wild-type and *eas1-1* mutant leaves for 25, 35, and 45 days under unstressed conditions in soil. SAGs included *SAG12*, *SAG13*, *SAG14*, *SAG18*, *SAG20*, *SAG21*, *SAG29*, *SAG101*, and *SAG113*. CRGs included *PSBA*, *RBCS*, *CAB3*, and *PETB*. *Actin* was used as an internal control. Total RNA was isolated from the sixth and seventh true leaves at the indicated time points. Bars indicate standard errors, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$); three experiments were performed with similar results.

senescence (Poovaiah and Leopold, 1973; Ma and Berkowitz, 2011). On the basis of our results, we hypothesized that ABA might activate calcium signaling as a means of affecting cell function via membrane deteriorative processes. To test this hypothesis, we examined whether calcium plays a role in the processes by which ABA inhibits senescence. Five-days-old seedlings grown on MS medium were then moved to MS agar plates containing nifedipine, a Ca^{2+} channel blocker. Although nifedipine can accelerate leaf senescence, leaves of *eas1-1* seedlings showed a stronger, more pronounced, and earlier senescent phenotype than those of the wild type, characterized by yellowish apoptotic leaves (**Figure 7A**). Further analysis showed that Fv/Fm and chlorophyll content decreased more rapidly in 8-days-old leaves of *eas1-1* seedlings than in those of wild-type plants (**Figures 7B,C**). In contrast, ion leakage was significantly increased and, on the fourth day of treatment, ion leakage of *eas1-1* mutant leaves reached 38.3%, while it was only 15.4% in the wild type (**Figure 7D**).

It has been reported that the concentration of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) play important roles in ABA signaling in plants (Jammes et al., 2011; Cheval et al., 2013). The changes of $[Ca^{2+}]_{cyt}$ in wild-type and *eas1-1* mutant seedlings under cold shock, osmotic, and H_2O_2 stresses were thus measured using an *Aequorea victoria* (Aq) bioluminescence-based Ca^{2+} imaging method (Bai et al., 2009). As shown in **Figure 7E**, more significant elevation of $[Ca^{2+}]_{cyt}$ was observed in wild-type plants than in *eas1-1* mutant plants. The average values of increased $[Ca^{2+}]_{cyt}$ promoted by different stresses in the wild type were 1.25- to 2.63-fold of those in *eas1-1* mutant plants (**Figure 7F**), which suggested that the *eas1* mutation decreased $[Ca^{2+}]_{cyt}$ elevation under cold, NaCl, mannitol, and H_2O_2 treatment.

Effects of ABA on the Expression of Calcium Channel Genes and Disruption of ABA-Activated Ca^{2+} Channel Activity in *eas1-1* Guard Cells

In order to explain why basal $[Ca^{2+}]_{cyt}$ and increased $[Ca^{2+}]_{cyt}$ in response to stresses were lower in *eas1-1* mutant plants than in wild-type plants, the expression levels of several calcium channel genes (*ACA3*, *CAX1*, *TPC1*, *CNGC1*, and *CAX2*) were determined by qRT-PCR. The expression of *CAX1* and *CAX2* was down-regulated, and that of *ACA3* and *TPC1* was slightly up-regulated

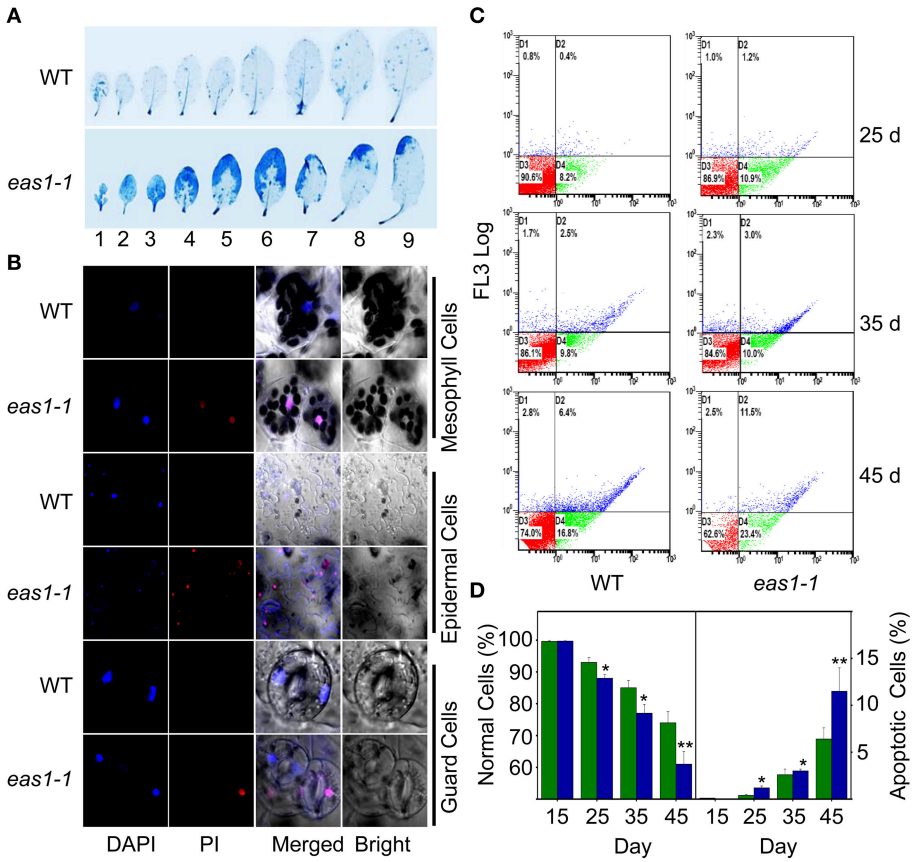

FIGURE 4 | EAS1 mutation causes earlier apoptosis in leaves of *eas1-1* mutant plants than in wild-type plants. (A) The first to ninth true leaves of wild-type and *eas1-1* mutant plants at 35 days after sowing under unstressed condition in soil. Leaves were stained with trypan blue for the detection of dead cells. **(B)** Estimation of cell membrane integrity by PI staining using confocal images. The sixth leaves of wild-type and *eas1-1* mutant plants at 35 days after sowing were used to isolate mesophyll cells, epidermal cells, and guard cells under unstressed condition in soil. Cells were subjected to 10 μM DAPI and 10 μg/mL PI staining for 0.5 h. **(C)** Quantification of age-dependent cell apoptosis by flow cytometry. Mesophyll protoplasts of sixth leaves were counted by cytometry in wild-type and *eas1-1* mutant plants at 25, 35, and 45 days after sowing under unstressed condition in soil. Cell counts in the four regions D1, D2, D3, and D4 represent the proportions of late-senescent cells, apoptotic cells, normal cells, and early-senescent cells, respectively. **(D)** Normal and apoptotic cell percentages of wild-type and *eas1* mutant protoplasts. The ratio of normal and apoptopic cells were the average of 15 results from three independent experiments. CXP ACQUISITION and CXP ANALYSIS software packages (Beckman-Coulter) were used for data acquisition and analysis, respectively. Bars indicate standard errors, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$).

in *eas1* mutant plants compared with that of wild-type plants at day 25. However, no difference in the expression of these genes was observed at day 35. At day 45, the expression of *CAX2* was apparently up-regulated (approximately seven-fold) and the expression of *TPC1* was down-regulated by ∼60% (**Figure 8A**) in *eas1-1* mutant plants compared with wild-type plants.

To examine whether endogenous ABA was responsible for the activation of Ca^{2+} influx currents, Ca^{2+} influx conductance in wild-type and *eas1-1* mutant plants was monitored by the patch-clamp technique. In whole-cell patch-clamp recording using conditions described previously (Bai et al., 2009), 50 μM ABA markedly evoked influx currents in guard cells of both wild-type and *eas1-1* mutant plants (**Figures 8Ba,c**). The average values of Ca^{2+} channel currents also confirmed these results (**Figures 8Bb,d**). In wild-type plants, treatment with ABA significantly induced Ca^{2+} channel activity for inward Ca^{2+}currents compared with the control, which rose from <30 pA at time zero to 205 pA (**Figure 8C**). In contrast, treatment of mutants with ABA had a minimal effect on Ca^{2+}channel activity compared with that in controls, with changes no greater than 50 pA being observed (**Figure 8C**).

DISCUSSION

ABA is an Inhibitor of Leaf Senescence

A crucial link between ABA and leaf senescence has yet to be discovered via genetic analysis. In this work, we have established a leaf senescence screening system based on chlorophyll fluorescence and successfully isolated *eas1*mutantsby chlorophyll fluorescence imaging. We were surprised to find that *eas1* is an *aba2* allelic mutant. It has long been known that ABA is a senescence promoter (Mizrahi et al., 1975; Gepstein and Thimann, 1980) and endogenous ABA levels play an important

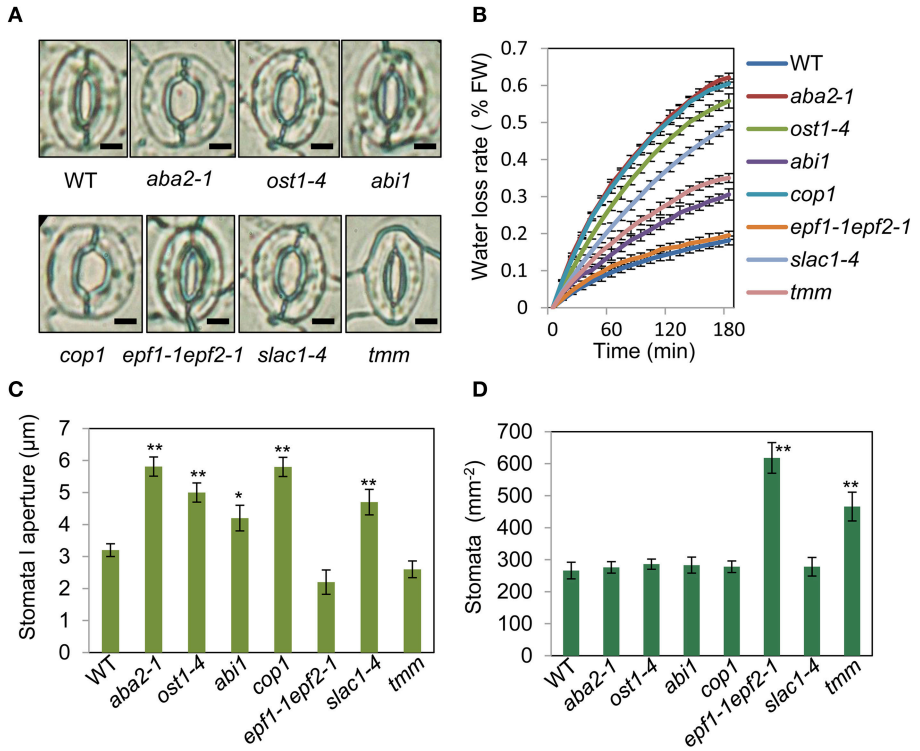

FIGURE 5 | Analysis of several stomatal mutants in terms of stomatal aperture size and stomatal density. (A) Representative images of stomata of wild-type and stomatal mutant plants at noon under unstressed conditions in soil. The epidermis of fifth leaf were quickly separated and observed by microscopy. Bar = 10 μm. **(B)** The *aba2-1*, *cop1*, *ost1-4*, *slac1-4, abi1*, and *tmm* mutants lost water more rapidly than the wild-type plants. Water loss from the detached leaves of plants in fresh weight (FW) at day 35. Leaves 5 and 6 were selected for experiments. The rate of water loss from high to low was as follows: *aba2* > *cop1* > *ost1-4* > *slac1-4* > *tmm* > *abi1* > *epf1-1epf2-1* > WT. **(C,D)** Abaxial stomatal densities and stomatal aperture sizes of wild-type plants and stomatal mutants with fifth fully expanded leaves. Bars indicate standard errors, while asterisks indicate significant differences from wild-type and mutant plants under Student's test (*$p < 0.05$, **$p < 0.01$); three experiments were performed with similar results.

role in the regulation of leaf senescence (Pourtau et al., 2004; Liang et al., 2014; Yang et al., 2014). Our genetic and physiological evidence indicates that *EAS1* mutations rapidly decreased the efficiency of leaf photosynthesis and caused early leaf senescence after day 35 in natural developmental conditions (**Figure 1**). Experiments on age-dependent PCD in leaves showed more significant and earlier apoptosis in *eas1* plants than in wild-type plants under unstressed conditions (**Figure 4**). In response to osmotic or oxidative stress, the detached leaves of *eas1* mutant also displayed phenotypes of higher sensitivity and earlier senescence than those of the wild type under dark treatment (**Figure 2**). Hence, it is suggested that ABA has a clear role in delaying leaf senescence, at least under dark-induced conditions. Recent studies showed that an Arabidopsis *NAC-LIKE, ACTIVATED BY AP3/PI* (NAP) transcription factor promotes chlorophyll degradation by enhancing transcription of *AAO3*, which leads to increased levels of the senescence-inducing ABA (Yang et al., 2014). However, our results clearly show that leaves of *eas1* plants have higher chloroplast density, chlorophyll concentration and appear greener at 25 days. This appears to be a concentration effect due to inhibited growth since the leaves are smaller (**Figure 1**). These contradictory results may be due to leave age and experimental condition. Moreover, *aao3* mutant seeds display normal seed dormancy (Seo et al., 2000; Finkelstein, 2013). It seems to imply that ABA2/EAS1 and AAO3 may play different roles in the regulation of leaf senescence.

The analysis of *eas1*/*aba2* mutants led us to the idea that ABA function is an age-dependent response in plant development and senescence. It appears that ABA controls both cellular protection activities and senescence activities. The balance between these two activities seems to be important in controlling the progression of leaf senescence and may be adjusted by other senescence-affecting factors such as age. In young plants, ABA is an internal orchestrator that balances the activities that promote morphogenesis and inhibition set of deterioration processes in plant growth and development. By contrast, in old plants (similar to stress conditions), ABA's protective effects decreased and its senescence activity increased. In fact, we found that several SAGs exhibited earlier and stronger expression in the early growth stage of *eas1* mutant leaves than in the wild type. For example, *SAG12*, an *Arabidopsis* gene encoding a cysteine protease, is expressed only in senescent tissues (Noh and Amasino, 1999). *SAG12* expression is specifically activated by developmentally controlled senescence pathways but not by stress- or hormone-controlled pathways (Noh and

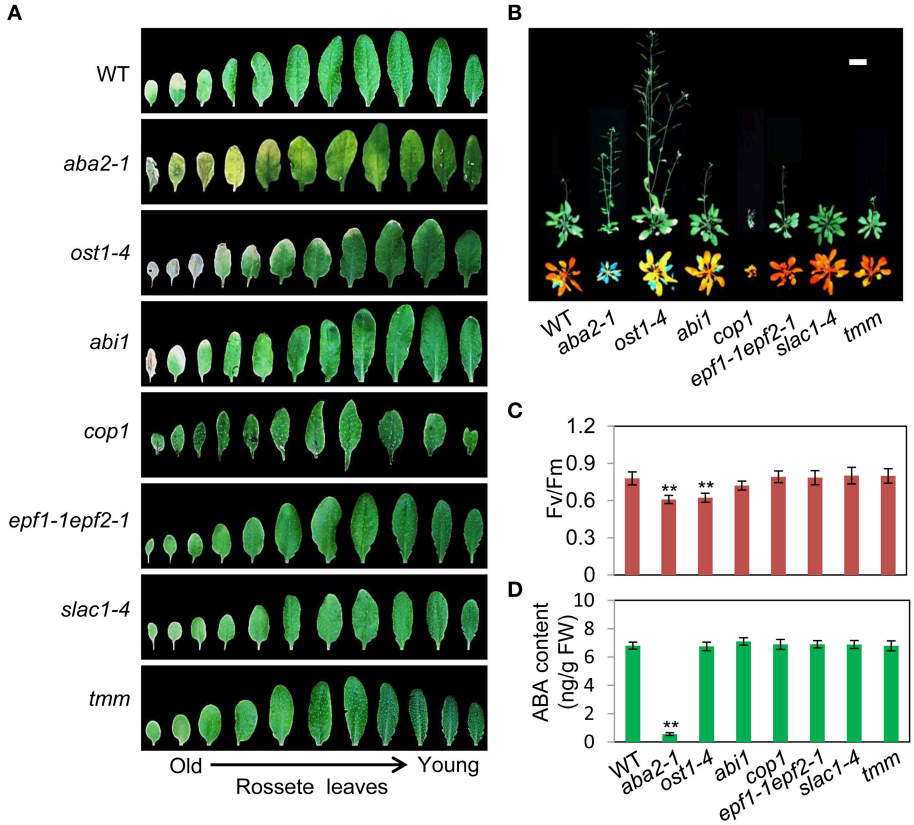

FIGURE 6 | OST1 mediates ABA regulation of leaf senescence, and stomatal behavior is not involved in this process. (A) The aging symptoms of rosette leaves at day 45 under unstressed conditions in soil. The first to eleventh rosette leaves are arranged from left to right. In order to highlight the leaf senescence phenotype, images are not to scale enlarge. **(B)** Representative images of wild-type and mutant plants at day 45 under unstressed conditions in soil, upper: bright; lower: chlorophyll fluorescence. The pictures show the aerial parts of plants. Bar = 5 cm. **(C)** Fv/Fm of wild-type and mutant rosette leaves under unstressed conditions in soil at day 45. **(D)** ABA content of the fifth rosette leaf of wild-type and mutant plants under unstressed conditions in soil at day 25. Bars indicate standard errors, while asterisks indicate significant differences from wild-type plants under Student's test (**$p < 0.01$); three experiments were performed with similar results.

Amasino, 1999). In contrast, the expression of *SAG12* in *eas1* mutant leaves was approximately 1500-fold higher than that in wild-type plants on day 25. Furthermore, the expression of *SAG29*, *SAG21*,and *SAG113* was also higher in *eas1* mutant leaves than in the wild type. In addition to these SAGs, other types (*SAG13*, *SAG14*, *SAG18*, *SAG101*) all displayed different degrees of up-regulation in *eas1* mutant plants. Interestingly, we found that the levels of RNA transcribed from most of the SAG genes examined (e.g., *SAG12*, *21*, *29*, and *113*) in leaves of *eas1* plants were significantly reduced in comparison to those of wild-type plants after day 45 (**Figure 3**). In addition, there was higher chlorophyll content and Fv/Fm ratio, as well as a significant increase in *eas1-1* plants before day 30. In contrast, these features were significantly attenuated after day 35, which suggested that day 30 is a turning point regarding ABA's function in *A. thaliana*. Over the course of development, the role of ABA decreases, at which time some stress-response genes and senescence-associated genes may start to function and produce senescence syndrome. Therefore, this integrated senescence response provides plants with optimal fitness by incorporating their environmental and endogenous status in a given ecological setting by fine-tuning the initiation timing, progression rate, and nature of leaf senescence.

ABA is the Internal Integrator of Leaf Senescence Onset Through $[Ca^{2+}]_{Cyt}$

Leaf senescence is basically governed by the developmental age. However, it is also influenced by various internal and environmental signals that are integrated into the age information. Our data provide genetic, molecular, and physiological evidence supporting the essential function of ABA in the onset of leaf senescence.

Similar to gibberellin and cytokinin (Zwack and Rashotte, 2013; Chen et al., 2014), a low concentration of calcium (0.1–1.0 μM) can delay leaf senescence by suppressing the decreases in chlorophyll and protein content, as well as the increase in hydraulic permeability (Poovaiah and Leopold, 1973). The application of a Ca^{2+} channel blocker hastened the senescence of detached wild-type leaves maintained in the dark, increasing the rate of chlorophyll loss, the expression of a senescence-associated gene, and lipid peroxidation (Ma and

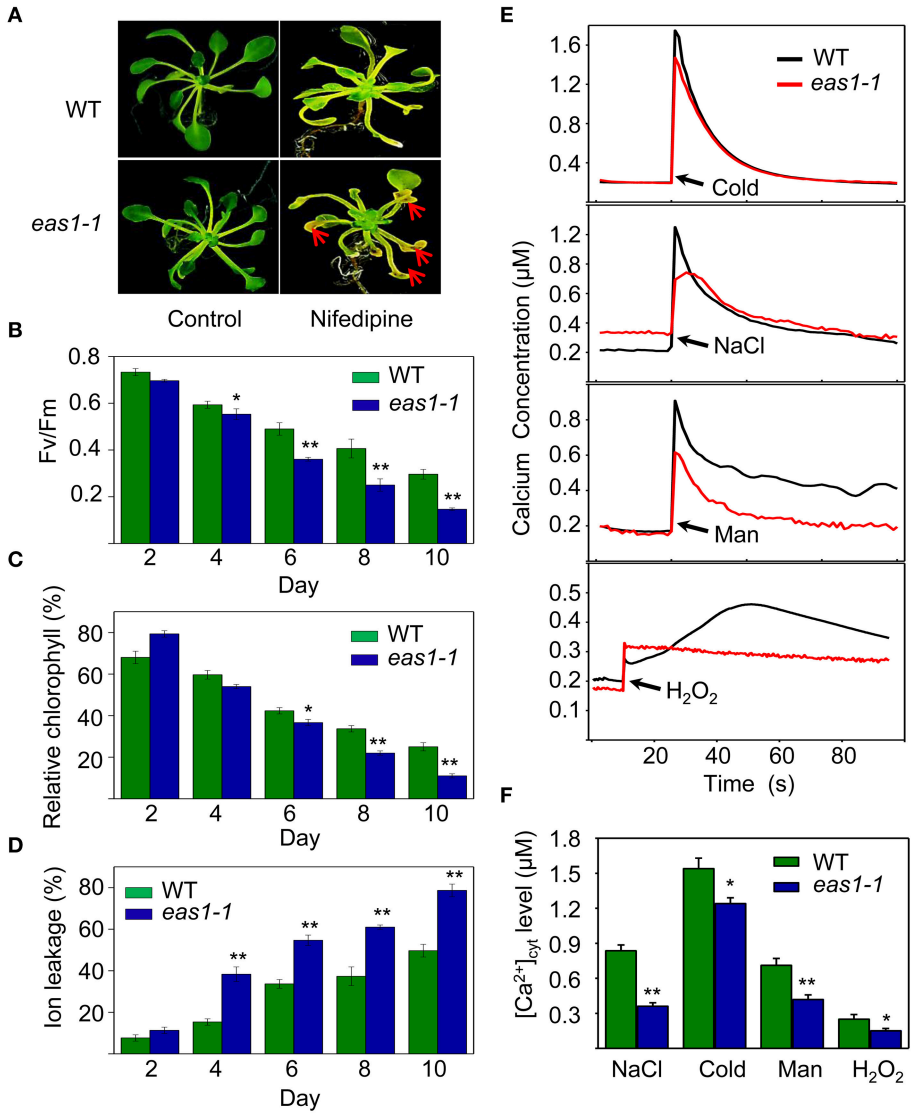

FIGURE 7 | Calcium deficiency accelerates leaf senescence of *eas1-1* mutant plants and analysis of kinetic changes in $[Ca^{2+}]_{cyt}$ in response to several stresses in wild-type and *eas1-1* mutant seedlings. (A) Seedlings grown on MS medium for 5 days were then moved to MS medium supplemented with 200 μM nifedipine for 8 days. Clear aging symptoms are shown by red arrows. **(B)** Time-dependent Fv/Fm values of wild-type and *eas1-1* mutant seedlings on medium supplemented with 200 μM nifedipine. **(C,D)** Relative chlorophyll contents and ion leakage at different times in wild-type and *eas1-1* leaves on medium supplemented with 200 μM nifedipine. **(E)** Elevation of $[Ca^{2+}]_{cyt}$ measured by Aq-emitted luminescence in response to cold shock (4°C), 400 mM NaCl, 500 mM mannitol, and 10 mM H_2O_2 in 7-days-old wild-type and *eas1-1* mutant seedlings. **(F)** Increased Ca^{2+} concentrations evoked by cold shock (4°C), 400 mM NaCl, 500 mM mannitol, and 10 mM H_2O_2 in 7-days-old wild-type and *eas1-1* mutant seedlings. Bars indicate standard errors, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$); three experiments were performed with similar results.

Berkowitz, 2011). Moreover, a calmodulin (CaM) antagonist enhanced the accumulation of the transcripts of senescence genes in detached leaves and CaM signaling could attenuate leaf senescence by inhibiting the expression of such genes (Fujiki et al., 2005). Similar results were obtained here in that *eas1* mutant plants displayed early senescence of leaves upon calcium blocker treatment (**Figures 7A–D**). The elevation in $[Ca^{2+}]_{cyt}$ was inhibited in *eas1-1* mutant plants in response to multiple stresses (**Figures 7E,F**). The expression of calcium channel genes was enhanced and ABA-activated Ca^{2+} channel activity was disrupted in *eas1-1* guard cells (**Figure 8**). Thus, it is possible that endogenous ABA-induced transient increase in $[Ca^{2+}]_{cyt}$ is an important component of early leaf senescence.

Earlier physiological observations that light-induced stomatal opening suppressed oat leaf senescence and stomatal closure accelerated or promoted senescence indicated that opening and closing of leaf stomata is the initial factor associated with senescence (Thimann and Satler, 1979a,b; Gepstein and Thimann, 1980). Mutants that are defective in ABA synthesis and stomatal response provide effective tools to dissect the

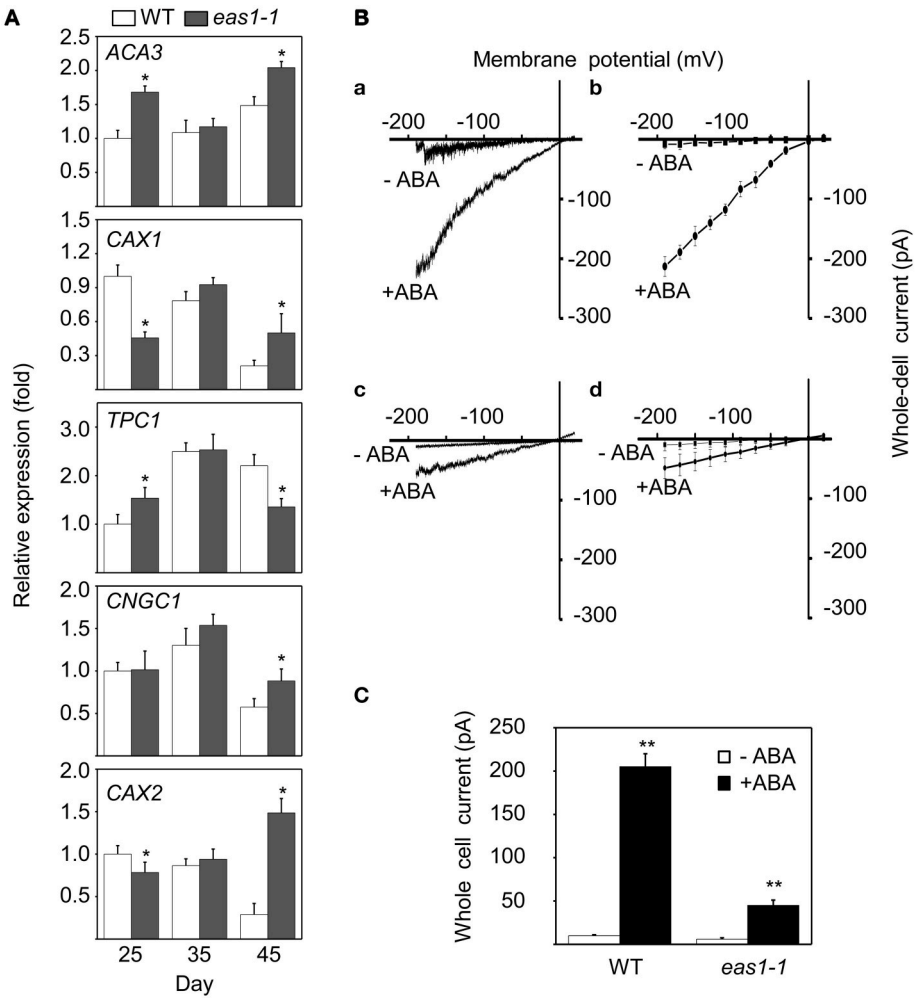

FIGURE 8 | Expression of several calcium channel genes and changes of Ca^{2+} influx currents activated by ABA in guard cells. (A) mRNA expression levels of *ACA3*, *CAX1*, *TPC1*, *CNGC1*, and *CAX2* in leaves from wild-type and *eas1-1* mutant plants at 25, 35, and 45 days under unstressed conditions in soil. *Actin* was used as an internal standard. Total RNA was isolated from the sixth and seventh true leaves at the indicated time points. **(B) (a,c)** Whole-cell Ca^{2+} influx currents in guard cells of wild-type and *eas1-1* mutant plants with or without 50 μM ABA; **(b,d)** Statistical analysis of Ca^{2+} channel currents in **(a,c)** ($n = 10$). **(C)** Average Ca^{2+} currents at 150 mV in wild-type and *eas1* mutant guard cells with or without 50 μM ABA treatment. Error bars indicate standard deviations, while asterisks indicate significant differences from wild-type plants under Student's test (*$p < 0.05$, **$p < 0.01$); three experiments were performed with similar results.

relationship between stomatal behavior and senescence onset. When the stomata open due to ABA deficiency, the release of blue signal in *cop1* mutant, or lowered ion transport activity, the results from these stomatal mutants are not the same in terms of leaf senescence: only the ABA-deficient mutants showed the promotion of senescence; Conversely, the *cop1* mutant did not display early-senescence syndrome, although it showed larger stomatal apertures compared with wild-type plants (**Figures 5**, **6**). In addition, transgenic *Arabidopsis* plants overexpressing RAP2.6L showed delayed water logging-induced early senescence by an increase of ABA content, stomatal closure, and antioxidant enzyme activity (Liu et al., 2012). Keeping ABA at the basal level is very important for plant development or stomatal regulation (Cheng et al., 2002). These results indicate that there is a close positive correlation between stomatal aperture size and plant senescence, but leaf senescence depends on the endogenous ABA level. This counters the assertion that stomata aperture size is the initial factor of senescence, and supports the fundamental role of the endogenous level of ABA in leaf senescence onset.

In summary, we suggest that ABA functions in development and senescence by orchestrating gene expression and the accumulation of physiological changes, which is similar to the theory of the yin-yang balance in traditional Chinese medicine (**Figure 9**). Keeping yin-yang in harmony is akin to attaining a homeostatic state (Ou et al., 2003), and the imbalance of yin-yang has been considered to be the cause of all disease. Similarly, plants enter senescence in yin, in which the role of ABA in the resistance to processes of cell deterioration gradually weakens. In detail, with developmental events taking place, cumulative physiological changes occur, such as the loss of

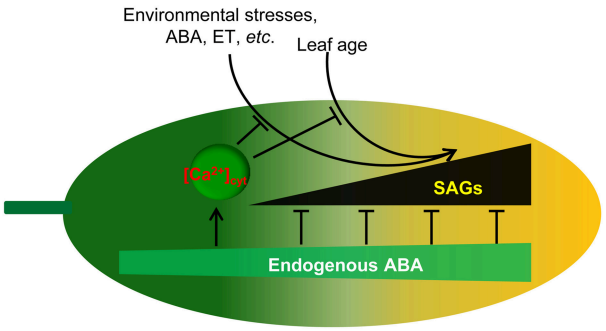

FIGURE 9 | Proposed model for endogenous ABA function in leaf senescence. Leaf age or environmental stresses trigger the onset of senescence through the promotion of SAG expression. Endogenous ABA not only promotes plant growth and development but also inhibits leaf senescence onset through the inhibition of SAG expression. Meanwhile, endogenous ABA content increases with environmental stresses and leaf age. $[Ca^{2+}]_{cyt}$ is involved in the regulation of this process. Thus, ABA appears to act as a key regulator linking internal and external factors and leaf senescence.

water from the senescing tissue, leakage of ions, transport of metabolites to different tissues, and biochemical changes, such as the generation of ROS, increases in membrane fluidity and peroxidation, and hydrolysis of proteins, nucleic acids, lipids, and carbohydrates. These downward conditions belong to yin. Those factors with protective properties, such as chlorophyll content, chloroplast number, antioxidant enzyme activities, known as upward conditions, pertain to yang. ABA is a key regulator for keeping yin-yang coordination in plant life. The senescence conditions lead to cumulative ABA with age. Under these conditions without ABA, leaf cells undergo rather orderly changes in cell structure, metabolism, and gene expression. The earliest and most significant change in cell structure is the breakdown of the chloroplast and the other organelles. Metabolically, carbon assimilation is replaced by the catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA. This could also explain why the application of exogenous ABA accelerates senescence in detached leaves, in which the high level of ABA is similar to that in stress conditions. Therefore, ABA is a factor controlling the onset of leaf senescence.

AUTHOR CONTRIBUTIONS

YS, YM, CM, and CS designed the research. YS, FX, and GZ. performed the research. YS and CS wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00181

Table S1 | List of primers used in this study.

Table S2 | Complementation tests of Arabidopsis *eas1* and *aba* mutants.

Figure S1 | EAS1 mutation accelerates plant senescence and flowering under unstressed conditions. (A) Comparison of flowering phenotype from 40-day-old wild-type and *OE-EAS1* and *eas1* mutant plants grown under unstressed conditions in soil. **(B)** Thirty-five-day-old wild-type (Col-0 background) and *aba2-1* (Col-0 background) mutant plants grown under unstressed conditions. **(C)** Seedlings of wild-type and *OE-EAS1* and *eas1* mutant plants grown for 30 days on agar plate in unstressed growth conditions. *eas1-1* and *eas1-2* mutant plants show the senescence phenotype of yellowing leaves.

Figure S2 | The *eas1* mutants are two new *aba2* alleles. (A) Positional cloning of *EAS1*. EAS1 was mapped to chromosome 1 between BAC clones F9I5 and F6D8. The *eas1-1* and *eas1-2* mutations were generated in the second exon in the *ABA2* gene (At1g52340). Closed boxes represent the open reading frame. The red dot is the centromere of chromosome 1. **(B)** ABA content of the fifth rosette leaf of wild-type and mutant plants at day 25 after sowing under unstressed condition in soil. **(C)** Germination of *eas1-1 and eas1-2* mutant seed is apparently insensitive to salt and mannitol treatment at different concentrations compared with wild-type seed. Seeds were germinated on agar medium supplemented with different concentration of NaCl or mannitol.The data collection were performed at day 5 after germination. **(D)** Infrared image of wild-type and *eas1-1* and *eas1-2* mutant plants at day 25 after sowing under unstressed conditions in soil. **(F)** Rate of water loss of detached leaves of wild-type and *eas1-1* and *eas1-2* mutant plants. Three experiments were performed with similar results. Error bars indicate standard deviations, while asterisks indicate significant differences from wild-type plants under Student's test (**$p < 0.01$).

Figure S3 | Determination of viability of wild-type and *eas1-1* mutant protoplasts in an age-dependent manner. (A,B) Estimation of mesophyll protoplast activity by FDA staining. Bars indicate standard errors, while asterisks indicate significant differences from wild-type plants (*$p < 0.05$, **$p < 0.01$); three experiments were performed with similar results. **(C)** Total protein extracted from the same weight of fresh leaf of the first to sixth true leaves of wild-type and *eas1-1* mutant plants. Ribulose 1,5-bisphosphate carboxylase/oxygenase (RBCL) is a protein marker. Proteins were visualized by coomassie blue staining.

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REVIEW
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Microbe Associated Molecular Pattern Signaling in Guard Cells

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Stomata, formed by pairs of guard cells in the epidermis of terrestrial plants, regulate gas exchange, thus playing a critical role in plant growth and stress responses. As natural openings, stomata are exploited by microbes as an entry route. Recent studies reveal that plants close stomata upon guard cell perception of molecular signatures from microbes, microbe associated molecular patterns (MAMPs), to prevent microbe invasion. The perception of MAMPs induces signal transduction including recruitment of second messengers, such as Ca^{2+} and H_2O_2, phosphorylation events, and change of transporter activity, leading to stomatal movement. In the present review, we summarize recent findings in signaling underlying MAMP-induced stomatal movement by comparing with other signalings.

Keywords: guard cell, microbe-associated molecular patterns, reactive oxygen species, ion channels, Ca^{2+} signaling, mitogen-activated protein kinase, open stomata 1, Ca^{2+}-dependent protein kinase

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INTRODUCTION

Stomata are microscopic pores formed by pairs of guard cells in the shoot epidermis of plants and regulate gas exchange, notably CO_2 uptake for photosynthesis and water loss during transpiration, thus playing a critical role in plant growth and stress responses such as drought stress. As natural openings, stomata are exploited as a major entry route by a wide range of microbes including bacteria, oomycetes, and fungi (Grimmer et al., 2012; Sawinski et al., 2013). On the other hand, plants proactively induce stomatal closure and inhibit stomatal opening to prevent microbe invasion, which is later termed as stomatal immunity (Melotto et al., 2008; Sawinski et al., 2013). Stomatal immunity is closely related to susceptibility to a variety of pathogenic microbe infections. For example, defection in stomatal closure in response to pathogenic bacteria increased infection in *Arabidopsis* (Melotto et al., 2006; Singh et al., 2012), while pre-closing stomata by abscisic acid (ABA) reduced infection in grapevine by oomycete, *Plasmopara viticola* (Allègre et al., 2009).

Microbe-associated molecular patterns (MAMPs) are molecular signatures that are highly conserved in whole classes of microbes but are absent from the host, such as chitin for fungi and flagellin for bacteria (Boller and Felix, 2009). Recognition of each MAMP is performed by specific surface-localized receptors containing various ligand-binding ectodomains in plants, which are termed as pattern-recognition receptors (PRRs). The perception of MAMPs by PRRs triggers signaling converging to common responses, such as ion fluxes including Ca^{2+} influx, K^+ efflux, and anion efflux, production of reactive oxygen species (ROS) and phosphorylation events, which are critical for plant innate immunity (Boller and Felix, 2009; Zipfel, 2014). Recent studies revealed that several MAMPs induce stomatal closure and inhibit stomatal opening, including flg22 (a conserved 22-amino-acid peptide near the N terminus of bacterial flagellin (Melotto et al., 2006; Zhang et al., 2008). Further results showed that plants with loss-of-function of the PRR for flg22,

FLAGELLIN-SENSITIVE 2 (FLS2), did not close stomata in response to flg22 and coronatine-deficient *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000, *Pst* DC3118 (Melotto et al., 2006; Zeng and He, 2010). As a result, the mutant is more susceptible to *Pst* DC3118 than wild type. These results indicate that plants mainly sense MAMPs to induce stomatal closure to prevent microbe invasion. The past decade has seen increasing efforts in elucidating MAMP signaling in guard cells and exciting findings every year. Several excellent reviews have been published covering this topic (Melotto et al., 2008; Zeng et al., 2010; Sawinski et al., 2013; McLachlan et al., 2014; Arnaud and Hwang, 2015). In the present review, we concentrate on the current knowledge of MAMP signaling in guard cells and discuss the latest findings by comparing with other signalings.

ABSCISIC ACID SIGNALING IN GUARD CELLS

Phytohormones play critical roles in regulating stomatal movement. Almost all the phytohormones are reported to be involved in stomatal movement, among which ABA, methyl jasmonic (MeJA), and salicylic acid (SA) are believed to induce stomatal closure in various plants (Acharya and Assmann, 2009; Murata et al., 2015). Particularly, mechanism of stomatal movement has been well characterized in the context of ABA signaling in guard cells. In this section, we briefly overview ABA signaling in *Arabidopsis* guard cells. For details on this topic, we refer readers to excellent reviews (Hubbard et al., 2010; Kim et al., 2010; Joshi-Saha et al., 2011).

Abscisic acid is mainly produced in response to drought stress and the ABA synthesized in guard cells plays a critical role in regulation of stomatal movement (Bauer et al., 2013). An Snf1-related protein kinase 2 (SnRK2), SnRK2.6 also known as Open stomata 1 (OST1), is a Ca^{2+}-independent protein kinase and an essential positive regulator in ABA signaling in *Arabidopsis* guard cells. In resting condition, OST1 kinase activity is inhibited by clade A Type 2C protein phosphatases (PP2Cs). Upon ABA perception, the interaction of ABA receptors, PYR/PYL/RCAR, and PP2Cs releases the inhibition of OST1, resulting in increment of OST1 kinase activity (Cutler et al., 2010; Hubbard et al., 2010; Joshi-Saha et al., 2011). In guard cell ABA signaling, OST1 is essential for recruitment of second messengers, such as H_2O_2, NO, and Ca^{2+}, which are important for regulation of transporters in the plasma membrane including S-type anion channels and H^+-ATPases (Mustilli et al., 2002; Bright et al., 2006; Acharya et al., 2013; Yin et al., 2013). OST1 has been reported to directly regulate ion channels including S-type anion channel SLAC1 (Geiger et al., 2009; Lee et al., 2009; Brandt et al., 2012), R-type anion channel ALMT12 (Meyer et al., 2010; Sasaki et al., 2010; Imes et al., 2013), inward-rectifying K^+ channels (K_{in} channels) KAT1 (Sato et al., 2009; Siegel et al., 2009; Uraji et al., 2012), K^+ uptake transporter 6 (KUP6; Osakabe et al., 2013), vacuolar anion exchanger CLCa (Wege et al., 2014) and Plasma membrane Intrinsic Protein 2;1 (PIP2;1; Grondin et al., 2015). Downstream components are also involved in ABA regulation of transporters including several Ca^{2+}-dependent protein kinases (CDPK), CPK3, CPK4, CPK5, CPK6, CPK10, CPK11, and CPK23 (Mori et al., 2006; Zhu et al., 2007; Zou et al., 2010; Brandt et al., 2015), and mitogen-activated kinases (MAPKs), MPK9 and MPK12 (Jammes et al., 2009). The regulation of transporters by ABA, especially the activation of anion channels and suppression of H^+-ATPases, causes depolarization of plasma membrane. For open stomata, this leads to efflux of anion, K^+ and water, resulting in stomatal closure. For closed stomata, this keeps stomata closed in response to light.

MAMPs KNOWN TO INDUCE STOMATAL CLOSURE

A wide range of MAMPs from bacteria, fungi and oomycetes have been identified and characterized (Zipfel, 2014). New types of MAMPs are emerging such as peptides from Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (Oome et al., 2014) and glycoside hydrolase family 12 (GH12) protein (Ma et al., 2015). The well-known MAMPs that induce stomatal movement are bacterium-derived MAMPs including flg22, elf18/elf26 (the first 18/26-amino-acid peptide of the N terminus of bacterial elongation factor Tu) and bacterial lipopolysaccharide (LPS), and fungus-derived MAMPs including chitin, chitosan and YEL. β-1→3-linked glucans are major components in oomycete cell wall and have been used to study stomatal movement (Allègre et al., 2009; Fu et al., 2011; Robinson and Bostock, 2014). In this section, we briefly introduce the molecular nature and perception of these MAMPs. For detail, we refer readers to excellent reviews (Boller and Felix, 2009; Zipfel, 2014; Sánchez-Vallet et al., 2015; Shinya et al., 2015).

MAMPs Derived from Bacteria

The widely used flg22, QRLSTGSRINSAKDDAAGLQIA, is an epitope in the flagellin of *P. syringae* pv. *tabaci* 6605, and induces stomatal closure and inhibits light-trigged stomatal opening in *Arabidopsis* (Melotto et al., 2006; Zhang et al., 2008). Though this epitope is widely conserved, studies have shown that variations of this epitope that are not sensed by *Arabidopsis* exist in several pathogenic bacteria (Pfund et al., 2004; Robatzek et al., 2006). The flg22 used here only refers to the one from *P. syringae* pv. *tabaci* 6605, which has been used in most of studies on stomatal movement. The perception of flg22 is mediated by a plasma membrane-localized leucine-rich repeat type receptor-like kinase (LRR-RLK), Flagellin-sensitive 2 (FLS2; Gomez-Gomez and Boller, 2000), which is expressed in guard cells (Melotto et al., 2006; Beck et al., 2014). Loss-of-function mutation of *FLS2* abolishes flg22-induced stomatal movement (Melotto et al., 2006; Zhang et al., 2008). Recent studies identified another epitope in the flagellin from various *P. syringae* pv. *tomato*, 28 amino acid long peptides (flgII-28), which induces stomatal closure in tomato (Cai et al., 2011). However, the PRR for flgII-28 remains to be identified.

Elongation factor is one of the most abundant proteins in bacteria. The widely used elf18, *N*-acetylated SKEKFERTKPHVNVGTIG, and elf26, *N*-acetylated SKEKF ERTKPHVNVGTIGHVDHGKTT, are the bioactive N terminals

of elongation factor with the first 18 and 26 amino acid residues from *Escherichia coli* and both induce stomatal closure in *Arabidopsis* (Zeng and He, 2010). The PRR for these two peptides in *Arabidopsis* is Elongation factor Tu receptor (EFR), which is also an LRR-RLK expressed in guard cells (Zipfel et al., 2006; Liu et al., 2009). Plants outside of the Brassicaceae are believed not to respond to this epitope of EF-Tu due to lack of EFR (Zipfel et al., 2006). Recently, a new epitope from the middle region comprising Lys176 to Gly225 of the *Acidovorax avenae* EF-Tu, termed as EFa50, is identified as a MAMP sensed by rice (Furukawa et al., 2014). It remains to be clarified whether EFa50 regulates stomatal movement in rice.

Upon binding of flg22 or elf18/elf26, FLS2 or EFR forms receptor complexes with LRR-RLKs belonging to Somatic-embryogenesis receptor-like kinase (SERK) family, among which Brassinosteroid insensitive1-associated kinase1/SERK3 (BAK1/SERK3) plays a dominant role (Chinchilla et al., 2007; Roux et al., 2011). Structure data revealed that the C-terminal region of flg22 functions as a molecular glue between ectodomains of FLS2 and BAK1 (Sun et al., 2013). Recent studies revealed that receptor-like cytoplasmic kinases (RLCKs) are direct substrates of the receptors complexes and transduce the signal of MAMPs to downstream events (Macho and Zipfel, 2014). A RLCKs, Botrytisinduced kinase1 (BIK1), is essential for stomatal closure induced by flg22 but not ABA (Li et al., 2014).

Lipopolysaccharides are characteristic components of Gram-negative bacteria and composed of conserved lipid A, core oligosaccharide regions and a long-chain poly saccharide (the O antigen) that can have variable composition, length, and branching of its carbohydrate subunits. Due to variety in structure and easy contamination during preparation, LPS used in many of the plant researches can be consider a mixture of MAMPs and is derived from human pathogen *P. aeruginosa.* The LPS induces stomatal closure in *Arabidopsis* and tomato (Melotto et al., 2006; Liu et al., 2009; Desclos-Theveniau et al., 2012). Lipopolysaccharides from *E. coli* O55:B5 induced stomatal closure in *Arabidopsis* (Melotto et al., 2006). The PRRs for LPS are still under investigation. It has been shown that plants sense the O antigen, core oligosaccharide and lipid A (Bedini et al., 2005; Silipo et al., 2005). Recent studies further revealed that a bulb-type (B-type) lectin S-domain (SD)-1 RLK, Lipooligosaccharide-specific reduced elicitation (LORE), functions as a putative PRR for LPS from *Pseudomonas* and *Xanthomonas* species including *P. aeruginosa* by sensing lipid A moiety, and is restricted to the Brassicaceae family of plants (Ranf et al., 2015). Interestingly, *Arabidopsis* responds to LPS from *E. coli* K12 and *E. coli* O111:B4 in an LORE-independent manner (Ranf et al., 2015). These results suggest that multiple PRRs including LORE are involved in LPS-induced stomatal closure.

MAMPs Derived from Fungi and Oomycetes

Chitin is an insoluble polymer of β-1,4-linked *N*-acetylglucosamineone and one of the major components of fungal cell wall. Chitosan is the deacetylated form of chitin. Caution has to be paid that commercially available chitosan can be only partially deacetylated. For example, some chitosan from Sigma is 75 to 85% deacetylated. Chitin induces stomatal closure in *Arabidopsis* (Lozano-Duran et al., 2014; Bourdais et al., 2015). Chitosan induces stomatal closure and inhibits light-induced stomatal opening in various plant species, such as *Arabidopsis* (Klüsener et al., 2002), tomato (Lee et al., 1999), pea (Srivastava et al., 2009), rapeseed (Li et al., 2009), tobacco (Fu et al., 2011), and barley (Koers et al., 2011). In these studies, both chitin and chitosan are a mixture of polymers with different degree of polymerization (DP). Actually, the degree of polymerization is critical for the plant responses to chitin and chitosan (Kauss et al., 1989; Yamada et al., 1993; Vander et al., 1998; Liu et al., 2012). A current model shows that perception of chitin in *Arabidopsis* involves three lysin motif type RLKs (LysM-RLKs), Lysin motif receptor kinase 5 (LYK5), LYK4 and chitin elicitor receptor kinase 1 (CERK1) (Miya et al., 2007; Cao et al., 2014). It has been reported that LYK5 has much higher affinity to chitin than CERK1 does, interacts with CERK1, and is indispensable for chitin-triggered CERK1 phosphorylation (Cao et al., 2014). These results suggest that LYK5 and CERK1 form a receptor complex for chitin. Recent studies also identified several RLCKs as downstream components of the receptor complex (Zhang et al., 2010; Shinya et al., 2014). For the perception of chitosan, high DP of chitosan seems to be critical (Kauss et al., 1989; Vander et al., 1998; Iriti and Faoro, 2009). Chitosan oligomers only weakly bind to CERK1 and is unlikely to induce several plant responses including ROS production (Kauss et al., 1989; Vander et al., 1998; Petutschnig et al., 2010). It is further suggested that surface charge of fully deacetylated chitosan polymers is responsible for their effects on plants (Kauss et al., 1989).

Mixtures of β-1→3-linked glucans with different DP have been shown to induce stomatal closure and inhibits stomatal opening in grapevine and tobacco (Allègre et al., 2009; Fu et al., 2011). The strength of stomatal closure induced by the glucans showed dependency on DP, which may reflect the different perceptions of these glucans. However, the molecular mechanism of β-1→3-linked glucan perception is poorly understood.

Elicitors from baker's yeast (YEL) extracted by ethanol precipitation mainly contains fungal cell wall fraction including mannan, β-1→3-linked glucans, chitin, and glycopeptides, and has been widely used as a fungal MAMP to induce plant immune response including stomatal responses (Hahn and Albersheim, 1978; Schumacher et al., 1987; Gundlach et al., 1992; Blechert et al., 1995; Kollar et al., 1997; Klüsener et al., 2002; Zhao et al., 2004; Ge and Wu, 2005; Khokon et al., 2010a; Salam et al., 2013; Ye et al., 2013b, 2015; Moon et al., 2015; Narusaka et al., 2015). YEL induces stomatal closure and inhibits light-induced stomatal opening in *Arabidopsis* (Klüsener et al., 2002; Khokon et al., 2010a; Salam et al., 2013; Ye et al., 2013b, 2015).

CORE SIGNALING EVENTS DOWNSTREAM OF MAMP PERCEPTION IN GUARD CELLS

Guard cell signaling induced by MAMPs involves in recruitment of second messengers, such as H_2O_2, NO, and Ca^{2+},

phosphorylation events mediated by CDPKs, OST1, and MAPKs, and changes of transporter activity. In this section, we review the main signaling events upon perception of MAMPs with focus on findings from *Arabidopsis* guard cells.

Production of Reactive Oxygen Species in Guard Cell MAMP Signaling

Reactive oxygen species, particularly H_2O_2, are important second messengers in guard cell signaling induced by abiotic and biotic factors (Pei et al., 2000; Zhang et al., 2001; Khokon et al., 2010a; Hoque et al., 2012; Hossain et al., 2013; Ye et al., 2013a; Kadota et al., 2014; Sobahan et al., 2015). Researches using leaf disks have provided most of the current knowledge of MAMP-induced H_2O_2 production. Recent studies further revealed that MAMPs including flg22, elf26, LPS, chitosan, β-1→3-linked glucans and YEL induce accumulation of H_2O_2 in guard cells (Desikan et al., 2008; Allègre et al., 2009; Ma W. et al., 2009; Desclos-Theveniau et al., 2012; Ma et al., 2013; Salam et al., 2013; Ye et al., 2015).

One of the main mechanisms involved in H_2O_2 production is mediated by plasma membrane-localized NAD(P)H oxidases, RBOHD and RBOHF. NAD(P)H oxidases transfer electrons from cytosolic NAD(P)H to apoplastic oxygen, leading to superoxide production. The superoxide can be converted to H_2O_2 through dismutation by unknown superoxide dismutase (Jannat et al., 2011; Suzuki et al., 2011). The produced H_2O_2 can accumulate in guard cells through diffusion and water channels (Henzler and Steudle, 2000; Bienert et al., 2007; Grondin et al., 2015). In ABA and MeJA signaling, both RBOHD and RBOHF redundantly regulate H_2O_2 production and stomatal closure (Kwak et al., 2003; Suhita et al., 2004; Munemasa et al., 2007). On the other hand, it seems that RBOHD plays a prominent role in flg22- and elf18-induced stomatal closure and H_2O_2 production in leaf (Mersmann et al., 2010; Macho et al., 2012; Kadota et al., 2014; **Figure 1**). Regarding the activation of NAD(P)H oxidases, studies have shown that elevation of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and phosphorylation by CDPKs are important for flg22- and elf18-induced H_2O_2 production in leaf disks (Boudsocq et al., 2010; Dubiella et al., 2013; Kadota et al., 2014), but not for ABA- and MeJA-induced H_2O_2 production in guard cells (Suhita et al., 2004; Munemasa et al., 2011; Brandt et al., 2015). Ca^{2+}-independent phosphorylation is essential to the activation of NAD(P)H oxidases. In ABA signaling, the Ca^{2+}-independent protein kinase, OST1, phosphorylates RBOHF at Ser13 and Ser174 and interacts with RBOHD (Sirichandra et al., 2009; Acharya et al., 2013) and is essential for H_2O_2 production in guard cells (Mustilli et al., 2002; Yin et al., 2013). Recent studies revealed that phosphorylation of several phosphorylation sites at the N-terminal part of RBOHD including Ser39 and Ser343 is increased in response to flg22 and elf18, which is independent on the elevation of $[Ca^{2+}]_{cyt}$ but dependent on the Ca^{2+}-independent activation of RLCKs, particularly BIK1 (Kadota et al., 2014; Li et al., 2014). Further results showed that phosphor-dead mutations of these phosphorylation sites suppress H_2O_2 production and stomatal closure induced by flg22 and elf18 and phosphor-mimetic mutations of the sites do not induce H_2O_2 production and stomatal closure but complement the flg22- and elf18-induced H_2O_2 production and stomatal closure in plants of loss-of-function mutations of *BIK1* and another *RLCK*, *PBL1*. These results indicate that phosphorylation of these sites by RLCKs is essential but not sufficient to induce H_2O_2 production in leaf disks. To integrate the Ca^{2+}-dependent and -independent regulation of RBOHD, the authors proposed that the BIK1-mediated phosphorylation primes RBOHD activation by increasing the sensitivity to the Ca^{2+}-based regulation (Kadota et al., 2014, 2015; Li et al., 2014). Future work is needed to elucidate this hypothesis.

Another important mechanism involved in H_2O_2 production in guard cell is mediated by class III peroxidases (PRXs; **Figure 1**). Both internal factors, such as SA (Mori et al., 2001; Khokon et al., 2011), isothiocyanates (ITCs; Hossain et al., 2013; Sobahan et al., 2015) and methylglyoxal (Hoque et al., 2012), and external factors, such as YEL (Khokon et al., 2010a), chitosan (Khokon et al., 2010b), flg22 (O'Brien et al., 2012), and elf26 (O'Brien et al., 2012), induce H_2O_2 production mediated by PRXs. Pharmacological and genetic studies further showed that salicylhydroxamic acid (SHAM)-sensitive PRXs but not RBOHD or RBOHF are essential for stomatal closure and H_2O_2 production in guard cells induced by SA, ITCs, methylglyoxal, chitosan and YEL (Khokon et al., 2010a,b, 2011; Hoque et al., 2012; Hossain et al., 2013). The chemistry of H_2O_2 production by PRXs can be affected by factors including pH and reductants. For example, under an acidic reaction condition, SA itself functions as electron donor to produce superoxide catalyzed by PRXs, which then can be converted to H_2O_2 by dismutases (Mori et al., 2001). Under an alkali condition, PRXs can directly utilize electron donors to produce H_2O_2, in which it seems that thiol compounds are preferred as the electron donors (Bolwell et al., 1995; Bolwell et al., 2002). While the identification of reductants in a physiological context remains challenging, apoplast alkalization is widely observed in plants treated by MAMPs such as flg22, elf18, and chitin (Felix et al., 1999; Felle et al., 2004; Zipfel et al., 2006; Boller and Felix, 2009). Recent studies have tried to identify the PRXs involved in the H_2O_2 production induced by MAMPs. Two PRXs, PRX33, and PRX34, were shown to be involved in H_2O_2 production induced by flg22, elf26, and fungal derived MAMPs (Daudi et al., 2012; O'Brien et al., 2012). However, it remains unknown whether these PRXs are involved in stomatal movement.

Production of Nitric Oxide Induced by MAMPs in Guard Cells

Nitric oxide has been widely shown to be involved in stomatal movement induced by various biotic and abiotic stimuli such as ABA, MeJA, SA, allyl isothiocyanate (AITC), and flg22 (Neill et al., 2002; Melotto et al., 2006; Garcia-Mata and

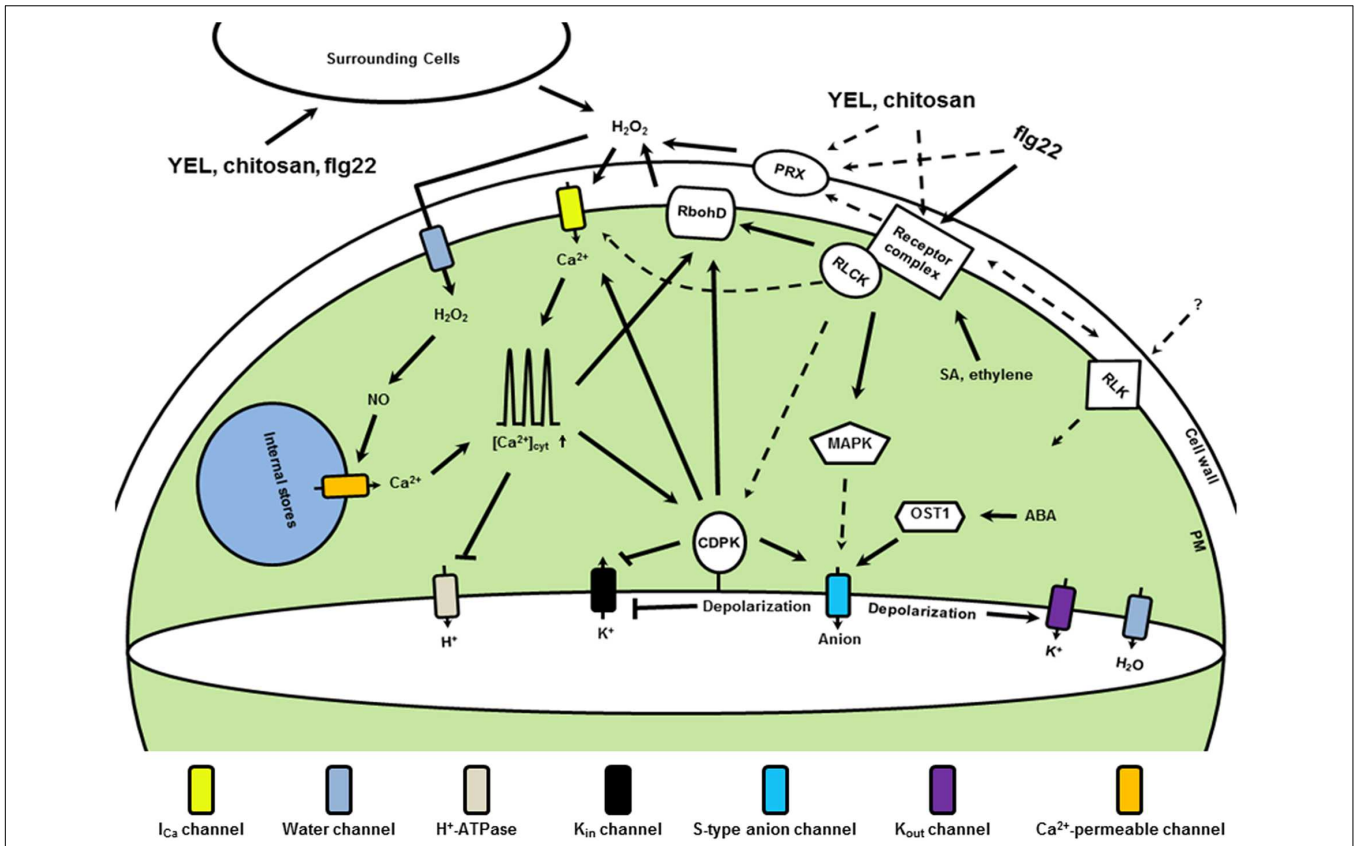

FIGURE 1 | Simplified microbe associated molecular patterns (MAMP) signaling in *Arabidopsis* guard cells. Upon perception of MAMPs, such as YEL, chitosan and flg22, receptor complexes are formed in the plasma membrane, which activate receptor-like cytoplasmic kinases (RLCKs). Downstream events are H_2O_2 production mediated by RbohD and PRXs, and activation of MAPKs and unidentified I_{Ca} channels. H_2O_2 accumulates in the cytosol and induces production of NO, which regulates Ca^{2+} release from internal stores. Elevation of $[Ca^{2+}]_{cyt}$ negatively regulates the activity of H^+-ATPases and activates CDPKs. Regulation by upstream components is also essential for CDPK activation. CDPKs negatively regulate K_{in} channel activity but positively regulate I_{Ca} channels and S-type anion channels. MAPK and resting OST1 activity are essential for S-type anion channel activation. Ethylene and SA at resting level regulate receptor levels. ABA at resting level may contribute to the resting OST1 activity. Increasing RLKs, such as GHR1, CRKs, and LecRKs, are found to be involved in MAMP signaling, but their activation is largely unknown. Arrowheads designate positive regulation and bars show negative regulation. Dashed lines indicate possible pathways need to be clarified, and question marks unconfirmed components.

Lamattina, 2007; Munemasa et al., 2007; Khokon et al., 2010a, 2011). Flg22, LPS, YEL and chitosan induce NO production in guard cells, which is required for stomatal closure (Melotto et al., 2006; Srivastava et al., 2009; Khokon et al., 2010a,b). Application of H_2O_2 has been shown to induce NO production in *Arabidopsis* guard cells (Bright et al., 2006). It is likely that H_2O_2 contributes to NO production induced by MAMPs. Genetic and pharmacological studies have shown that H_2O_2 is involved in ABA-induced NO production (Bright et al., 2006). Pharmacological studies have also shown that H_2O_2 is involved in NO production induced by YEL and chitosan (Srivastava et al., 2009; Khokon et al., 2010a,b). However, the mechanism how H_2O_2 is involved in NO production remains unknown. Cyclic AMP has been shown to be involved in LPS-induced NO production (Ma W. et al., 2009). Enzymatic mechanism mediated by nitrate reductases and NO synthase-like enzymes and non-enzymatic mechanism are involved in generation of NO in plant cells (Desikan et al., 2002; Moreau et al., 2008; Neill et al., 2008; Gayatri et al., 2013). Pharmacological studies have suggested that NO synthase-like enzymes were involved in flg22-, LPS-, and chitosan-induced stomatal closure (Melotto et al., 2006; Srivastava et al., 2009). However, the genes of NO synthase-like enzymes remain to be identified. It has been suggested that nitrate reductases were involved in chitosan-induced NO production (Srivastava et al., 2009). Two *Arabidopsis*, NIA1 and NIA2, were reported to be involved in ABA- and SA-induced NO production and stomatal closure (Desikan et al., 2002; Hao et al., 2010). Further investigation of the roles of NIA1 and NIA2 in MAMP-induced NO production and stomatal closure may move the field forward.

Elevation of $[Ca^{2+}]_{cyt}$ Induced by MAMPs in Guard Cells

Cytosolic Ca^{2+} has long been considered as an important second messenger in guard cell signaling induced by various internal and external stimuli (Shimazaki et al., 2007; Kim et al., 2010;

Roelfsema and Hedrich, 2010). A lot of methods have been introduced to monitor the $[Ca^{2+}]_{cyt}$ in guard cells including fluorescent dyes such as fura-2 and fluo-3, and genetically encoded indicators such as aequorin, yellow cameleon and R-GECO1 (Irving et al., 1992; Allen et al., 1999a,b; Harada and Shimazaki, 2009; Keinath et al., 2015). Among these methods, real-time imaging of live guard cells expressing the fluorescence resonance energy transfer-based indicator, yellow cameleon, has greatly advanced our understanding of Ca^{2+} signaling (Allen et al., 1999b; Mori et al., 2006). Based on this technique, we learn that spontaneous repetitive elevation of $[Ca^{2+}]_{cyt}$ occurs in guard cells, that stomata respond differently to different patterns of elevation of $[Ca^{2+}]_{cyt}$ (Allen et al., 2000, 2001), and that Ca^{2+} influx from the apoplast and Ca^{2+} releasing from internal stores are essential for the elevation of $[Ca^{2+}]_{cyt}$ induced by stimuli such as ABA and MeJA (Allen et al., 2000; Hamilton et al., 2000; Pei et al., 2000; Klüsener et al., 2002; Garcia-Mata et al., 2003; Munemasa et al., 2007; Akter et al., 2012; Hossain et al., 2014). Several MAMPs including chitosan, YEL, flg22 and chitin have been demonstrated to induce elevation of $[Ca^{2+}]_{cyt}$ in guard cells (Klüsener et al., 2002; Khokon et al., 2010a; Salam et al., 2013; Ye et al., 2013b; Thor and Peiter, 2014; Keinath et al., 2015). Pharmacological and genetic studies reveal that Ca^{2+} influx and Ca^{2+} release mechanisms are essential for elevation of $[Ca^{2+}]_{cyt}$ induced by chitosan, YEL and flg22 (Klüsener et al., 2002; Ye et al., 2013b; Thor and Peiter, 2014). Influx of Ca^{2+} is mediated by plasma membrane non-selective Ca^{2+}-permeable cation channels (I_{Ca} channels), which are activated by hyperpolarization (Hamilton et al., 2000; Pei et al., 2000). It has been shown that LPS, chitosan and YEL activate I_{Ca} channels in guard cells (Klüsener et al., 2002; Ali et al., 2007; Ye et al., 2013b). The identification of I_{Ca} channels involved in MAMP signaling remains challenging. Studies have suggested that glutamate receptor-like channels (GLRs) are involved in elevation of $[Ca^{2+}]_{cyt}$ in seedlings induced by flg22, elf18, and chitin (Kwaaitaal et al., 2011) and that cyclic nucleotide-gated channels (CNGCs) are involved in elevation of $[Ca^{2+}]_{cyt}$ induced by LPS (Ali et al., 2007). On the other hand, recent studies suggest that neither GLRs nor CNGCs are essential for flg22-induced elevation of $[Ca^{2+}]_{cyt}$ (Thor and Peiter, 2014). Recently, OSCA1 and its homologs have been identified as an I_{Ca} channel involved in hyperosmolality response (Hou et al., 2014; Yuan et al., 2014). It remains unknown whether OSCA1 is involved in MAMP signaling. Pharmacological studies suggested that many factors involved in Ca^{2+} releasing in guard cells, such as NO, cGMP, cADPR, and IP3 (Klüsener et al., 2002; Garcia-Mata et al., 2003; Hossain et al., 2014). Recent studies showed that Ca^{2+} releasing is essential for flg22-induced elevation of $[Ca^{2+}]_{cyt}$ (Thor and Peiter, 2014). Application of NO does not activate I_{Ca} channels in the plasma membrane, but induces Ca^{2+} releasing, probably through cGMP-cADPR pathway (Garcia-Mata et al., 2003; Joudoi et al., 2013; Hossain et al., 2014). Therefore, it is likely that NO produced by MAMPs contributes to MAMP-induced elevation of $[Ca^{2+}]_{cyt}$ in guard cells. The identification of the transporters involved in Ca^{2+} releasing remains challenging.

Sensing of Ca^{2+} in MAMP-induced Stomatal Movement

In *Arabidopsis*, there are a maximum of 250 proteins possibly having the Ca^{2+}-binding EF-hand motif (Day et al., 2002), which play important roles in transducing the signal of Ca^{2+} to downstream events. In addition to the ubiquitous eukaryotic Ca^{2+} sensor calmodulin (CaM), other proteins containing EF-hand are CDPKs, CaM-like proteins (CML) and calcineurin B-like protein (CBL). CBLs can form complexes with CBL-interacting protein kinases (CIPKs) to convert the Ca^{2+} signal to phosphorylation events (Steinhorst and Kudla, 2013).

Ca^{2+}-dependent protein kinases comprise a gene family of 34 members in *Arabidopsis*. The roles of the 34 CDPKs in plant growth and stress response can differ from each other due to their specificities in tissue expression, subcellular localization, $[Ca^{2+}]_{cyt}$ dependency, substrates and regulation mechanisms (Boudsocq and Sheen, 2013; Liese and Romeis, 2013). So far, several members of CDPKs including CPK3, CPK4, CPK5, CPK6, CPK8, CPK10, CPK11, and CPK23 are identified as positive regulators in stomatal movement induced by ABA, MeJA, CO_2 and exogenous H_2O_2 (Mori et al., 2006; Zhu et al., 2007; Zou et al., 2010, 2015; Munemasa et al., 2011; Hubbard et al., 2012; Merilo et al., 2013; Brandt et al., 2015). Recent works showed that CPK6 positively functions in YEL-induced stomatal closure and inhibition of light-induced stomatal opening (Ye et al., 2013b). On the other hand, CPK6 shows a negative effect on H_2O_2 scavenging mechanism induced by YEL. Since the CPK6 also positively functions in stomatal closure induced by ABA and MeJA (Mori et al., 2006; Munemasa et al., 2011; Brandt et al., 2015), the results suggest that CPK6 is a convergent point of signaling for stomatal closure induced by abiotic and biotic stimuli. The important role of CPK6 is related to its regulation of S-type anion channels (see Activation of S-type Anion Channels in Response to MAMPs in Guard Cells). CDPKs including CPK6 are activated by flg22 and involved in flg22-induced H_2O_2 production in leaf disks and mesophyll cells (Boudsocq et al., 2010; Dubiella et al., 2013; Guzel et al., 2015). Recent studies showed that *cpk3 cpk6 cpk5 cpk11* loss-of-function mutation shows a trend to partly impair flg22-induced stomatal closure in a nanoinfusion experiment (Guzel et al., 2015). Nevertheless, the quadruple mutation did not strongly impair flg22-induced stomatal closure, which may attribute to the compensatory mechanism by other Ca^{2+} sensing mechanisms. A recent study showed that CPK28 negatively regulates flg22-induced stomatal closure and that BIK1 is a target of CPK28 (Monaghan et al., 2014). Results from *in vitro* experiments have shown that activation of CDPKs is dependent on $[Ca^{2+}]_{cyt}$ (Boudsocq et al., 2012). In ABA signaling, the activation of CDPKs including CPK6 seems to be solely dependent on the elevation of $[Ca^{2+}]_{cyt}$ induced by ABA (Laanemets et al., 2013; Brandt et al., 2015). On the other hand, the activation of CDPKs including CPK6 in response to flg22 is dependent on both elevation of $[Ca^{2+}]_{cyt}$ and FLS2-dependent signaling (Boudsocq et al., 2010; Dubiella et al., 2013). These

results suggest the activation of CDPK differs in different signalings.

Research on involvement of CMLs and CBL-CIPK pairs in MAMP signaling in guard cells is scarce. In *Arabidopsis*, CML24 has been shown to be involved in LPS-induced stomatal closure, probably by regulating NO production (Ma et al., 2008; Walker, 2011). Recent studies have suggested that CBL-CIPK pairs are involved in stomatal response (Maierhofer et al., 2014). Future research is needed to identify CMLs and CBL-CIPKs involved in guard cell MAMP signaling.

Ca^{2+}-sensing receptor (CAS) represents a Ca^{2+} sensor of low Ca^{2+} affinity/high-capacity that does not contain EF-hand motif and is associated with thylakoid membranes (Han et al., 2003; Nomura et al., 2008; Weinl et al., 2008). Studies have revealed that CAS is involved in stomatal closure induced by high extracellular Ca^{2+} and flg22, but not ABA (Han et al., 2003; Weinl et al., 2008; Nomura et al., 2012). In these studies, CAS has been shown to be important for elevation of $[Ca^{2+}]_{cyt}$ induced by high extracellular Ca^{2+} in guard cells and is required for flg22-induced elevation of $[Ca^{2+}]_{cyt}$ in *Arabidopsis* plants. These results suggest that Ca^{2+} releasing from chloroplast involving CAS is essential for flg22-induced stomatal closure.

Open Stomata 1 Involvement in MAMP-induced Stomatal Closure

There are increasing results showing that OST1 plays a central role in guard cell signaling induced by various stimuli such as high CO_2, low humidity, and ozone (Xie et al., 2006; Ache et al., 2010; Vahisalu et al., 2010; Xue et al., 2011; Merilo et al., 2013). Recent studies have revealed that OST1 kinase is also involved in stomatal closure induced by MAMPs including flg22, LPS, and YEL (Melotto et al., 2006; Montillet et al., 2013; Guzel et al., 2015; Ye et al., 2015). The importance of OST1 seems to be related to its essential role in activation of S-type anion channels (see Activation of S-type Anion Channels in Response to MAMPs in Guard Cells), because OST1 is not involved in elevation of $[Ca^{2+}]_{cyt}$ and H_2O_2 accumulation induced by YEL but is involved in S-type anion channel activation induced by ABA, CO_2, YEL, and high Ca^{2+} (Xue et al., 2011; Hua et al., 2012; Brandt et al., 2015; Ye et al., 2015).

To our knowledge, there is no evidence that kinase activity of OST1 is increased by stimuli other than ABA in guard cells. On the other hand, recent studies have shown that flg22 does not increase OST1 kinase in *Arabidopsis* suspension cells and YEL does not increase OST1 kinase in *Arabidopsis* guard cells (Montillet et al., 2013; Ye et al., 2015). OST1 is mainly activated upon inhibition of PP2Cs by ABA perception in plants (Mustilli et al., 2002; Ma Y. et al., 2009; Park et al., 2009). Flg22 does not increase ABA content in plant and YEL does not induce transcription of ABA responsive gene, *RD29B,* in guard cells (Nomura et al., 2012; Ye et al., 2015), suggesting that flg22 and YEL do not increase ABA content in guard cells. These results taken together raise the possibility that resting activity of OST1 kinase, which itself is very weak, is involved in stomatal closure induced by stimuli including flg22 and YEL (**Figure 1**). Further research is needed to validate this possibility.

Mitogen-activated Protein Kinase Involvement in MAMP-induced Stomatal Movement

Activation of MAPKs including MPK3, MPK4, and MPK6, is one of the early response induced by MAMPs such as flg22, elf18, LPS, and chitin (Zipfel et al., 2006; Boller and Felix, 2009; Ranf et al., 2015). It has been shown that MPK3 and MPK6 are essential for stomatal closure induced by flg22 and LPS (Gudesblat et al., 2009; Montillet et al., 2013). On the other hand, MPK3 and MPK6 seem not to be activated by ABA or involved in ABA-induced stomatal closure (Gudesblat et al., 2007; Montillet et al., 2013). Interestingly, MPK3 is involved in ABA inhibition of light-induced stomatal opening (Gudesblat et al., 2007), which is reminiscent of the involvement of OST1 in stomatal closure induced by flg22 and YEL. MPK4 is reported to be negatively involved in stomatal closure induced by *Pst* DC3000, in which flg22 is the dominant MAMP sensed by guard cells (Zeng and He, 2010; Hettenhausen et al., 2012). MPK9 and MPK12 function redundantly in stomatal closure induced by ABA, MeJA, YEL, and chitosan (Jammes et al., 2009; Salam et al., 2012, 2013). Further results reveal that mutation of *mpk9 mpk12* increased the susceptibility of *Arabidopsis* to spray-inoculated *Pst* DC3000 (Zeng and He, 2010; Jammes et al., 2011). On the other hand, it has been reported that MPK9 and MPK12 were not involved in stomatal closure induced by flg22 at 5 μM (Montillet et al., 2013). These results suggest that different MAMPs recruit different MAPKs to induce stomatal closure. MPK9 and MPK12 function redundantly in inhibition of light-induced stomatal opening induced by YEL while only mutation in MPK12 impaired ABA inhibition of light-induced stomatal opening (Salam et al., 2013; Des Marais et al., 2014). These results suggest that the regulation of MPK9 and MPK12 are differently regulated by ABA and YEL.

It has been reported that MPK3 and MPK6 are not involved in elevation of $[Ca^{2+}]_{cyt}$ induced by flg22 and elf18 and that MPK9 and MPK12 are not involved in elevation of $[Ca^{2+}]_{cyt}$ induced by YEL and chitosan (Salam et al., 2012, 2013). The activation of MAPKs can be induced by flg22 in a Ca^{2+}-independent manner (Boudsocq et al., 2010; Ranf et al., 2011). These results suggest that MAPKs can function in parallel with Ca^{2+}-dependent pathways to regulate stomatal movement induced by MAMPs. Note that activation of MAPKs can differs in mechanism among different MAMPs, as seen in recent studies that a RLCK, PBL27, is specifically required for activation of MAPKs by chitin but not flg22 (Shinya et al., 2014). MPK9 and MPK12 have been shown to be essential for activation of S-type anion channels induced by ABA, MeJA, and high extracellular Ca^{2+} (Jammes et al., 2009; Brandt et al., 2015; Khokon et al., 2015). It is likely that the function of MAPKs is related to regulation of S-type anion channels in MAMP-induced stomata movement.

Regulation of Plasma Membrane Transporters in MAMP-induced Stomatal Movement

Guard cell volume is tightly regulated by transporters, especially the ones in plasma membrane. Recent studies have revealed that

MAMPs regulate plasma membrane transporters, including I_{Ca} channels, K_{in} channels, S-type anion channels and H^+-ATPases in guard cells (Klüsener et al., 2002; Zhang et al., 2008; Liu et al., 2009; Koers et al., 2011; Ye et al., 2013b, 2015; Guzel et al., 2015). In this section, we review these findings.

Activation of I_{Ca} Channels in Response to MAMPs in Guard Cells

I_{Ca} channels in the plasma membrane function as a pathway for Ca^{2+} influx, which is activated at hyperpolarization condition (Hamilton et al., 2000; Pei et al., 2000). Patch clamp results have shown that I_{Ca} channels are activated by ABA, MeJA, exogenous H_2O_2, and MAMPs including YEL, chitosan and LPS in *Arabidopsis* guard cells (Pei et al., 2000; Murata et al., 2001; Klüsener et al., 2002; Ali et al., 2007; Munemasa et al., 2011; Ye et al., 2013b, 2015). Intriguingly, activation of I_{Ca} channels is observed with cytosol dialyzed with ATP-free solution. These results suggest that MAMPs including YEL, chitosan and LPS can activate I_{Ca} channels in a phosphorylation-independent manner. Since application of H_2O_2 can activate I_{Ca} channels, it is likely that MAMP-induced H_2O_2 contributes to activation of I_{Ca} channels. YEL and chitosan activate I_{Ca} channels in guard cells that have most of the cell wall-bind peroxidases removed and LPS activate I_{Ca} channels in guard cells with cytosol dialyzed with NAD(P)H-free solution (Klüsener et al., 2002; Ali et al., 2007; Ye et al., 2013b). These results suggest that MAMPs can activate I_{Ca} channels in a H_2O_2-independent manner and/or that I_{Ca} channels activated by exogenous H_2O_2 are different from the ones by MAMPs in these experimental conditions (**Figure 1**). Further studies reveal that CPK6 is required for activation of I_{Ca} channels induced by ABA, MeJA, and YEL (Mori et al., 2006; Munemasa et al., 2011; Ye et al., 2013b). On the other hand, it has been shown that suppression of EF-hand-containing proteins by an inhibitor, W7, is required for LPS activation of I_{Ca} channels (Ali et al., 2007). These results suggest that Ca^{2+}-dependent mechanism can play both negative and positive roles in activation of I_{Ca} channels induced by different MAMPs.

Activation of S-type Anion Channels in Response to MAMPs in Guard Cells

Early studies have identified two types of anion channels in guard cells, S-type and R-type anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Schroeder and Keller, 1992). While R-type anion channel is activated transiently and shows strong voltage dependency, S-type is weakly voltage-dependent and lack of time-dependent inactivation. Therefore, activation of S-type anion channel is likely to induce long-term anion efflux and sustained depolarization, representing a hallmark of stomatal closure. In *Arabidopsis* guard cells, several genes have been identified as S-type anion channels including SLAC1 and SLAC1 homolog 3 (SLAH3), and R-type channels, ALMT12. However, SLAC1 shows the most prominent role in regulation of stomatal closure induced by various stimuli, such as ABA, CO_2, darkness and high extracellular Ca^{2+} (Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010; Sasaki et al., 2010). Electrophysiological studies have shown that S-type anion channels are activated by chitosan, YEL and flg22 in guard cells (Koers et al., 2011; Ye et al., 2013b, 2015; Guzel et al., 2015). Further results have shown that both SLAC1 and SLAH3 were required for flg22-induced stomatal closure and activation of S-type anion channel (Montillet et al., 2013; Guzel et al., 2015). It is unknown whether ALMT12 is involved in MAMP-induced stomatal movement. While the regulation R-type anion channels remains largely unknown, the regulation of S-type anion channels has been extensively studied. Here, we briefly review the regulation of SLAC1.

In *Arabidopsis* guard cells, elevation of $[Ca^{2+}]_{cyt}$ is essential for S-type anion channel activation induced by ABA, high extracellular Ca^{2+} and CO_2, but itself is not sufficient for activation of S-type anion channels (Allen et al., 2002; Siegel et al., 2009; Xue et al., 2011). These results raised the hypothesis that external and internal stimuli enhance/prime guard cells to respond to increased $[Ca^{2+}]_{cyt}$ levels and to activate S-type anion channels (Young et al., 2006; Siegel et al., 2009; Kim et al., 2010; Hubbard et al., 2012). Since the identification of SLAC1 as the main S-type anion channel in guard cells, many regulators have been revealed by *in vitro* experiments including CDPKs, CBL-CIPK complexes, OST1 and GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) as positive regulators and PP2Cs as negative regulators (Geiger et al., 2009, 2010, 2011; Lee et al., 2009; Brandt et al., 2012, 2015; Hua et al., 2012; Scherzer et al., 2012; Maierhofer et al., 2014). However, only a few of the regulators have been shown to positively function in stomatal closure, including CPK3, CPK5, CPK6, CPK23, OST1, and GHR1 (Mustilli et al., 2002; Mori et al., 2006; Hua et al., 2012; Merilo et al., 2013; Brandt et al., 2015). In case of CPK23 there is also evidence that CPK23 negatively regulates salt and drought response (Ma and Wu, 2007). These results point out that the regulation of S-type anion channels can be complicated in guard cells in response to different stimuli. CPK6 and OST1 are required for stomatal closure induced by YEL and flg22 and activation by YEL of S-type anion channel (Melotto et al., 2006; Hua et al., 2012; Montillet et al., 2013; Ye et al., 2013b, 2015; Guzel et al., 2015). CPK6 does not seem to be engaged in activation of OST1 kinase by ABA and OST1 is not involved in $[Ca^{2+}]_{cyt}$ elevation in guard cells induced by YEL (Brandt et al., 2015; Ye et al., 2015). These results raise the possibility that CPK6 and OST1 directly regulate S-type anion channels in flg22 and YEL signaling as they do *in vitro*. Note that OST1 may function at resting activity. Further research on identification of phosphorylation sites of SLAC1 and their regulation by components such as CDPKs and OST1 is needed in order to further elucidate the regulation of S-type anion channels by MAMPs (**Figure 2**).

Studies have demonstrated that CPK6 and OST1 both can phosphorylate SLAC1 at Ser59 and Ser120 *in vitro*, which are probably dephosphorylated by PP2Cs (Geiger et al., 2009; Vahisalu et al., 2010; Brandt et al., 2012, 2015; Maierhofer et al., 2014). Recent studies showed that phosphorylation of Ser59 and S120 redundantly function in S-type anion channel activation by ABA in guard cells (Maierhofer et al., 2014; Brandt et al., 2015). On the other hand, mutation of Ser120 impaired stomatal closure induced by various stimuli other than ABA (Vahisalu et al., 2010; Merilo et al., 2013). These results suggest that different stimuli induce different phosphorylation pattern

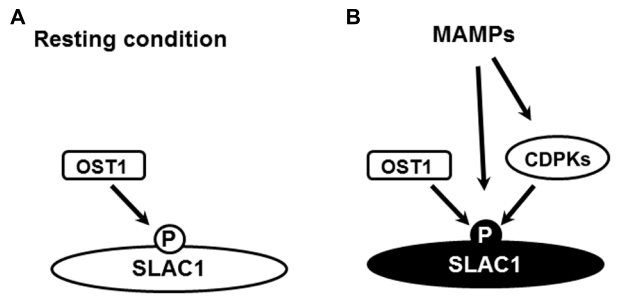

FIGURE 2 | Hypothetical regulation of SLAC1 by phosphorylation in guard cell MAMP signaling. (A) Resting OST1 activity contributes to resting level of SLAC1 phosphorylation, particularly to the level of S120 phosphorylation, which is essential for the activation of SLAC1 by MAMPs. **(B)** In response to MAMPs, CDPK-dependent and –independent mechanisms change the phosphorylation status of SLAC1 including the phosphorylation of S59, resulting in SLAC1 activation. The change of color of letter "P" and "SLAC1" from black in **(A)** to white in **(B)** indicates a changed phosphorylation status and activation status, respectively.

of S-type anion channels leading to stomatal closure. Further results showed that phosphorylation of Thr531 in SLAC1 results in constitutive activation of anion currents and phosphorylation of Ser59 and Ser120 are not sufficient for SLAC1 activation in oocytes (Maierhofer et al., 2014). These phosphorylation sites are strong candidates for the regulation by kinases and phosphatases in guard cells. The exist of different sites with different functions provides the molecular basis for Ca^{2+} priming model mentioned above.

Regulation of Potassium Channels and H^+-ATPases by MAMPs in Guard Cells

Inward-rectifying K^+ channels function as the main gate for K^+ influx to the cytosol, while outward-rectifying K^+ channels (K_{out} channels) function as the main gate for K^+ efflux to the apoplast. Both K_{in} channels and K_{out} channels are voltage-dependent and do not inactivate with time (Schroeder, 1988; Blatt, 1990). The non-inactivation property of potassium channels allows long-term efflux of K^+ needed during stomatal closure and influx of K^+ needed during stomatal opening. K_{out} channels are activated but K_{in} channels are deactivated by depolarization of plasma membrane. In addition to the plasma membrane potential, flg22 suppressed both K_{in} channels, which was mediated by FLS2 and G-protein (Zhang et al., 2008). Recent studies also showed that YEL suppresses K_{in} channels, which is mediated by CPK6 (Ye et al., 2013b). Intriguingly, flg22 seems not to affect the voltage-dependency of K_{in} channels (Zhang et al., 2008), while ABA shifts the voltage dependency of K_{in} channels to more hyperpolarization (Armstrong et al., 1995). These results indicate that the suppression of K_{in} channel activity by ABA and MAMPs can be different in mechanism and suggest that decrease in number of active K_{in} channels contributes to the inhibition by MAMPs. It has been shown that KAT1, the dominant K_{in} channel in guard cells, undergoes internalization during stomatal closure (Sutter et al., 2007; Eisenach et al., 2012), and traffic system is important for flg22-induced stomatal closure (Spallek et al., 2013). Further studies suggested that phosphorylation of CDPK recognition sites in KAT1 by protein kinase C activator suppresses KAT1-mediated currents with voltage dependency unchanged (Sato et al., 2010). These results raise the possibility that phosphorylation by CPK6 of KAT1 contributes to YEL suppression of K_{in} channel activity. OST1 has been shown to phosphorylate KAT1 at Ter306 *in vitro*, which is critical for KAT1 activation in oocytes (Sato et al., 2009). It is likely that OST1 is involved in suppression of K_{in} channel activity by MAMPs. Flg22 also suppresses K_{out} channels in *Arabidopsis* guard cell (Zhang et al., 2008). It remains unknown whether this is common response for other MAMPs. Though activity of K_{out} channels is decreased, K^+ efflux is sufficient for stomatal closure induced by flg22.

Plasma membrane H^+-ATPases transport H^+ into the apoplast at the expense of ATP, leading to hyperpolarization of plasma membrane, the driving force for stomatal opening in the light. It is known that ABA inhibits H^+-ATPases in guard cells, which is essential for stomatal closure and inhibition of light-induced stomatal opening (Goh et al., 1996; Merlot et al., 2007). It has been shown that H_2O_2, NO, Ca^{2+}, and phosphatidic acid contribute to ABA inhibition of H^+-ATPases (Kinoshita et al., 1995; Zhang et al., 2004, 2007; Takemiya and Shimazaki, 2010; Uraji et al., 2012; Yin et al., 2013). Various MAMPs including flg22, chitosan, YEL and β-1→3-linked glucan inhibit light-induced stomatal opening. Studies have also shown that constitutive activation of AHA1 by *ost2* mutation, the dominant H^+-ATPase in guard cells, impairs stomatal closure induced by flg22 and LPS (Liu et al., 2009). These results suggest that H^+-ATPases are suppressed by MAMPs in guard cells.

Involvement of Phytohormones in MAMP-induced Stomatal Response

It has been reported that stomatal closure induced by flg22 and LPS was impaired in ABA-deficient mutant (Melotto et al., 2006; Montillet et al., 2013; Du et al., 2014), suggesting endogenous ABA is involved in flg22 and LPS signaling in guard cells. This idea is supported by the fact that the master regulator of ABA signaling, OST1, is required for stomatal closure induced by flg22 and LPS. On the other hand, flg22 was shown not to induce ABA synthesis in *Arabidopsis* leaves and activation of OST1 in *Arabidopsis* suspension cells (Nomura et al., 2012; Montillet et al., 2013). These results suggest that resting level of ABA but not the elevating level of ABA induced by flg22 is required for signaling in guard cells. A possible function of the resting level of ABA is to produce the resting activity of OST1 (**Figure 1**). On the other hand, YEL and chitosan induced stomatal closure in ABA-deficient mutant (Issak et al., 2013). However, we may not be able to exclude the possibility that endogenous ABA is not required for stomatal closure induced by YEL and chitosan, since there is considerable ABA in these ABA-deficient mutants. For example the content of ABA in *aba2-2* is around 23% of those in wild type (Nambara et al., 1998). These results raise the possibility that the remained ABA is enough for stomatal closure induced by YEL and chitosan. Future work is needed to elucidate

the role of endogenous ABA in stomatal closure induced by MAMPs.

Stomatal closure induced by LPS and *Pst* DC3000 is impaired in SA-deficient mutant (Melotto et al., 2006; Zeng and He, 2010), suggesting that endogenous SA is involved in stomatal closure induced by LPS and flg22. It seems that SA functions through NPR1, a master regulator of SA signaling, in flg22 signaling, since stomatal closure induced by *Pst* DC3000 and *Pst* DC3118 was impaired in *npr1-1* mutant (Zeng and He, 2010). Flg22 and LPS also induces SA production and expression of SA-responsive gene, *PR1*, in *Arabidopsis* leaves (Denoux et al., 2008; Tsuda et al., 2008; Nomura et al., 2012). A role of SA may be related to the regulation of PRR levels in guard cells, since recent works have shown that SA finely regulates levels of PRRs including FLS2 in *Arabidopsis* (Tateda et al., 2014). The endogenous SA may be recruited as a substrate for PRXs that are involved in flg22-indued stomatal closure (Mori et al., 2001). Future work is needed to elucidate how endogenous SA functions in guard cell MAMP signaling.

Studies have shown that ethylene signaling components, ETR1 and EIN2, are involved in flg22-induced stomatal closure probably by regulating FLS2 transcription and protein level and H_2O_2 production, but only in an unwounded condition (Mersmann et al., 2010). These results suggest that ethylene signaling is required for guard cell flg22 signaling in a stimulus-dependent manner. Ethylene production has been widely observed to be induced by various MAMPs including flg22, elf18, elf26, chitin, and YEL (Felix et al., 1991; Kunze et al., 2004; Zipfel et al., 2006). It is likely that ethylene signaling is also involved in stomatal closure induced by other MAMPs.

Other Components Involved in MAMP Signaling in Guard Cells

Increasing evidences are emerging that reactive carbonyl species (RCS), such as 4-hydroxy-2-nonenal and acrolein, is produced by both enzymatic and non-enzymatic mechanisms and regulates guard cell signalings (Montillet et al., 2013; Islam et al., 2015). Recent studies have shown that RCS production mediated by a lipoxygenase (LOX), LOX1, is required for stomatal closure induced by flg22 but not ABA (Montillet et al., 2013). It remains unknown how the RCS is involved in flg22 signaling.

In addition to GHR1, recent studies identified several RLKs, including L-type lectin receptor kinase-VI.2 (LecRK-VI.2), LecRK-V.5, and Cysteine-rich receptor-like kinases (CRKs), which are involved in guard cell MAMP signaling. LecRK-V.5 has been reported to negatively regulate stomatal closure and H_2O_2 accumulation in guard cells induced by flg22, LPS, elf26 and ABA (Desclos-Theveniau et al., 2012). LecRK-VI.2 positively regulates stomatal closure induced by flg22 and elf26 but not ABA (Singh et al., 2012). Further results show LecRK-VI.2 is not involved in H_2O_2 production but activation of MAPKs induced by flg22 in leaves. Recent phenotypic studies of the T-DNA insertion mutants of CRK family have identified many of its members are involved in stomatal closure induced by stimuli including flg22, chitin and ABA, and shown that CRKs provide signaling specificity (Bourdais et al., 2015). For these RLKs, the future challenge is to elucidate their regulation and substrates in MAMP signaling.

REDUNDANCY IN GUARD CELL MAMP SIGNALING

Great advance in understanding guard cell signaling including the one induced by MAMPs has been made based on genetic methods, particularly using mutant plants. In these studies, functionally redundant mechanisms are suggested. Typical examples can be found in H_2O_2 production, sensing of Ca^{2+}, MAPK function and regulation of anion channels. However, these mechanisms do have their own specificity. For example, CPK3 and CPK6 have different Ca^{2+} sensitivity, with CPK6 activated at lower Ca^{2+} concentration, but both can phosphorylate SLAC1 (Boudsocq et al., 2012; Scherzer et al., 2012; Laanemets et al., 2013). Theoretically, CPK6 functions at lower Ca^{2+} concentration, while CPK6 and CPK3 both function at higher concentration, which are therefore considered functionally redundant at higher concentration. It can be expected that functional redundancy appears depending on conditions and the strength of stimuli is an important factor to determine the occurrence of redundancy. A challenge for future dissection of signaling is to define the biological conditions and mimic them in the labs. It is also needed to mention that the functional redundancy in signaling should not be confused with compensatory mechanisms that have been widely observed in extreme experimental conditions for plants, such as constitutive loss-of-function mutations. It is likely that to change the properties of functionally redundant components is a common mechanism for compensation. For example, the gene expression level of *SLAH3* doubles in *slac1* mutant, which may account for the partial flg22-induced stomatal closure in *slac1* mutant (Geiger et al., 2011; Guzel et al., 2015). On the other hand, flg22-induced stomatal closure was abolished in *ost1* mutant in the same study but OST1 seems not to activate SLAH3. It is therefore possible that SLAC1 plays a dominant role but not function redundantly with SLAH3 in flg22-induced stomatal closure in wild-type plants. In the future, the challenge is to validate the contribution of these suggested redundant components to MAMP signaling in wild-type plants. Elucidation of the compensatory mechanisms in these mutants may also contribute to our understanding and is of particular importance in practical aspect.

COMPARISON OF MAMP SIGNALINGS IN LEAF EPIDERMAL CELLS, MESOPHYLL CELLS AND GUARD CELLS

Unlike epidermal and mesophyll cells, guard cells do not have plasmodesmata but function autonomously. FLS2 and co-receptor, BAK1, are expressed in epidermal cells, mesophyll cells and guard cells, suggesting that the similar perception mechanism of flg22 exist in these three cell types (Robatzek et al., 2006; Shang et al., 2015). Future biochemical studies in

a cell-specific context are needed to validate this suggestion. While many downstream components such as RbohD, CPK6, MPK3, and MPK6 seem to be expressed ubiquitously in the leaf, components, such as MPK9, MPK12, OST1, SLAC1, and ALMT12 are mainly expressed in guard cells (Mustilli et al., 2002; Mori et al., 2006; Negi et al., 2008; Vahisalu et al., 2008; Jammes et al., 2009; Meyer et al., 2010; Sasaki et al., 2010). Endogenous hormone ABA concentration is much higher in guard cells than epidermal and mesophyll cells (Waadt et al., 2014). These differences in signaling component levels determine the output of MAMP responses in different cell types. For example, flg22 induces H_2O_2 production and $[Ca^{2+}]_{cyt}$ elevation in guard cells, epidermal cells and mesophyll cells (Ranf et al., 2008; Jeworutzki et al., 2010; Desclos-Theveniau et al., 2012; Macho et al., 2012; Thor and Peiter, 2014; Guzel et al., 2015; Keinath et al., 2015). Flg22-induced depolarization of plasma membrane, the driven force of stomatal closure, is not impaired in *rboh*D, *slah3* and fusicoccin-treated mesophyll protoplasts, but flg22-induced stomatal closure is impaired in *rboh*D, *slah3,* and *ost2* plants, suggesting that flg22-induced depolarization of plasma membrane is different in mechanism in the two cell types (Jeworutzki et al., 2010; Macho et al., 2012; Kadota et al., 2014; Li et al., 2014; Guzel et al., 2015).

CONCLUDING REMARKS AND OUTLOOKS

Since 2006, stomatal immunity has emerged as an important part of plant immunity. The output is stomatal closure and inhibition of stomatal opening to prevent microbe invasion. Though it is known that MAMPs are important signals to trigger stomatal immunity, we know little about how and how much MAMPs are exposed to the surveillance of guard cells. For example, stomatal closure by *Pst* DC3118 but not *E. coli* is abolished in *fls2* mutant (Melotto et al., 2006; Zeng and He, 2010), indicating that guard cells differently sense different pathogens even though it seems that they have the same set of MAMPs. It also remains unclear whether MAMPs including elf18/26, normally considered to exist inside the cells, are exposed to cell surface. A recent study suggests that *Arabidopsis* plants sense elongation factor Tu in the outer membrane vesicles secreted by Gram-negative bacteria (Bahar et al., 2016).

Though plasma membrane-localized PRRs in guard cells are widely accepted to function to perceive MAMPs, MAMP signaling also happens in the cell wall. It remains unknown whether the apoplast signaling is regulated by the plasm membrane-localized PRRs or there are PRRs in the apoplasts. Upon binding of PRRs and ligands, co-receptors are immediately recruited to form receptor complexes, which determine the specificity of signalings. The core signaling downstream to induce stomatal closure is that MAMP perception induces Ca^{2+}-dependent mechanisms to activate S-type anion channels, which is dependent on Ca^{2+}-independent mechanisms (**Figures 1** and **2**). Future research is needed to unravel new Ca^{2+}-dependent mechanisms. Emergent challenge is to elucidate how Ca^{2+}-independent mechanisms, such as the ones mediated by OST1 and MAPKs, are involved in MAMP signaling in guard cells. Signaling specificity provided by RLCKs and CRKs has been observed in MAMP-induced stomatal response. Further elucidation of these specificities is essential for dissecting MAMP signaling in guard cells.

The roles of phytohormones in guard cell MAMP signaling are still unclear. Though increase of phytohormone by methods such as direct application can induce stomatal movement, it is less likely that MAMPs increase the level of phytohormone to trigger stomatal movement, as seen in the case that flg22 does not increase ABA content. Therefore, it is important to note that the function of elevating level of phytohormone can be different from that of phytohormone in unstressed plants. An emerging role is that phytohormones, such as ethylene and SA, in unstressed plants regulate the level of PRRs. A possible role is that phytohormones, such as ABA, are important for providing basal activity of important signaling components. The future challenge is to elucidate the roles of endogenous phytohormones in guard cell MAMP signaling.

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Convergence and Divergence of Signaling Events in Guard Cells during Stomatal Closure by Plant Hormones or Microbial Elicitors

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Dynamic regulation of stomatal aperture is essential for plants to optimize water use and CO_2 uptake. Stomatal opening or closure is accompanied by the modulation of guard cell turgor. Among the events leading to stomatal closure by plant hormones or microbial elicitors, three signaling components stand out as the major converging points. These are reactive oxygen species (ROS), cytosolic free Ca^{2+}, and ion channels. Once formed, the ROS and free Ca^{2+} of guard cells regulate both downstream and upstream events. A major influence of ROS is to increase the levels of NO and cytosolic free Ca^{2+} in guard cells. Although the rise in NO is an important event during stomatal closure, the available evidences do not support the description of NO as the point of convergence. The rise in ROS and NO would cause an increase of free Ca^{2+} and modulate ion channels, through a network of events, in such a way that the guard cells lose K^+/Cl^-/anions. The efflux of these ions decreases the turgor of guard cells and leads to stomatal closure. Thus, ROS, NO, and cytosolic free Ca^{2+} act as points of divergence. The other guard cell components, which are modulated during stomatal closure are G-proteins, cytosolic pH, phospholipids, and sphingolipids. However, the current information on the role of these components is not convincing so as to assign them as the points of convergence or divergence. The interrelationships and interactions of ROS, NO, cytosolic pH, and free Ca^{2+} are quite complex and need further detailed examination. Our review is an attempt to critically assess the current status of information on guard cells, while emphasizing the convergence and divergence of signaling components during stomatal closure. The existing gaps in our knowledge are identified to stimulate further research.

Keywords: ABA, cytosolic free Ca^{2+}, cytosolic pH, ROS, guard cells, ion channels, nitric oxide, secondary messengers

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INTRODUCTION

Stomata are tiny pores found on the leaf surface of higher plants, which facilitate the evaporation of H_2O via transpiration and intake of CO_2 for photosynthetic carbon assimilation (Acharya and Assmann, 2009). Stomata are also major points of entry for pathogens into the plants (Melotto et al., 2006, 2008). Therefore, the regulation of stomatal aperture is essential for limiting the loss of H_2O as well as restricting pathogen entry. The guard cells are quite sensitive to several internal and external stimuli, including abiotic (drought, light, temperature, high CO_2, humidity) or

biotic factors (pathogens and elicitors). Plant hormones (such as abscisic acid, ABA, methyl jasmonate, MJ) and polyamines (PAs) induce stomatal closure. Elicitors such as salicylic acid (SA), chitosan, and Flg22 also cause stomatal closure (Alcázar et al., 2010; Jing et al., 2012; Gayatri et al., 2013; Ye et al., 2013; Agurla et al., 2014). Stomata open when guard cells are turgid and close when the guard cells are flaccid (Blatt, 2000). During stomatal opening, guard cells accumulate osmotically active components, such as potassium ions, anions, malate and sucrose, leading a decrease in water potential, influx of water, and increase in turgor. In contrast, the reversal of these events leads to flaccidity in guard cells and stomatal closure (Vavasseur and Raghavendra, 2005; Bright et al., 2006; Roelfsema et al., 2012).

Among several effectors, the effects of ABA (a phytohormone) on stomatal movements have been studied in detail. ABA induced stomatal closure is mediated by many signaling components like cytoplasmic pH, reactive oxygen species (ROS), reactive nitrogen species (nitric oxide, NO), cytosolic free Ca^{2+}, G-proteins, protein kinases, protein phosphatases, phospholipids, phospholipases, and sphingolipids (Wang and Song, 2008; Raghavendra et al., 2010; Umezawa et al., 2010; García-Mata and Lamattina, 2013; Song et al., 2014). The diverse spectrum of signaling components during stomatal closure have been reviewed frequently (Kim et al., 2010; Joshi-Saha et al., 2011; Gayatri et al., 2013; Agurla et al., 2014; Kollist et al., 2014; Song et al., 2014; Murata et al., 2015; Lee et al., 2016).

There are yet questions about the sequence of the signaling events during stomatal closure. For e.g., cytosolic free Ca^{2+} may act at either downstream or upstream of ROS/NO. The changes in cytosolic pH of guard cells may be important at either downstream or upstream of ROS or NO. The production of NO precedes that of ROS, but NO can act as antioxidant as well. Despite these ambiguities, it is clear that a rise in ROS or NO triggers a rise in free Ca^{2+} of guard cells, modulate the ion channels and cause an efflux of K^+/Cl^-/malate, leading to loss in turgor of guard cells. We emphasize that the signaling events during stomatal closure converge at ROS, cytosolic Ca^{2+}, and ion channels. Similarly, ROS, NO, and Ca^{2+} form the points of divergence.

Points of Convergence: ROS, Cytosolic Free Ca^{2+}, and Ion Channels

When guard cells are exposed to signals originating from abiotic or biotic factors the process of signal transduction is initiated. During this process, three points can be recognized as those of convergence: ROS, cytosolic free Ca^{2+}, and anion channels. For e.g., plant hormones (such as ABA or MJ) and microbial elicitors invariably cause an increase in the levels of ROS or NO in guard cells, leading to rise in free Ca^{2+} within the guard cells (**Table 1**). There are excellent reviews, emphasizing the role of ROS (Kollist et al., 2014; Song et al., 2014; Murata et al., 2015), NO (Hancock et al., 2011; García-Mata and Lamattina, 2013; Gayatri et al., 2013; Agurla et al., 2014), and cytosolic free Ca^{2+} in guard cells (Kim et al., 2010; Roelfsema and Hedrich, 2010). Hormones and elicitors interact with different receptor entities, but the subsequent steps converge to activate NADPH oxidase, increase ROS, NO, and Ca^{2+} in guard cells (**Figure 1**). Although NO in guard cells is a key signaling component, there is no sufficient evidence to describe it as point of convergence. While it is clear that ROS can cause an increase in NO of guard cells, no other components that can raise NO levels has been described.

Reactive Oxygen Species (ROS)

A marked rise in ROS of guard cells is a consistent feature of stomatal closure induced by ABA, MJ, and even microbial elicitors (Zhang H. et al., 2009; Song et al., 2014). While the effect of ABA on NADPH oxidase is mediated by ABA-receptors-protein phosphatase interactions (Raghavendra et al., 2010), the mechanism of NADPH oxidase stimulation by elicitors is ambiguous. Certain MAP kinases activated by elicitors could in turn activate NADPH oxidase (Zhang H. et al., 2009).

There has been overwhelming evidence that NADPH oxidase is the major ROS source in ABA, MJ, or SA induced stomatal closure. However, the source of ROS may not always be NADPH oxidase, as ROS production in response to elicitors, such as SA, yeast elicitor, and chitosan can occur through a salicylhydroxamic acid (SHAM) sensitive peroxidase or amine oxidases (e.g., copper amine oxidase or polyamine oxidase) (Khokon et al., 2011; Gao et al., 2013; Murata et al., 2015). During stomatal closure induced by methylglyoxal (MG), isothiocyanates or thiocyanates, the rise in ROS of *Arabidopsis* guard cells was mediated by a SHAM sensitive peroxidase (Hoque et al., 2012; Hossain et al., 2013). Activation of NADPH oxidase can occur also by phosphatidic acid (PA) (Zhang H. et al., 2009). Thus, the ROS of guard cells is a major point of convergence. The ROS production by different systems, involving NADPH oxidase or peroxidase has been reviewed recently by Murata et al. (2015).

Cytosolic Free Calcium

Calcium (Ca^{2+}) is an important secondary messenger during stomatal closure (McAinsh et al., 1990; Hubbard et al., 2012). The role of Ca^{2+} is confirmed by monitoring of Ca^{2+} in guard cells by fluorescent probes, the Ca^{2+} chelators, and Ca^{2+} channel blockers (Pei et al., 2000; Kim et al., 2010). The rise in Ca^{2+}, due to influx or release from internal sources like endoplasmic reticulum, further activates anion channels and inhibits the K^+_{in} channels, all leading to stomatal closure. There are suggestions that Ca^{2+} may act also upstream of ROS and NO (Garcia-Mata et al., 2003). In contrast, Zhang et al. (2011) observed that calcium channels functioned downstream of H_2O_2 in G-protein α-subunit (*gpa1*) mutants. In *gpa1* mutants, ABA-induced ROS production was disrupted, but Ca^{2+} channels were activated by exogenous H_2O_2 application.

Ion Channels

The ion channels represent the last step of signal transduction, leading to stomatal closure. The ionic status driven by the activity of cation/anion channels determines the turgor state of guard cells. Rise in free Ca^{2+} of guard cells causes the efflux of K^+/Cl^-/other ions. The detailed descriptions of ion channels, their intracellular location, encoding genes, along with mutants are made in a few reviews (Hedrich, 2012; Roelfsema et al., 2012; Kollist et al., 2014). Plants have several types of K^+ channels,

TABLE 1 | Major points of convergence as well as divergence during signal transduction leading to stomatal closure by hormones or elicitors.

Convergence	Upstream component	References
ROS		
	NADPH oxidase	Kwak et al., 2003
	Peroxidase	Khokon et al., 2010
	Copper amine oxidase	An et al., 2008
	G-protein alpha subunit (GPA)	Zhang et al., 2011
	OST1 protein kinase	Mustilli et al., 2002
	Cytosolic free Ca^{2+}	Kobayashi et al., 2007
	Phosphatidic acid	Zhang et al., 2004
	MAPK	Meng and Zhang, 2013
	PI3K/PI4K	Park et al., 2003
	S1P	Ma et al., 2012
	PA/ Phospholipase Dα1	Zhang Y. et al., 2009
	Cytosolic pH	Suhita et al., 2004
CYTOSOLIC FREE Ca^{2+}		
	ROS	Pei et al., 2000
	NO	Hossain et al., 2014
	Inositol 1,4,5-trisphosphate	Gilroy et al., 1990
	Cyclic ADP ribose	Leckie et al., 1998
	Calcineurin-B like proteins	Drerup et al., 2013
ION CHANNELS		
Ca^{2+}_{in} channels		
	Ca^{2+}	Mori et al., 2006
	NO	Garcia-Mata et al., 2003
Inward-rectifying K^+ channels (KAT1)		
	PA	Uraji et al., 2012
	Cytosolic free Ca^{2+}	Grabov and Blatt, 1999
	NO	Sokolovski and Blatt, 2004
Outward rectifying K^+ channel (GORK)		
	pH	Hosy et al., 2003
	Cytosolic free Ca^{2+}	Pei et al., 1998
	NO	Sokolovski and Blatt, 2004
Slow anion channel 1 (SLAC1)		
	MAPK9/12	Danquah et al., 2014
	Cytosolic free Ca^{2+}	Geiger et al., 2010
Slow anion channel Homolog 3 (SLAH3)		
	Cytosolic free Ca^{2+}	Geiger et al., 2010
Quick anion channels (QUAC1/ALMT6)		
	OST1	Engineer et al., 2016
Divergence	**Downstream component**	**References**
ROS		
	NO	Bright et al., 2006
	MAPK9/12	Jammes et al., 2009
	Cytosolic free Ca^{2+}	Pei et al., 2000
	Cytosolic pH	Zhang et al., 2001
NO		
	PLDδ	Distéfano et al., 2012
	Cytosolic free Ca^{2+}	Zhao et al., 2013
	Cytosolic free Ca^{2+}_{in}	Garcia-Mata et al., 2003
	K^+_{in} channels	Garcia-Mata et al., 2003
	K^+_{out} channels	Sokolovski and Blatt, 2004
CYTOSOLIC FREE Ca^{2+}		
	NADPH oxidase	Kimura et al., 2012
	NO	Garcia-Mata and Lamattina, 2007
	Cytosolic pH	Islam et al., 2010
	SLAC1	Laanemets et al., 2013
	SLAH3	Geiger et al., 2011

The convergence is illustrated by the multiple upstream elements leading to an increase in the given component. Similarly, the divergence occurs when multiple components are modulated by the given signaling element. An illustration is given in ***Figure 1****.*
ROS, reactive oxygen species; NO, nitric oxide; MAPK, mitogen-activated protein kinases; SLAC1, slow anion channel-associated 1; SLAH3, slow anion channel homolog 3; Ca^{2+}, calcium; H_2O_2, hydrogen peroxide; K_{in} channel, K^+ inward rectifying channel; K_{out} channel, K^+ outward rectifying channel; PA, phosphatidic acid; OST1, open stomata 1; QUAC1, quick anion channel 1; ALMT, aluminum activated malate transporters; PLD, phospholipase D; S1P, sphingosine-1-phosphate.

which can allow either inward or outward movement of K^+. The K^+_{in} channels open up, when the membrane potential becomes hyperpolarized. In contrast, outward-rectifying K^+channels (K^+_{out}) open when the membrane potential is depolarized.

Guard cell Ca^{2+}-permeable cation channels are stimulated by H_2O_2 and NO, whose levels are raised by ABA or MJ during stomatal closure (Mori et al., 2006; Rienmüller et al., 2010). Elevated free Ca^{2+} in guard cells can be due to the activation of Ca^{2+} channels in not only plasma membrane but also vacuolar or internal membrane network. The activation of ion channels would promote efflux of malate and other anions make the guard cells lose turgor and cause stomatal closure. But, there is considerable ambiguity on the relative dominance and specificity of different ion channels. Guard cells are known to contain slow anion channel-associated 1 (SLAC), quick anion channel 1 (QUAC), slow anion channel homolog 3 (SLAH), and even aluminum activated malate transporters (ALMT) (Roelfsema et al., 2012). Further work is required to elucidate the role of each of these different types of anion channels and their interactions.

Points of Divergence: ROS, NO, and Cytosolic Free Ca^{2+}

The rise in levels of ROS, NO, or cytosolic free Ca^{2+} in guard cells trigger multiple events downstream (**Table 1**). The ability to induce diverse effects makes these three signaling components qualified to be the points of divergence (**Figure 1**). The rise in ROS of guard cells initiates several downstream events: NO production, elevation of cytosolic free Ca^{2+}, and rise in cytosolic pH (Wang and Song, 2008; Song et al., 2014). Kinetic studies indicated that ROS production was prior to the NO production (Gonugunta et al., 2008). The positioning of the ROS was further confirmed by using Arabidopsis mutants and hydrogen-rich water (HRW) (Xie et al., 2014). The impaired NO synthesis and stomatal closure in response by HRW and rescue of closure by exogenous application of NO in *rbohF* mutant indicated that ROS functioned as an upstream signaling component. The importance of ROS in NO production was also demonstrated

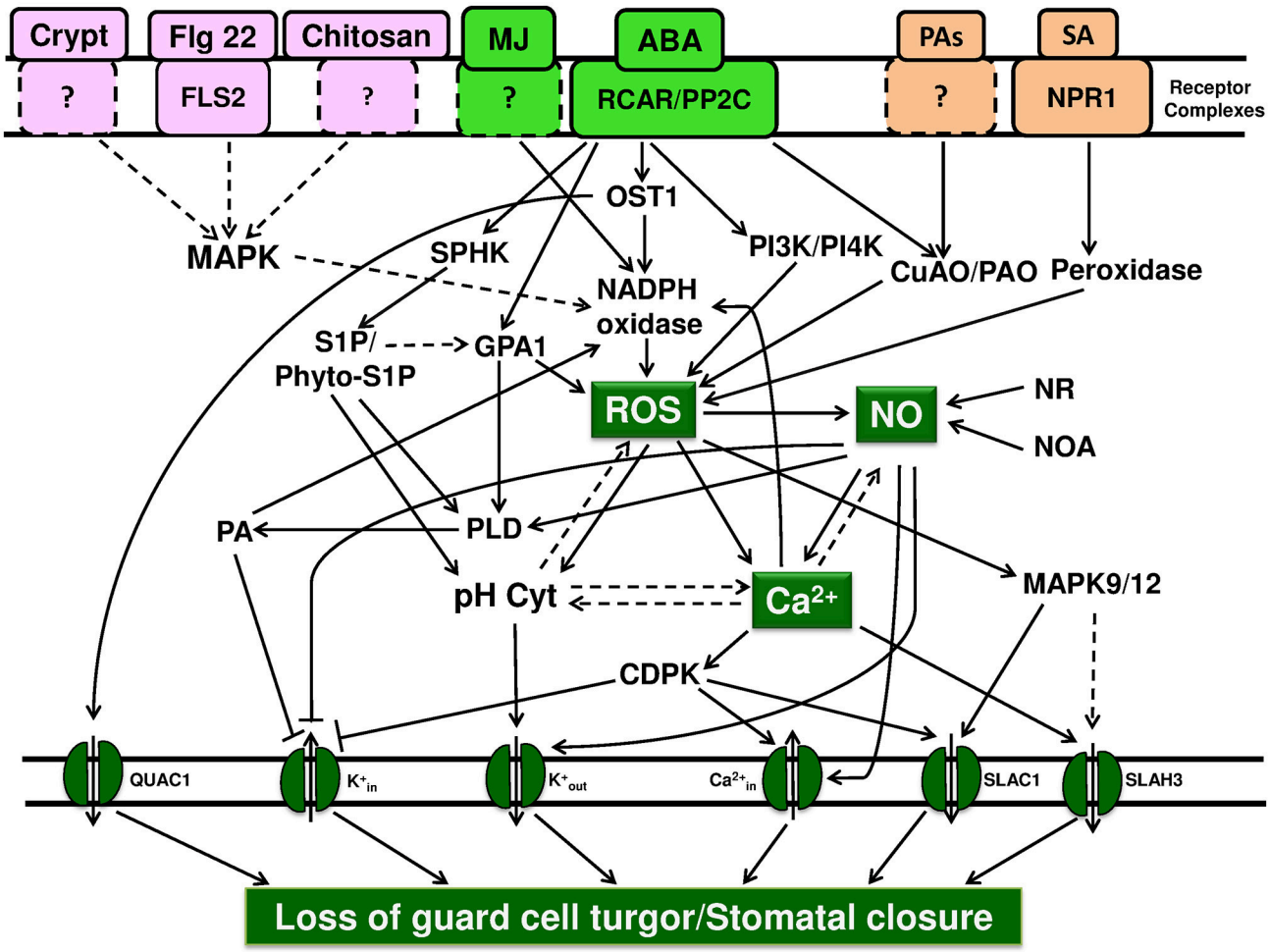

FIGURE 1 | Key points of convergence and divergence during stomatal closure in response to plant hormones and elicitors. Stomatal closure is the result of ion efflux out of guard cells, loss of their turgor, and forms the ultimate step during signal transduction. We suggest that ROS, cytosolic free Ca^{2+}, and ion channels form points of convergence during stomatal closure by a variety of abiotic/abiotic factors. Similarly, ROS, NO, and cytosolic Ca^{2+} are identified as points of divergence. The activation of NADPH oxidase and ROS production are among the earliest events. Similarly, the modulation of ion channels, influx of free Ca^{2+} along with efflux of K^+ and anions, are the final steps, leading to the loss of ions/turgor of guard cells. The binding of ABA to RCAR/PYR or Flg22 to FLS2 or SA to S-receptor are well established, while receptors of cryptogein, chitosan, and PAs are yet to be characterized. ROS: When ABA binds to the receptor (RCAR/PYR/PYL), PP2C becomes non-functional, leading to phosphorylation, and activation of OST1 protein kinase. The elevated kinase activity along with Ca^{2+}, activates NADPH oxidase, and subsequently elevates ROS production. Besides NADPH oxidase, CuAO/PAO are also involved in the increase of ROS in guard cells. The levels of ROS can be elevated by also peroxidase, for e.g., upon salicylic acid binding to its receptor. Further, G-protein alpha subunit induces the ROS production through the activation of NADPH oxidase. Modulation of ROS levels by NO, cytosolic Ca^{2+}, cytosolic pH can occur by direct or indirect mechanisms but these reactions need to be established. Cytosolic free Ca^{2+}: the rise in the levels of ROS and NO, can increase the levels of cytosolic free Ca^{2+}, by either release of $Ca^{2+}$from internal stores or influx of external Ca^{2+} through plasma membrane Ca^{2+}_{in}channels. Ca^{2+} also activates SLAH3 and SLAC1 ion channels, while inhibiting K^+_{in} ion channels. Ion channels: the modulation of cation/anion channels results in the net efflux of K^+/Cl^-/ malate and influx of Ca^{2+}, making guard cells to lose turgor and causing stomatal closure. NO: NR, nitrate reductase; NOA, nitric oxide associated 1 are the sources of NO. Although there are suggestions that ROS, cytosolic Ca^{2+} or cytosolic pH can elevate NO levels, the mechanism is not known. The rise in NO leads to divergent actions, namely the rise in cytosolic Ca^{2+}, activation of PLD, and subsequently NADPH oxidase. Further, NO activates K^+_{out} ion channels, inhibits K^+ channels, and activates Ca^{2+}_{in} ion channels. Other components: The role of cytosolic pH is not completely understood. The available evidence suggests that the cytosolic pH may act parallely with the events involving ROS/NO/cytosolic free Ca^{2+}. Similarly, G-proteins, phospholipids, phospholipases, phosphatidyl inositol kinases, sphingolipids, and MAP kinases also act in such a way to cause the loss of turgor in guard cells and stomatal closure. Solid arrows represent the events which are documented, while broken arrows represent the possible effects/suggestions.

in mutants deficient in G-proteins and nitrate reductase (Bright et al., 2006; He et al., 2013).

Nitric oxide (NO) is a small, gaseous molecule involved in growth, development and even disease resistance of plants (Domingos et al., 2015). Studies using modulators (scavengers/inhibitors/donors) of NO production emphasized the importance of NO during stomatal closure (Gayatri et al., 2013; Agurla et al., 2014). NO production in guard cells of *Arabidopsis* and *Vicia faba* is essential for stomatal closure by SA and yeast elicitor (Sun et al., 2010; Khokon et al., 2011). Real time

monitoring studies suggested that NO acted as a downstream signaling component to the ROS as well as pH (Gonugunta et al., 2008; Srivastava et al., 2009). Nitric oxide synthase (NOS) is the source of NO in animal cells, but the presence/operation of NOS in plant cells is quite uncertain. Both nitrate reductase (NR) and NOA1 (nitric oxide associated) are shown to be the sources of NO in guard cells of *V. faba* and Arabidopsis (Hao et al., 2010; Gao et al., 2013).

The interaction of NO with the other signaling components is quite crucial (Gayatri et al., 2013). In guard cells, NO can cause multiple effects, namely rise in internal Ca^{2+}, cytosolic alkalization, and activation of K^+_{out} channels (Gonugunta et al., 2008; Jing et al., 2010). NO is also essential for the elevation of the signaling components, like PLDα1 and PLDδ, during PA induced stomatal closure (Distéfano et al., 2008, 2010; Uraji et al., 2012).

The components of downstream signaling by Ca^{2+} in guard cells are quite intriguing. The changes in Ca^{2+} are sensed and mediated by the different types of intracellular calcium binding proteins like calmodulins, calcium dependent protein kinases (CDPKs, particularly, CPK3, and CPK6) and calcium sensing receptors (CAS) (Mori et al., 2006). Ca^{2+}-dependent CPK6, CPK21, and CPK23 activate SLAC1 in oocytes (Geiger et al., 2010; Brandt et al., 2012). In contrast, Ca^{2+}-independent protein kinases like OST1 are involved in ABA activation of intracellular calcium channels (Murata et al., 2015). Ca^{2+}-independent SnRK2 protein kinases such as OST1, have been shown to activate SLAC1 in *Xenopus leavis* oocytes (Geiger et al., 2009; Lee et al., 2009; Brandt et al., 2012). Such Ca^{2+} activation of S-type anion currents is an early and essential step during stomatal closure (Siegel et al., 2009; Chen et al., 2010).

Other Components

Cytosolic pH

Cytoplasmic pH is a signaling component in developmental processes, such as root growth (Scott and Allen, 1999). A marked rise in cytoplasmic pH is a common feature during stomatal closure by ABA, MJ, elicitors, and even S1P (Suhita et al., 2004; Gonugunta et al., 2008). Cytosolic alkalization and production of NO in the guard cells and stomatal closure were observed on exposure to ethephon (source of ethylene) and pyrabactin (Jing et al., 2010; Puli and Raghavendra, 2012). Similarly, darkness or ultraviolet B (UV-B) exogenous Ca^{2+} induced stomatal closure was also accompanied by the increase in cytoplasmic pH and ROS (Ma et al., 2013; Zhu et al., 2014). In a reverse of the situation, fusicoccin (a fungal phytotoxin, produced by *Fusicoccum amygdale*) induced stomatal opening, by causing cytoplasmic acidification, and lowering of NO levels, even in presence of ABA (Huang et al., 2013).

Among the upstream components leading to the alkalization of cytoplasm in guard cells are the elevated ROS, PA/PLD, NO, and S1P/phytoS1P. However, the exact trigger of guard cell alkalization on exposure to ABA or MJ or elicitors and the downstream events of cytoplasmic pH change are not clear. A possibility is that on cytoplasmic alkalization, the K^+_{out} channels are activated, triggering K^+ efflux and collapse of turgor in guard cells (Blatt and Armstrong, 1993). Cytosolic alkalization needs to coordinate with the increase in cytosolic free Ca^{2+} during ABA or MJ induced stomatal closure (Islam et al., 2010). Unlike the role of ROS, NO, and cytosolic Ca^{2+}as points of convergence and divergence, the action of cytoplasmic pH seems to be parallel. Further experiments are needed to make cytoplasmic pH qualified to be called as a point of convergence.

G-Proteins

Although the modulation of heterotrimeric G proteins is known to be an important component leading to stomatal closure, the exact mode of G-protein action is ambiguous. Ge et al. (2015) suggested that ethylene induced stomatal closure was mediated through Gα induced ROS production in *Arabidopsis thaliana*. In similar case, Arabidopsis *gpa1* mutants, deficient in G-protein α subunit, are impaired in Ca^{2+}-channel activation, and ROS production, in response to ABA (Zhang et al., 2011). G-proteins were essential for the production of ROS as well as NO during the effects of UV-B irradiation or external Ca^{2+} (Li et al., 2009; Zhang et al., 2012; He et al., 2013). Most of these evidences suggest that G-proteins induce an increase in the levels of ROS in guard cells. It is not clear if ROS production is due to or independent of NADPH oxidase.

Phospho- and Sphingolipids

Phosphatidic acid (PA), the product of phospholipase C/D (PLC/PLD) induced stomatal closure by inhibiting K^+_{in}channel in the guard cells, besides interacting with ABI1 and activating NADPH oxidase (Jacob et al., 1999; Zhang et al., 2004). NO induced stomatal closure was restricted by PLC/PLD inhibitors (Distéfano et al., 2008), suggesting that PA acts downstream of the NO during stomatal closure in *V. faba*. Furthermore, ABA-induced NO production was impaired in *pldα1* mutant guard cells (Distéfano et al., 2008). Phosphoshingolipids such as sphingosine-1-phosphate (S1P) and phytosphingosine-1-phosphate (phytoS1P) regulate multiple functions in plants besides stomatal closure (Ng et al., 2001; Coursol et al., 2005; Puli et al., 2016). ABA activates sphingosine kinases (SHPKs), leading to the production of S1P. However, our knowledge of downstream signaling components of S1P is limited (Coursol et al., 2003).

Interactions among Signaling Components and with Environmental Factors

Signaling components, particularly ROS and NO, play an important role in not only stomatal closure but also in integrating stimuli from abiotic or biotic stress (Song et al., 2014; Saxena et al., 2016). The marked interactions between ROS, NO, Ca^{2+}, and pH are pointed out (Zhang et al., 2001; Gonugunta et al., 2009; Song et al., 2014). ROS and NO interact with each other and can increase cytosolic Ca^{2+} and modulate ion channels. However, the feedback relationship between NO and ROS is obscure. Similarly, cytoplasmic pH may act directly on ion channels, particularly K^+_{out} or indirectly by modulating ROS and/or NO, yet the mechanism of such action is not completely clear. Further, Ca^{2+} also can interact with NO and pH (Wang et al., 2011; Gayatri et al., 2013). It is likely that ABA plays a key role in these interactions. Endogenous ABA is involved during MJ-induced stomatal closure (Munemasa et al., 2007, 2011; Ye

et al., 2013). Both the Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways are considered to function during stomatal closure (Kim et al., 2010; Roelfsema et al., 2012). However, the interrelationships of such Ca^{2+}-dependent and independent pathways during guard cell signal transduction are yet to be elucidated.

Interactions of guard cell signaling components with environmental factors are not only interesting but are essential for adaptation. Drought raises the levels of ROS and ABA levels in plant tissues, with both these phenomena leading to stomatal closure (Saxena et al., 2016). The effects of CO_2 induced stomatal closure can also be mediated by ABA (Chater et al., 2015). Further experiments are needed to identify the exact link between CO_2 and ABA. An increase in ROS due to elevated CO2 in guard cells (Kolla et al., 2007) could raise the endogenous ABA levels and amplify the signaling events leading to stomatal closure. Similar involvement and interactions of ROS, NO, and pH are reported during UV-B induced stomatal closure (He et al., 2013; Zhu et al., 2014).

CONCLUDING REMARKS

The patterns and action sequence of signaling components during stomatal closure have been worked out using different triggers, such as ABA, MJ, and chitosan (Gonugunta et al., 2009). Both plant hormones or microbial elicitors cause an increase in ROS, NO, pH, and free Ca^{2+} of guard cells, modulate ion channels, and cause an efflux of K^+/Cl^-/malate from guard cells, leading to stomatal closure. We emphasize that ROS, cytosolic Ca^{2+},and ion channels are the points of convergence (**Figure 1**). The cytosolic pH, G-proteins, and phospho-/sphingolipids are also important components during stomatal closure but they may be acting in parallel. Further work required to elucidate the perception of signals, such as methyl jasmonate or elicitors and how they activate NADPH oxidase leading to ROS production. Several of the unresolved questions make the stomatal guard cells an ideal system for studying signal transduction mechanism in plant cells.

AUTHOR CONTRIBUTIONS

AR proposed the topic. AR and AS collected the literature, critically assessed the information, and wrote the manuscript together.

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The Dual Role of Nitric Oxide in Guard Cells: Promoting and Attenuating the ABA and Phospholipid-Derived Signals Leading to the Stomatal Closure

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OVERVIEW OF THE ABA-INDUCED SIGNALING LEADING TO THE REGULATION OF STOMATAL MOVEMENT

Plants regulate the gas exchange with the environment through microscopic pores formed by specialized cells called guard cells that constitute the stomata. The control of water loss and CO_2 uptake of plants relies on the size of the stomatal pore. Abscisic acid (ABA) is the master hormone governing the intricate network of molecular switches and physiological responses of guard cells that determine the degree of stomatal aperture. Once plants sense water deficit, ABA is synthesized, and enters the guard cells triggering a series of signals that result in stomatal closure and preservation of the water status of the whole plant. ABA signaling in guard cells involves several mechanisms sustained by enzymes, small molecules, and second messengers that finally promote the inactivation of inward-rectifying K^+ ($I_{K,\ in}$) channels, activation of outward-rectifying K^+ ($I_{K,\ out}$) channel, and activation of slow and rapid-anion channels (MacRobbie, 2006), resulting in the facilitation of solute efflux from guard cells and stomatal closure. The ABA receptor is a complex structure formed by a family of soluble proteins known as pyrabactin resistance/regulatory component of ABA receptor (PYR/PYL/RCAR) (Ma et al., 2009; Park et al., 2009), which interacts with a protein phosphatase-kinase complex, functioning as a double negative regulatory system (Umezawa et al., 2009; Vlad et al., 2009). The phosphatases ABA insensitive 1 (ABI1), ABA insensitive 2 (ABI2), and homology to ABI1 (HAB1) belong to clade A type 2C protein phosphatase (PP2C) and the kinases belong to the group III of the sucrose non-fermenting 1 (SNF1)-related protein kinase 2 SnRK2.2; 2.3; and the 2.6, the last one also known as open-stomata 1 (OST1) (Kulik et al., 2011). Once ABA binds to its receptor, it generates a conformational change of the PYR/PYL/RCAR-ABA complex that promotes the binding of PP2C allowing the phosphorylation, and hence the activation, of SnRK2. Downstream, SnRK2 phosphorylates numerous target proteins involved in ABA responses, including the NADPH oxidase (NADPHox) respiratory burst oxidase homolog F (RbohF) (Sirichandra et al., 2009). Plant NADPHox RbohD and RbohF play an active role in the production of reactive oxygen species (ROS) during ABA-induction of stomatal closure. Furthermore, it has been recently found that activated OST1 interacts with type 2A protein phosphatase (PP2A)-subunits (Waadt et al., 2015), which are functional proteins proposed to positively and negatively regulate the ABA signaling in guard cells (Kwak et al., 2002; Pernas et al., 2007).

The production of the second messenger nitric oxide (NO) is required for ABA-dependent induction of stomatal closure (Desikan et al., 2002; Garcia-Mata and Lamattina, 2002; Neill et al., 2002; Suhita et al., 2004; He et al., 2005; Kolla et al., 2007). NO regulates a subset of ABA-evoked responses by inactivating $I_{K,\ in}$ channels via a cGMP/cADPR-dependent increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Garcia-Mata et al., 2003). NO also induces the production of the lipid second messenger phosphatidic acid (PA) in guard cells (Distefano et al., 2008). PA is generated by phospholipase D (PLD) or by PLC through the hydrolysis of polyphosphoinositides (PPIs) in concerted action with diacylglycerol kinases. In addition, the hydrolysis of PPIs by PLCs also produces water-soluble inositol polyphosphates (InsPPs), that diffuses to the cytosol, promoting the release of Ca^{2+} from intracellular stores in guard cells (Lemtiri-Chlieh et al., 2003) and contributing to the increase of $[Ca^{2+}]_{cyt}$. Results have shown that NO-induction of stomatal closure was impaired when either PLC or PLD activity was inhibited (Distefano et al., 2008). These evidences suggest that PLD and PLC are participating in the NO-signaling pathway in guard cells (Distéfano et al., 2010). Regarding PA, it binds to both RbohD and RbohF, increasing their activity and leading to superoxide ($O_2^{\cdot-}$) production and H_2O_2 formation, and thereby contributing to the induction of stomatal closure (Zhang et al., 2009). In addition, it has been shown that PA interacts with and inhibits ABI1 (PP2C) (Zhang et al., 2004), and activates SnRK2s type I SnRK2.4 and 2.10 (Testerink et al., 2004) and PP2A (Gao et al., 2013), all of them components of the ABA signaling. Yet, there is no conclusive evidence supporting that both NO and PA production is via the activation of the PYL/PYR/RCAR receptor. **Figure 1** summarizes the core of the signaling components under the control of NO and PA downstream ABA that, once integrated, determine the control of stomatal movements. There, it is highlighted the dual and compensatory mechanisms exerted by NO in the promotion and attenuation of the ABA-stimulated stomatal closure.

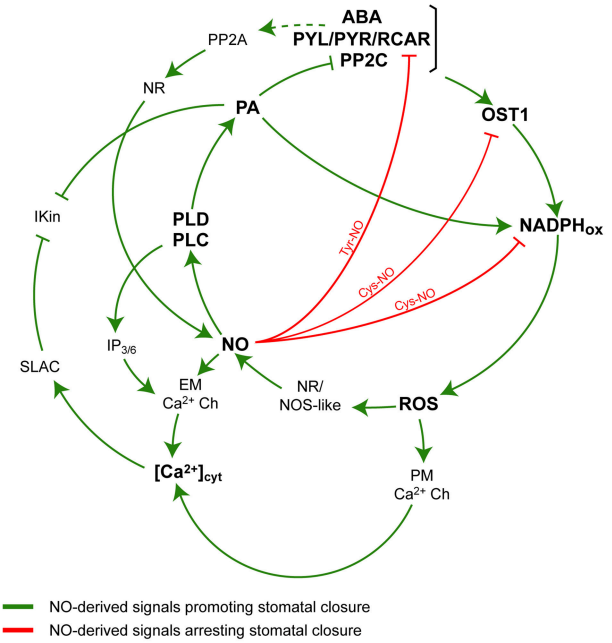

FIGURE 1 | A simplified model of ABA signaling in guard cells. Nitric oxide (NO) promotes and attenuates the ABA-induced and phospholipid-mediated stomatal closure. The positive ABA-stimulus inducing the stomatal closure and involving NO and phospholipid-derived signals are in green. The negative effects of NO linked to post-translational modifications of proteins and attenuating the ABA signaling are in red. The model shows that ABA binds to its receptor pyrabactin resistance/regulatory component and recruits the protein phosphatase 2C [ABA-PYL/PYR/RCAR-PP2C], resulting in the activation of the kinase open stomata 1 (OST1). Then, OST1 phosphorylates and activates NADPH oxidase (NADPHox), with the consequent generation of reactive oxygen species (ROS) and downstream, the formation of NO through the enzymatic activities nitrate reductase (NR) and NO synthase-like (NOS-like). NO induces the formation of phosphatidic acid (PA) via the activation of phospholipase C (PLC) and phospholipase D (PLD) by a still unknown mechanism. PA in turn activates $NADPH_{ox}$ and inhibits PP2C and inward-rectifying K^+ ($I_{K,\ in}$) channels. The activity of PLC also generates inositol phosphates ($IP_{3/6}$) contributing to the release of Ca^{2+} from intracellular stores through endomembranes Ca^{2+}-channels (EM Ca^{2+} Ch). The increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) activates slow -anion channels (SLAC) which also inhibits $I_{K,\ in}$. The production of ROS also participates in the regulation of $[Ca^{2+}]_{cyt}$ through the activation of plasma membrane Ca^{2+} channels (PM Ca^{2+} Ch). The model also shows a pathway proposing that ABA is able to induce the production of NO via the dephosphorylation and activation of NR through the activity of protein phosphatase 2A (PP2A) (Heidari et al., 2011). The attenuating effects of NO by breaking the ABA stimulus include the inhibition and degradation of the ABA receptor PYL/PYR/RCAR through the nitration of Tyr residues (Tyr-NO), and the inactivation of OST1 and $NADPH_{ox}$ via S-nitrosylation (S-NO).

BREAKING THE SENSE OF THE IMPULSE, THE NO-MEDIATED ATTENUATION OF ABA SIGNALING IN GUARD CELLS

One of the most intriguing and less understood processes in signal transduction is how do cells put a brake to multi-directional signal cascades with just one output. New available evidences suggest that NO could also function as blocker of the ABA-induced stomatal closure through the inhibition of the signaling by post-translational modifications of some key components of the cascade. The S-nitrosylation of Cysteine residues by NO-derived compounds is considered the most important NO-dependent post-translational modification of proteins due to its versatility and occurrence under physiological conditions (Astier and Lindermayr, 2012). It was demonstrated that Arabidopsis RbohD ability to form ROS is negatively regulated by the *S*-nitrosylation in cell death processes and immunity (Yun et al., 2011). The S-nitrosylation of Cys 890 of the Arabidopsis RbohD was sufficient to abolish its activity of forming ROS intermediates and consistently, its mutation also blocks any possibility of regulating NADPHox enzymatic activity. Moreover, Cys890 is conserved and also S-nitrosylated in humans and fly, suggesting a conserved post-translational regulatory pathway of NADPHox during evolution (Yun et al., 2011). As stated above PA binds to RbohD, and the PA-binding motif localizes in amino acid residues 101–330 (Zhang et al., 2009). In this region, mutation of the arginine residues 149, 150, 156, and 157 in RbohD resulted in the loss of PA binding and

the loss of the activation of RbohD by PA (Zhang et al., 2009). It would be interesting to know if there exists any structural interference between the binding of PA and the S-nitrosylation of RbohD.

In a general view of the regulating process governing ABA-induced stomatal movement, NO could first induce lipid and lipid-derived molecules which activate NADPHox, but at a later time point, and probably based on increased and damaging concentrations of H_2O_2 and NO, NO is able to stop ROS production by inhibiting NADPHox activity directly by S-nitrosylation (Yun et al., 2011). Nevertheless, the NO-dependent post-translational modifications on RbohD still need to be proven in guard cells.

As stated, OST1 is a serine/threonine protein kinase that acts as a positive regulator mediating the ABA-induced stomatal closure through the activation of downstream effectors (Wang et al., 2013). In a very nice piece of work, two years later, Wang et al. (2015) demonstrated that NO negatively regulates ABA signaling in guard cells through the S-nitrosylation of OST1. NO can S-nitrosylates OST1 *in vitro* and *in vivo* at cysteine 137, a residue adjacent to the kinase catalytic site, provoking the dysfunction of its phosphorilating activity (Wang et al., 2015). At a first glance, it can be perceived that NO possesses a multitasking capacity of modulating ABA signaling in guard cells through a complex biological activity. It includes both positive and negative effects that can be summarized as an attenuated mechanism for the regulation of stomatal closure induced by ABA, in a smooth and continuously highly controlled adjustment. **Figure 1** details the interactions occurring in guard cells highlighting the positive and negative effects of NO on the phospholipid-derived signals and the ABA-induced signaling resulting in stomatal closure. It includes (A) direct positive effects (increase of $[Ca^{2+}]_{cyt}$ and PA) and (B) negative effects leading to the attenuation of the ABA signaling through the inhibition of key effectors of stomatal closure (inhibition of NADPHox and OST1 by S-nitrosylation). A recently published article adds new *in vitro* and *in vivo* evidences showing that the family of ABA receptors PYR/PYL/RCAR is inactivated by nitration of tyrosine residues leading to the degradation of the receptor via proteasome. The non-reversible nitration of tyrosine residues is a post-translational modification of proteins that requires the formation of the strong oxidant peroxynitrite, a compound formed from the fast reaction between superoxide ($O_2^{\cdot-}$) and NO. In addition, the article shows that the ABA receptor is also S-nitrosylated, resulting in a full capacity of the receptor of inhibiting PP2C activity (Castillo et al., 2015). Even if authors speculate about the relevance of the S-nitrosylation and Tyr-nitration as a NO-mediated mechanism that modulates the ABA receptor biological activity, it was not yet proved whether it is functionally active in guard cells under physiological conditions associated to drought stress. It would be interesting to see if an increase of ABA concentration after perceiving the drought stress is enough to promote the nitration and degradation of the ABA receptor, leading to the loss of the response to ABA and to the brake of ABA-induced stomatal closure.

Overall, this opinion article tries to recall the already known two sides of the NO "coin" as a ubiquitous, homeostatic, and synchronizer molecule in cell physiology. Thereby, we highlight here the rationale of NO acting both in promoting and arresting the ABA-induced/phospholipid-mediated signals triggering the stomatal closure, as a way to avoid the exacerbation of a hormonal stimulus. In future investigations, however, it remains to be deciphered if the multi targets of NO are reached simultaneously or through a temporal and spatial pattern of its actions.

AUTHOR CONTRIBUTIONS

The analysis, revision of the bibliography and the discussion of the data were conducted by AL, CG, and LL. The manuscript was prepared and written, including round of corrections, by AL, CG, and LL. The design and general supervision was performed by LL.

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OPINION
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Gasotransmitters and Stomatal Closure: Is There Redundancy, Concerted Action, or Both?

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The epidermis of the aerial part of land plants is pierced by pores through which plants perform gas exchange with environment. The guard cells (GCs), the specialized cells that surround the pore, have the capacity to sense diverse environmental and endogenous stimuli and integrate them into a single output which is the regulation of the stomatal pore width. The stomatal pore size is modulated by changes of the guard cell volume, driven by variations in the osmotic potential of the GCs.

The stress hormone abscisic acid (ABA), the master regulator of stomatal movement, induces stomatal closure by the inhibition of H^+-ATPases and activation of rapid and slow anion channels, producing the depolarization of the plasma membrane (PM) in GCs, and by an increase in the cytosolic Ca^{2+} concentratrion $[Ca^{2+}]_{cyt}$. While the rise of the $[Ca^{2+}]_{cyt}$ blocks the influx of K^+ by the inactivation of the inward rectifying K^+ channels (K^+_{in}), the depolarization of the PM, in turn promotes K^+ efflux driven by outward rectifying K^+ channels (K^+_{out}; Blatt, 2000). This process is closely regulated by a complex signaling network that involves the participation of numerous ubiquitous signaling components like ROS, protein kinases, phospholipases, and protein phosphatases (Kim et al., 2010; Song et al., 2014); and by other signaling components that are emerging as active players in this signaling network, such is the case of gasotransmitters (García-Mata and Lamattina, 2013).

A gasotransmitter is a small gas molecule that: (i) can freely permeates biological membranes; (ii) it is endogenously generated by specific enzymes; (iii) it has specific functions at physiologically relevant concentrations; (iv) it functions can be mimicked by exogenous application of a donor; and (v) it has specific cellular and molecular targets (Wang, 2002). The group of gasotransmitters is, so far, composed by Nitric Oxide (NO), Carbon Monoxide (CO), and Hydrogen Sulfide (H_2S) and the three of them have been reported to participate in the promotion of stomatal closure (García-Mata and Lamattina, 2013), however, the biology of CO in this physiological process is less known than that of NO and H_2S. Therefore, this opinion will be focused mainly on the action and interaction of NO and H_2S. The two of them are accepted as active players in the regulation of stomatal movement, however there are still obscure points and some of them will be discussed in this opinion article: (i) their specific molecular targets; (ii) the molecular mechanisms underpinning their action; (iii) the interplay between them during the stomatal closure induction; and (iv) the crossed-regulation of their metabolism.

All the three gasotransmitters are synthesized during the promotion of stomatal closure. CO is synthesized via the activity of heme oxygenase (Shekhawat and Verma, 2010). In *Vicia faba* CO induces stomatal closure in a dose-dependent manner and acts upstream of the production of NO during ABA-dependent stomatal closure (Cao et al., 2007; She and Song, 2008). It has been reported that the hormones ABA and ethylene (Eth) require the production of NO for the regulation of stomatal movement (García-Mata and Lamattina, 2002; Neill et al., 2002; He et al., 2011; Song et al., 2011). NO can be synthesized either from NO_2 by two genes, *NIA1*

and *NIA2*, that code for a nitrate reductase (NR), or from L-arginine in a reaction catalyzed by an enzyme with nitric oxide synthase (NOS)-like activity, even though the involved enzyme named AtNOA1 possesses GTPase activity (Moreau et al., 2008). However, it was reported that the Arabidopsis triple mutant *nia1/nia2/atnoa1*, which produces very low levels of NO, is hypersensitive to ABA (Lozano-Juste and León, 2010), suggesting that NO could have a dual role in ABA-dependent responses. Moreover, it has been found that increased levels of NO are dependent on the NADPHox-dependent production of H_2O_2 (Bright et al., 2006). NO levels can also be modified by an Alternative Oxidase (AOX). Tobacco plants lacking AOX show high NO levels that impacts in stomatal function (Cvetkovska et al., 2014). Recently García et al. (2010) have shown that Arabidopsis mutant plants in β-Cyanoalanine synthase (Cys-C1), a mitochondrial enzymatic source of H_2S, show higher *AOX1a* transcript levels than wild type, however exogenous application of H_2S to rice cell culture induced AOX expression (Xiao et al., 2010). Further physiological studies are needed to clarify this interaction.

H_2S is produced during the passage of L-cysteine to pyruvate and ammonia in a reaction catalyzed by L-cysteine desulfhydrase (DES1; Alvarez et al., 2010). In Arabidopsis there are three different genes involved in this reaction: the *DES1* gene (Alvarez et al., 2010; Scuffi et al., 2014), the *At-LCDES* gene (Jin et al., 2011), and *L-CDes* gene (Hou et al., 2013). Recently, it has been shown that the expression of these genes is upregulated in response to ABA, Eth, JA, and SA, all hormones that modulate stomatal movement (Hou et al., 2013). Even if sequence analysis has shown that the promoter region of the *DES1* gene contains ABA-responsive elements (Scuffi et al., 2014), further work is needed in order to have a better understanding about the mechanism by which these hormones induce the expression of those genes. Although, DES1 was reported to mediate ABA-dependent stomatal closure (Scuffi et al., 2014), it was recently reported that H_2S regulates the activity of K^+_{in} channel mostly in an ABA- and Ca^{2+}-independent manner, suggesting the existence of ABA-regulated signaling pathways that can be, alternatively, activated in response to other stimuli (Papanatsiou et al., 2015).

NO PHYSIOLOGY IN GUARD CELLS

More than a decade of work on the participation of NO on the regulation of stomatal movement resulted in a more or less bounded idea of its mechanism of action, in particular in those events triggered by ABA. As stated above, ABA-dependent ROS production induces NO synthesis via NR/NOS-like activities. NO regulates the activity of K^+_{in} either via the Ca^{2+} release from intracellular Ca^{2+} stores, or through the production of phospholipase D (PLD)-dependent inositol phosphates. Another molecular target of NO is the soluble guanylate ciclase (sGC) that generates cyclic guanosine monophosphate (cGMP) which is converted to 8-nitroguanosine 3′,5′-cyclic monosphosphate (8nitro-cGMP) by NO, modulating the $[Ca^{2+}]_{cyt}$ (Joudoi et al., 2013). Recently, it has been proposed that NO may break the ABA signaling in guard cells. On the one hand NO mediates ABA-dependent stomatal closure via the regulation of K^+ and Cl^- channels (Garcia-Mata et al., 2003; Sokolovski et al., 2005), on the other hand it was suggested that NO can act as a negative regulator of ABA pathway via *S*-nitrosylation of the SnrK2,6/OST1 (Wang et al., 2015) and through the nitration of a Tyr residue of the PYR/PYL/RCAR ABA receptor complex (Castillo et al., 2015).

H_2S PHYSIOLOGY IN GUARD CELLS

This first report of the participation of H_2S in guard cell signaling appeared in 2010, and since then several works have shown that this gasotransmitter induces stomatal closure in different plant species (García-Mata and Lamattina, 2010; Hu et al., 2014; Papanatsiou et al., 2015). In Arabidopsis, DES1 produces H_2S in response to ABA. H_2S, in turn, increases endogenous NO production (Scuffi et al., 2014). H_2S-dependent stomatal closure is impaired in the *nia1/nia2* double mutant. Moreover, the expression of both genes was reported to be upregulated by H_2S donors, suggesting that NR is involved in H_2S-dependent NO production (Scuffi et al., 2014). However, Lisjak et al. (2010, 2011) showed that exogenous addition of H_2S decreased ABA-dependent NO production and thus induced stomatal opening. Interestingly, it was reported that H_2S also modulates Eth-dependent stomatal closure, but in this particular case, NO was reported to act upstream of H_2S (Liu et al., 2011; Hou et al., 2013).There are other components, beside NR, that were pointed as targets of H_2S during stomatal closure induction, among them: (i) the member of the multidrug resistance protein family AtMRP5, which was proposed as a modulator of Ca^{2+} and anion channels (Suh et al., 2007; García-Mata and Lamattina, 2010); (ii) 8-nitro cGMP, which reacts with H_2S to form 8-mercapto cGMP to modulate $[Ca^{2+}]_{cyt}$ (Honda et al., 2015); and (iii) K^+_{in} channels, which are inactivated by H_2S in an ABA-independent manner (Papanatsiou et al., 2015).

THE INTERPLAY BETWEEN NO AND H_2S

The gasotransmitters NO and H_2S not only share physicochemical similarities but they can also interact with each other in different biological systems and physiological conditions. Even though, there is still much to learn about: (i) the chemical nature of these interactions, (ii) the different products that can be potentially formed *in vivo* from the interaction, and (iii) the different biological outcomes. The study of the interaction between different gasotransmitters has kept the attention of researchers from different fields. As a result, different kinds of interactions have been described. There are interactions in which different gasotransmitters can act on the same molecular targets but having either the same or sometimes opposite outcomes (Mustafa et al., 2009b). An example of this in plants is the case of ABA-dependent induction of stomatal closure, where it has been reported that, on the one hand, ABA induces H_2S production which in turn increases endogenous NO levels triggering stomatal closure (García-Mata and Lamattina,

2010; Scuffi et al., 2014), while on the other hand, it is reported that exogenous addition of H_2S decreases ABA-dependent NO production, thereby producing the opening of the stomatal pore (Lisjak et al., 2010, 2011). There are other crosstalks in which different gasotransmitters produce the same outcome but acting on different molecular targets (Coletta et al., 2012). Such is the case of the regulation of guard cell K^+ channels, where both NO and H_2S selectively inactivate K^+_{in}, however NO does this through a response that involves the release of Ca^{2+} from intracellular stores (Garcia-Mata et al., 2003), while H_2S inactivates K^+_{in}, mostly in an ABA and Ca^{2+} independent manner (Papanatsiou et al., 2015).

H_2S and NO can also regulate each other source, by modulating the enzymatic production of the other. In animal systems H_2S is able to down regulate NO production by inhibiting both constitutive and inducible NOS isoforms (Kubo et al., 2007) or to upregulate endothelial NOS (eNOS) dependent NO production (Predmore et al., 2011). In plants, it has been suggested that H_2S induces NR-dependent NO production via the regulation of both *NIA1* and *NIA2* genes (data accessible at NCBI GEO database, accession GSE32566). The cross regulation of gasotransmitter sources has been also shown in the opposite sense. Zhao et al. (2001) have shown that NO donors upregulate the expression of the animal enzymatic H_2S source, cystathionine-γ-lyase (CSE) and its consequent H_2S production. In plants NO also upregulates H_2S production during Eth-induced stomatal closure by increasing the expression of *AtL-CDes/AtD-CDes* genes (Liu et al., 2011, 2012).

The gasotransmitters can directly modify their specific targets by posttranslational modification (PTM) of the target protein. Nitric oxide can react with the thiol group of a cysteine residue to form S-nitrosocysteines residues (R-SNO) in a process known as S-nitrosylation, while H_2S forms a persulfide group (R-SSH) in a process known as S-sulfhydration. There are proteins that can be modified by both gasotransmitters at the same cysteine residue. Interestingly, sulfhydration and nitrosylation can influence the protein function in different manners, S-nitrosylation modifications usually results in the inactivation of the protein while S-sulfhydration of the protein in many cases results in activating the biological function of the protein (Mustafa et al., 2009a; Jiang et al., 2010). Two examples of the different effects of the PTM in plants are the enzymes ascorbate peroxidase (APX) and glyceraldehydes-3P-dehydrogenase (GAPDH) whose activities are affected in different senses when they are S-nitrosylated or S-sulfhydrated, enabling enzymes play additional functions due to PTMs processes (Aroca et al., 2015).

The study of the interaction between H_2S and NO is one of the current challenges for understanding the biology of these two gasotransmitters. The current knowledge shows that these two gases can interact at different stages of the signaling process, at different levels of their biosynthetic pathways and depending of the metabolic and redox status of the target cells (Cortese-Krott et al., 2014, 2015a; Lo Faro et al., 2014).

The existence of a direct chemical reaction between NO and H_2S has gained strength in recent years. It is speculated that this interaction may result in the formation of some novel forms of nitrosothiols not yet fully characterized. In a recent paper, Cortese-Krott et al. (2015b) proposed that the interaction of NO and H_2S would result in the formation of bioactive products at physiological pHs, emphasizing the formation of nitrosopersulfide (NO^-), polysulfides (HS_n), and N-nitroso-hydroxylamine-N-sulfonate (SULFI/NO). These compounds could regulate the bioavailability of NO and H_2S by either the releasing or scavenging of each of them, which is depending on the relative concentrations of each one and the redox status of the cell (Cortese-Krott et al., 2015a) In a simplified schematic representation, **Figure 1**, we summarize the interplay and the close association existing between NO and H_2S, and the formation of potential intermediates that could be involved in the regulation of guard cell physiology. These new discovered molecular forms might potentially explain the conflicting results concerning the roles of NO and H_2S of influencing stomatal movement.

CONCLUSIONS

The details of the biochemical interaction between NO and H_2S are scarcely known in guard cells and in plant systems in general, in comparison to some physiological processes in animal systems (Kolluru et al., 2013; Lo Faro et al., 2014; Cortese-Krott et al., 2015a). However, the evidences presented in this study indicate that both gases can modulate stomatal movement acting independently, or in concerted action, in and ABA-triggered

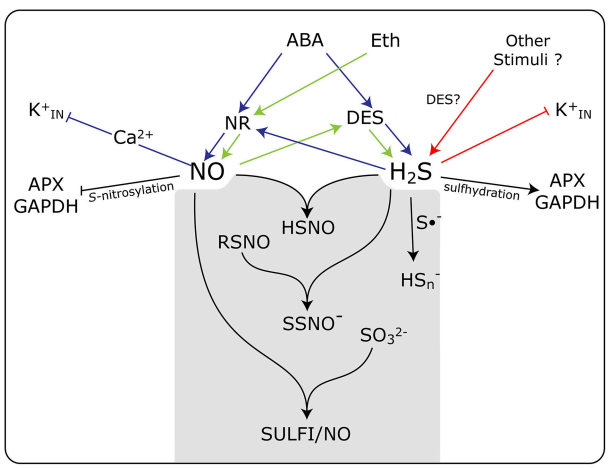

FIGURE 1 | Schematic model showing the action of NO and H_2S and the interplay between them in guard cell signaling in response to different stimuli. Both NO and H_2S are produced enzymatically by nitrate reductase (NR) and L-cysteine desulfhydrase (DES), respectively, in response to abscisic acid (ABA), ethylene (Eth), and other stimuli. Some of the known targets of NO and H_2S are inward rectifying K^+ channels (K^+_{in}), and the enzymes ascorbate peroxidase (APX) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gray box shows the chemical reactions between NO and H_2S, and the formation of potential intermediates that could be involved in the regulation of guard cell physiology. HSNO, thionitrous acid; $SSNO^-$, nitrosopersulfide; RSNO, nitrosothiols; Hs_n^-, polysulfide; $S\bullet^-$, radical anion; SO_3^{2-}, sulfite, and SULFI/NO, N-nitrosohydroxylamine-N-sulfonate. Blue arrows, ABA triggered events, green arrows Eth triggered events; red arrows participation of other stimuli. Arrow end, activation; blunt end, inactivation.

signaling cascade, or in ABA-independent manner. They can modulate the activity of the same molecular target by PTM of cysteine residues, and can even regulate the production and/or bioavailability of each other. In conclusion, it arises the point of the need to be cautious when drawing conclusions about the effects of either NO or H_2S, unless the effect of both are studied together at the same biological conditions.

AUTHOR CONTRIBUTIONS

Both DS and LL contributed in the writing of the manuscript, LL also contributed to give shape the idea of the opinion. CG made most of the writing and the figure and had the original idea.

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Expression of *Arabidopsis* Hexokinase in Citrus Guard Cells Controls Stomatal Aperture and Reduces Transpiration

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Hexokinase (HXK) is a sugar-phosphorylating enzyme involved in sugar-sensing. It has recently been shown that HXK in guard cells mediates stomatal closure and coordinates photosynthesis with transpiration in the annual species tomato and *Arabidopsis*. To examine the role of HXK in the control of the stomatal movement of perennial plants, we generated citrus plants that express *Arabidopsis* HXK1 (*AtHXK1*) under KST1, a guard cell-specific promoter. The expression of KST1 in the guard cells of citrus plants has been verified using GFP as a reporter gene. The expression of *AtHXK1* in the guard cells of citrus reduced stomatal conductance and transpiration with no negative effect on the rate of photosynthesis, leading to increased water-use efficiency. The effects of light intensity and humidity on stomatal behavior were examined in rooted leaves of the citrus plants. The optimal intensity of photosynthetically active radiation and lower humidity enhanced stomatal closure of *AtHXK1*-expressing leaves, supporting the role of sugar in the regulation of citrus stomata. These results suggest that HXK coordinates photosynthesis and transpiration and stimulates stomatal closure not only in annual species, but also in perennial species.

Keywords: sugar, hexokinase, stomata, transpiration, water-use efficiency, citrus rooted leaves, humidity, light intensity

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INTRODUCTION

Stomata, formed by two guard cells, open at dawn to allow the atmospheric carbon dioxide (CO_2) needed for photosynthesis to enter the leaf, at the cost of extensive transpirational water loss. When carbon fixation and utilization are less efficient, the stomata close to reduce the loss of water via transpiration (Assmann, 1993). Mechanistically, stomata open in response to increases in the osmolarity of the guard cells. These increases are followed by the movement of water into the guard cells, which opens the stomata (Taiz and Zeiger, 1998). Stomata close when the osmolarity of the guard cells is reduced and the water exits the guard cells.

At the start of the previous century, the prevailing paradigm was that sugars generated from starch degradation in guard cells at dawn are the primary osmolytes that open stomata (Lloyd, 1908). The discovery that K^+ ions, Cl^- ions and malate ions are the primary osmolytes that open

stomata (Schroeder et al., 2001; Roelfsema and Hedrich, 2005; Pandey et al., 2007) yielded a modified hypothesis suggesting that K^+ ions open stomata at dawn and that sugars generated from starch degradation, photosynthetic carbon fixation or the import of apoplastic (intercellular) sucrose replace K^+ ions over the course of the day and keep stomata open (Gotow et al., 1988; Tallman and Zeiger, 1988; Poffenroth et al., 1992; Talbott and Zeiger, 1993, 1996, 1998; Amodeo et al., 1996). A non-osmotic role for sugars in stomatal opening has been recently suggested. In the suggested scenario, sucrose is cleaved within guard cells by either sucrose synthase or invertase and this cleavage provides substrates for organic acid synthesis and respiration that open stomata (Antunes et al., 2012; Daloso et al., 2015a,b). Yet, recent studies in *Arabidopsis*, tomato and *Vicia faba* have shown that sugars close stomata (Kelly et al., 2013; Li et al., 2015).

Sugar is produced primarily in leaf mesophyll cells. In many plant species, sucrose - a glucose-fructose disaccharide – is the primary transported sugar exported to the intercellular space, the apoplast, prior to being loaded into the phloem (Rennie and Turgeon, 2009). Some of this apoplastic sucrose is carried toward the open stomata by the transpiration stream, so that the concentration of sucrose in the guard cells' apoplast may reach 150 mM (Lu et al., 1995, 1997; Ewert et al., 2000; Outlaw and De Vlieghere-He, 2001; Kang et al., 2007). It has been suggested that this accumulation of sucrose decreases stomatal apertures due to an extracellular osmotic effect (Outlaw, 2003; Kang et al., 2007). However, recent studies with tomato and *Arabidopsis* have shown that sucrose stimulates stomatal closure independent of its osmotic effect and that this closure is mediated by HXK within the guard cells (Kelly et al., 2013).

Hexokinase is an essential enzyme that phosphorylates glucose and fructose, the products of sucrose cleavage (Dennis and Blakeley, 2000). In plants, HXK is the only enzyme that can phosphorylate glucose and may also phosphorylate fructose (Granot, 2007, 2008). Most studies of HXK in plants have involved *Arabidopsis HXK1* (*AtHXK1*), which mediates sugar-sensing, in addition to its catalytic hexose-phosphorylation activity (Moore et al., 2003; Rolland et al., 2006). In a recent study, we found that HXK mediates stomatal closure in response to sugar levels (Kelly et al., 2013). Furthermore, expression of *AtHXK1* specifically in the guard cells of the annual species tomato and *Arabidopsis*, under the potato-derived KST1 guard cell-specific promoter, stimulated stomatal closure and reduced stomatal conductance and transpiration (Kelly et al., 2013). Yet, the role of HXK in the stomata of perennial species, including trees, is not known. In the current study, we examined the use of KST1 promoter to drive guard cell-specific expression of *AtHXK1* in citrus plants and the effect of HXK on citrus stomatal movement.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Experiments were conducted on the citrus Troyer citrange (*Citrus sinensis* 'Washington' sweet orange × *Poncirus trifoliata*). Troyer citrange explants were transformed with the *KSTpro::GFP* and *KSTpro::HXK1* constructs described in (Kelly et al., 2013) using agrobacterium-mediated transformation as described in (Luth and Moore, 1999). Regenerant plants were grafted onto new Troyer citrange rootstock and the graft points were wrapped in polyethylene. After several weeks, the polyethylene was removed and PCR was used to test each plant for the presence of the transgene. Plants transformed with *KSTpro::GFP* or *KSTpro::HXK1* are referred to as GCGFP and GCHXK, respectively. The transgenic plants were vegetatively propagated by grafting shoots onto Troyer citrange rootstocks. The grafted plants were grown in a temperature-controlled greenhouse under natural growth conditions.

Rooting Leaves

Detached mature leaves (about 2 months old) of WT and GCHXK plants were rooted in a high-humidity polystyrene chamber filled with perlite. The leaves generated roots from the edge of the petiole after approximately 45 days in perlite (**Figures 5A,B**). The rooted leaves were planted in 50-ml Falcon tubes filled with perlite and each tube was covered with Parafilm to prevent evaporation. WT and GCHXK rooted leaves were placed in a growth chamber that was kept at 25°C, with a 12-h light/12-h dark photoperiod (light turned on at 6:00 a.m. and turned off at 6:00 p.m.), humidity set at 35% and one of the following light intensities: 100, 400, 600, and 800 μmol m^{-2} s^{-1} of PAR (photosynthetic active radiation); comprised of 87.5% red light and 12.5% blue light (Led Lamp 300w, N.B. Advanced Solutions).

Confocal Microscopy Imaging

Images were acquired using the Olympus IX 81 inverted laser scanning confocal microscope (Fluoview 500) equipped with a 488-nm argon ion laser and a 60 × 1.0 numerical aperture PlanApo water immersion objective. Green fluorescent protein was excited by 488-nm light and the emission was collected using a BA 505–525 filter. A BA 660 IF emission filter was used to observe chlorophyll autofluorescence. Confocal optical sections were obtained in 0.5- to l-μm increments. The images were color-coded green for GFP and magenta for chlorophyll autofluorescence.

RNA Extraction, cDNA Preparation and Quantitative Real-Time PCR

Leaf tissue was harvested from WT and GCHXK plants and total RNA was extracted from that tissue using the Logspin method (Yaffe et al., 2012). In brief, samples were ground using a Geno/grinder (SPEX SamplePrep, Metuchen, NJ, USA) and RNA was extracted in 8 M guanidine hydrochloride buffer (Duchefa Biochemie) and transferred to tubes containing 96% EtOH (Bio Lab, Jerusalem, Israel). Then, samples were transferred through a plasmid DNA extraction column (RBC Bioscience, New Taipei City, Taiwan), followed by two washes in 3 M Na-acetate (BDH Chemicals, Mumbai, India) and two washes in 75% EtOH, and eluted with DEPC (diethylpyrocarbonate) water (Biological Industries, Co., Beit Haemek, Israel) that had been preheated to 65°C. The RNA was treated with

RQ1-DNase (ProMega, Madison, WI, USA) according to the manufacturer's instructions, to degrade any residual DNA. For the preparation of cDNA, total RNA (1 μg) was taken for reverse transcription-PCR using qScript^{TM} cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD, USA) following the manufacturer's instructions. cDNA samples were diluted 1:7 in double-distilled water. Quantitative real-time PCR reactions were performed using SYBR Green mix (Thermo-Scientific, Waltham, MA, USA) and reactions were run in a RotorGene 6000 cycler (Corbett, Mortlake, NSW, Australia). Following an initial pre-heating step at 95°C for 15 min, there were 40 cycles of amplification each consisting of 10 s at 95°C, 15 s at 55°C, 10 s at 60°C and 20 s at 72°C. Results were analyzed using the RotorGene software. Data were normalized using *Citrus sinensis* actin as a reference gene (accession no. XM_006464503). The following primers were used for amplification:

Actin_F: GTC TGG TCC ATC CAT TGT CCA
Actin_R: CAA TGG CCC CAA CCT TAG C
HK_F: GCC TTT GAA GAG GAT TGT GC
HK_R: CAT GAC ACG GAA GTT TGT CC

Gas-Exchange Measurements

Citrus stomatal conductance (g_s), photosynthesis and transpiration rates were measured on fully developed leaves of plants grown in a greenhouse, using a Li-6400 portable gas-exchange system (LI-COR, Lincoln, NE). All measurements were conducted between 9:00 a.m. and 12:00 p.m. Photosynthesis was induced under optimal light (600 μmol m^{-2} s^{-1}) with 400 μmol mol^{-1} CO_2 surrounding the leaf (Ca). The amount of blue light was set to 12.5% photosynthetically active photon flux density to optimize stomatal aperture. The leaf-to-air VPD (vapor pressure difference) was kept at around 1.3–1.63 kPa during all measurements. Leaf temperature for all measurements was approximately 25°C (ambient temperature).

Whole-Plant Relative Transpiration and Continuous Transpiration Rate Measurements

Whole-plant transpiration rates were determined using lysimeters, as described in detail in Sade et al. (2010). Wild-type plants and GCHXK transgenic plants grafted on WT rootstocks were planted in 3.9-L pots and grown under controlled conditions. Each pot was placed on a temperature-compensated load cell with digital output and was sealed to prevent evaporation from the surface of the growth medium. A wet vertical wick made of 0.14-m^2 cotton fibers partially submerged in a 1-L water tank was placed on a similar load cell and used as a reference for the temporal variations in the potential transpiration rate. The output of the load cells was monitored every 10 s and the average readings over 3 min were logged in a data-logger for further analysis. Whole-plant transpiration was calculated as a numerical derivative of the load cell output following a data-smoothing process (Sade et al., 2010). At the time of the experiment (sunrise was around 6:20 a.m. and sunset was around 7:00 p.m.). The plant's daily transpiration rate was normalized to the total leaf area (measured using LI-COR area meter model Li-3100) and the data for a neighboring submerged wick and these figures were averaged for each line (amount taken up by the wick daily = 100%).

RESULTS

Expression Analysis of the KST1 Promoter in Citrus Plants and Generation of *KST1pro::HXK1* Plants

The potential use of the KST1 promoter to drive guard-cell expression in citrus plants was examined using transgenic citrus plants that had been transformed with *KSTpro::GFP*, in which GFP served as a reporter gene (**Figure 1A**). These plants were referred to as GCGFP plants, with GC standing for guard cells. Unlike tomato and *Arabidopsis*, in which the activity of *KST1pro* was shown to be specific to guard cells (Kelly et al., 2013), transgenic GCGFP citrus plants displayed exclusive or preferred expression in guard cells that was associated with the leaf developmental stage (**Figure 1A**). Exclusive expression of GFP was observed in guard cells of young leaves (less than 1 month old; **Figure 1A4–6**). Meanwhile, in mature leaves (more than 1 month old; **Figure 1A7–9**), expression of GFP was observed primarily in guard cells, with a very small amount of expression in epidermal pavement cells (**Figure 1A8**, blue arrows). No expression was observed in any other tissues or plant parts such as mesophyll cells (**Figure 1A6,9**) or roots (not shown), indicating that the KST1 promoter might be an efficient tool for driving guard cell expression in citrus plants. We then created transgenic citrus plants that express the *Arabidopsis* HXK1 (*AtHXK1*) under the KST1 promoter. These plants were referred to as GCHXK plants, with GC standing for guard cells. The expression of *AtHXK1* in GCHXK plants was verified using quantitative real-time PCR (**Figure 1B**). The expression of *AtHXK1* seems low relative to the reference gene actin, perhaps because actin is expressed in all types of cells. Nevertheless, this result confirms that *AtHXK1* is expressed in GCHXK plants.

Expression of *AtHXK1* in Citrus Guard Cells Reduces Stomatal Conductance and Transpiration and Increases *WUEi*

GCHXK and WT plants were propagated by grafting transgenic or WT shoot parts onto WT scions and the grafted plants were analyzed using the LI-COR 6400 gas-exchange system. While net photosynthesis (A_N) remained unaffected (**Figure 2C**), the stomatal conductance (g_s) and transpiration of the GCHXK plants were reduced (**Figures 2A,B**), leading to increased intrinsic water-use efficiency (*WUEi*; **Figure 2D**), calculated as the ratio of A_N/g_s (Gago et al., 2014). No differences in stomatal density or specific leaf area were observed between WT and GCHXK leaves (Supplementary Tables S2 and S3), indicating that the lower stomatal conductance and transpiration could not be attributed to changes in leaf morphology (Gago et al., 2014). Following the increase in *WUEi*, we wished to examine the growth of the GCHXK plants. The growth of the GCHXK plants seemed to be slightly enhanced over several months

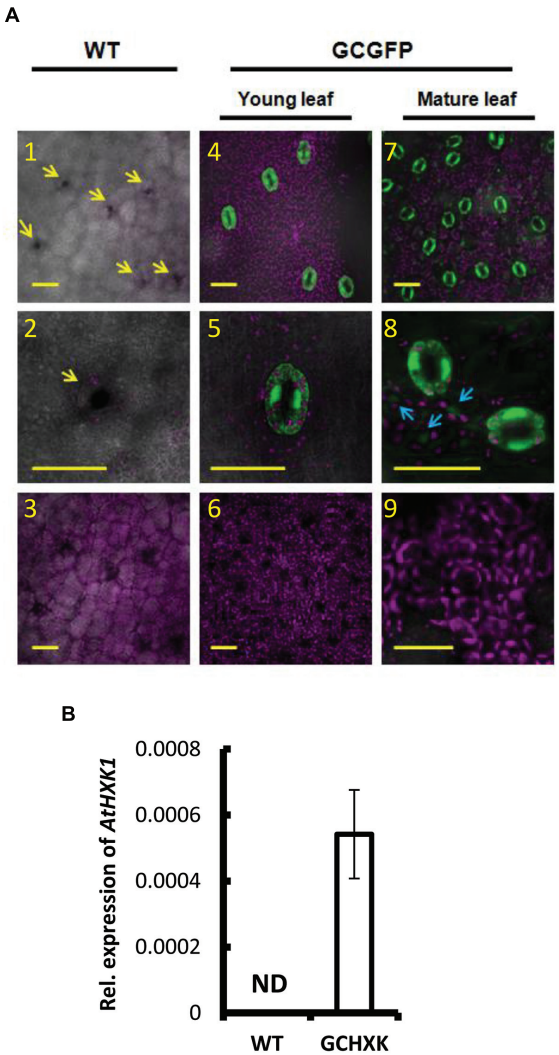

FIGURE 1 | Expression pattern of KST1::GFP in citrus and expression level AtHXK1 in KST::HXK1 lines. (A) Confocal images of leaves from wild-type citrus plants (WT; Panels 1–3) and from transgenic citrus plants (Panels 4–9) expressing GFP (designated GCGFP) under the control of the KST1 promoter (*KSTpro::GFP*). Panels 4–6 show young leaves (i.e., less than 1 month old); panels 7–9 show mature leaves (i.e., more than 1 month old). GFP fluorescence stained green and chlorophyll autofluorescence stained magenta. All images are merged with white light images. Panels 1, 4, and 7 show the epidermis level (with a small fraction of the mesophyll level underneath it). Panels 2, 5, and 8 show the enlarged epidermis region and panels 3, 6, and 9 show the mesophyll level. The arrows in panel 1 point toward the stomata and the arrows in panel 8 point toward GFP expression in epidermal pavement cells. Scale bars = 30 μM. **(B)** Quantitative real-time PCR was performed using RNA extracted from young leaves of the WT and transgenic plants expressing KST::HXK1 designated GCHXK ($n = 5$). β-*actin* was used for normalization. Data are means ± SE. ND, not detected.

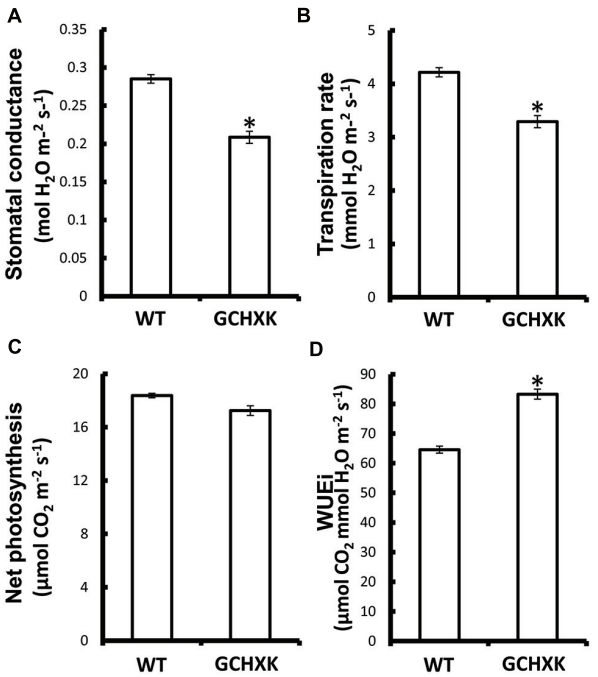

FIGURE 2 | Expression of *AtHXK1* in guard cells of citrus plants reduced stomatal conductance and transpiration with no negative effects on the rate of photosynthesis. WT and GCHXK plants were analyzed using the LI-COR 6400 gas-exchange measurement system. **(A)** Stomatal conductance. **(B)** Transpiration rate. **(C)** Net photosynthesis. **(D)** Intrinsic water-use efficiency (*WUEi*). Data are given as means (±SE) of 8 and 11 independent repeats for the WT and GCHXK lines, respectively. Asterisks denote significant differences relative to the WT (*t*-test, $P < 0.01$).

(**Figure 3A**). To avoid destructive measurements, we measured the perimeter of the stem just above the grafting point (as a parameter of growth). GCHXK plants had significantly wider stems 15 months after grafting (**Figure 3B**), indicating enhanced growth of GCHXK plants.

The Effect of *AtHXK1* on the Whole-Plant Transpiration Rate

The effect of *AtHXK1* on the transpiration rate was further examined with intact grafted GCHXK and WT plants using a precise and sensitive lysimeter-scale system (Sade et al., 2010; Wallach et al., 2010). Continuous measurement of the rate of transpiration over the course of the day revealed that the transpiration rate per unit leaf area was significantly reduced in GCHXK plants (**Figure 4A**) and that the cumulative whole-plant relative daily transpiration per unit leaf area (RDT) was reduced accordingly (**Figure 4B**). The transpiration rate of GCHXK was notably lower in the middle of the day (between 9:00 a.m. and 3:00 p.m.) (**Figure 4A**), when transpiration increases (Ribeiro and Machado, 2007) and more sucrose is supposedly being carried toward the guard cells. These results suggest that HXK plays a role in the regulation of citrus stomatal aperture over the course of the day, stimulating stomatal closure, probably in response to sugar levels.

New Rooted-Leaves System to Analyze GCHXK and WT Leaves

To further study the effect of increased expression of *AtHXK1* in citrus guard cells, we developed a new rooted-leaves system that

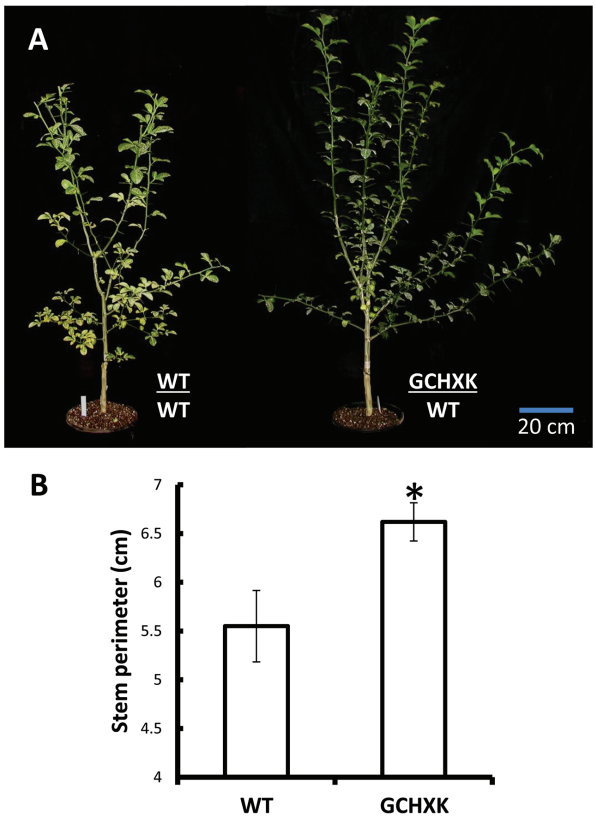

FIGURE 3 | Expression of *AtHXK1* in the guard cells of citrus plants improves plant growth. (A) Representative images of wild-type Troyer citrange and GCHXK transgenic line expressing *AtHXK1* specifically in guard cells, both grafted on Troyer citrange rootstocks. The grafts of these plants were performed on the same day using chip buds taken from GCHXK and WT plants. **(B)** Stem diameter just above the grafting point. Data are given as means (±SE) of 4 and 5 independent repeats for the WT and GCHXK lines, respectively, 15 months after grafting. Asterisks denote significant differences relative to the WT (*t*-test, $P < 0.05$).

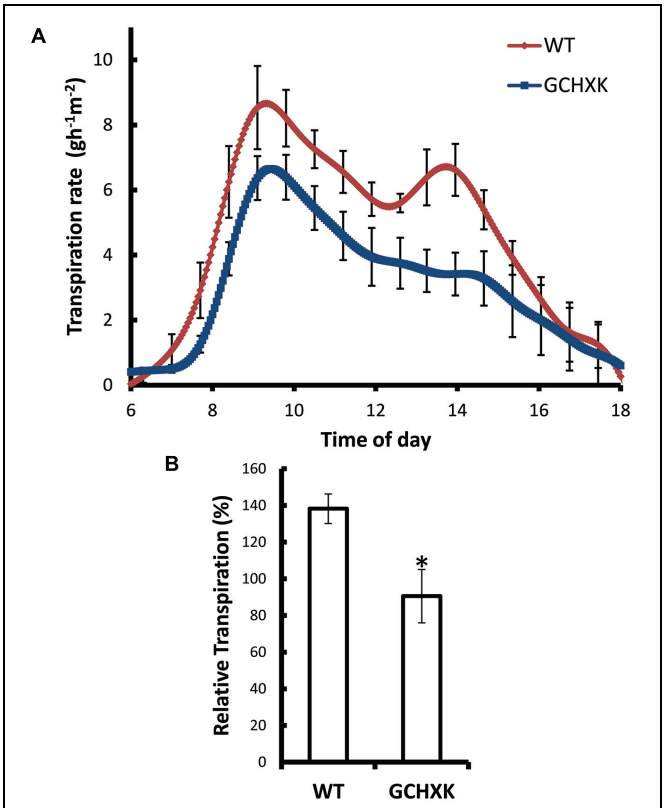

FIGURE 4 | Guard-cell expression of *AtHXK1* reduces the rate of transpiration. (A) Transpiration rates of the WT (red line), GCHXK (blue line) plants were monitored continuously throughout the day. The rate of transpiration was normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. **(B)** Whole-plant average relative daily transpiration per unit leaf area of the WT and GCHXK plants. Data points are means ± SE ($n = 4$ for WT, $n = 4$ for GCHXK). The asterisk denotes a significant difference relative to the WT (*t*-test; $P < 0.01$).

allowed us perform physiological experiments under controlled stable conditions (for detailed description of the method please see the "Rooting leaves" subsection of the Material and Methods). Leaves of WT and GCHXK plants were rooted (**Figures 5A,B**) and the rooted leaves were planted in 50-ml plastic tubes filled with perlite, which were covered with Parafilm to reduce water evaporation (**Figures 5C,D**). The rooted leaves were placed in a temperature-controlled growth chamber, which provided adjustable uniform growth conditions such as temperature and light intensity (**Figure 5E**). Since reduced transpiration is known to increase leaf temperature (Merlot et al., 2002), we used infrared thermal imaging to compare the temperatures of WT and GCHXK leaves (**Figure 6A**). The temperatures of the GCHXK leaves were significantly higher than those of the WT leaves (**Figure 6B**), indicating a reduced rate of transpiration in rooted GCHXK leaves.

The stomatal-closure effect of HXK is thought to be dependent on the amount of sugar produced through photosynthesis. Accordingly, stomata closure and transpiration might be affected by light intensity. We, therefore, used the rooted-leaf system to follow the rate of transpiration at various light intensities with the assumption that, at optimal light-intensity levels, GCHXK leaves may exhibit a lower rate of transpiration than WT leaves. The transpiration rates of GCHXK and WT leaves were measured at various light intensities (100, 400, 600, and 800 μmol/m2 s) by consecutively weighing the tubes throughout the day. It appeared that the transpiration rate at each light intensity remained quite consistent over the course of the day (**Figures 7A–D**), perhaps due to the constant conditions within the growth chamber. At low light intensity (100 $\mu mol/m^2 \cdot s$), the transpiration rate of the rooted GCHXK leaves was slightly higher than that of the WT rooted leaves and, at 800 μmol/m2·s, it was similar to that of the WT. Yet, at 400 $\mu mol/m^2 \cdot s$, the transpiration rate of the GCHXK rooted leaves was slightly, but significantly lower than that of the WT leaves and at 600 μmol/m2·s, [considered an optimal light intensity for citrus (Pimentel et al., 2004)], the transpiration rate of the rooted GCHXK leaves was half that of the WT (**Figure 7C**), indicating a significant reduction in stomatal aperture. The constant transpiration rate at each light intensity allowed us to

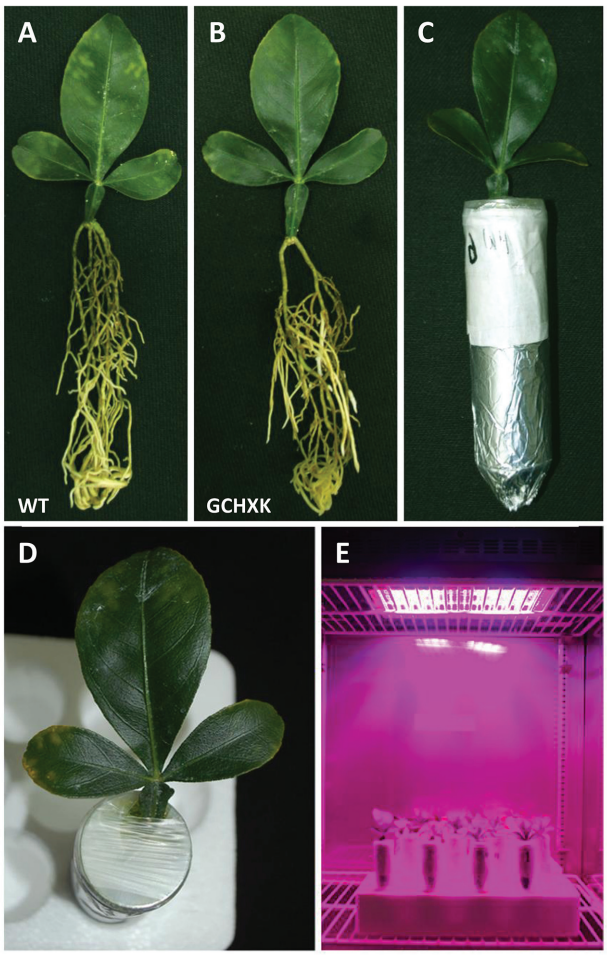

FIGURE 5 | A new rooted-leaves model system. (A) Rooted WT citrus leaf. **(B)** Rooted GCHXK leaf. **(C)** Rooted leaves planted in a 50-ml Falcon tube filled with perlite. **(D)** The tube around the leaf was covered with Parafilm to reduce evaporation. **(E)** Rooted WT and rooted GCHXK leaves were placed in a growth chamber with controlled temperature, humidity and lighting.

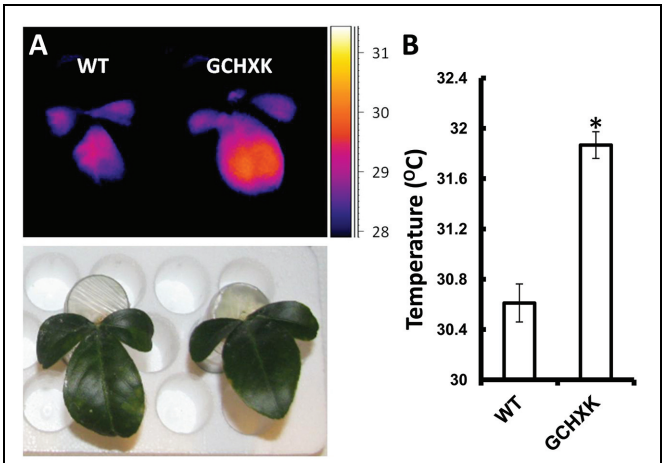

FIGURE 6 | Guard-cell expression of *AtHXK1* increases leaf temperature. (A) Images of rooted leaves under a light intensity of 600 μmol/m^2·s were captured using a thermal camera (ThermaCAM model SC655; FLIR Systems); warm colors represent high temperatures (scale is shown at right). **(B)** Leaf temperatures of the WT and GCHXK lines determined using ThermaCAM researcher pro 2.10 software. Data are means ± SE from 9 and 12 biological repeats of WT and GCHXK, respectively. The asterisk denotes a significant difference relative to the WT (*t*-test; $P < 0.01$).

plot the mean transpiration rate at each light intensity versus light intensity (**Figure 7E**). While the transpiration rate of the WT leaves was significantly affected by light intensity, being very low under low light-intensity conditions and peaking at 600 μmol/m^2·s, the transpiration rates of the GCHXK leaves were relatively constant at the various light intensities, with the lowest transpiration rate noted at 600 μmol/m^2·s. These results suggest that HXK moderates the stomatal response to light intensity and may even increase stomatal opening at low light intensities.

The Effect of Humidity on the Transpiration Rate of GCHXK Leaves

It is assumed that HXK within guard cells stimulates stomatal closure in response to the amounts of sugars that are carried to guard cells by the transpiration stream. We hypothesized that under low-humidity conditions (high VPD), the transpiration rate increases transiently and more sugars are carried toward the guard cells, which may enhance the closure effect in GCHXK stomata and reduce the transpiration rate. To examine this hypothesis, we exposed rooted WT and rooted GCHXK leaves to high (70%) and low (35%) levels of humidity and measured their respective transpiration rates. Under high-humidity conditions, the transpiration rates of WT and GCXK leaves were similar, but, at the low level of humidity, the transpiration rate of GCHXK was significantly lower than that of the WT (**Figure 8**). These results support our hypothesis that VPD is a central component of the stomatal-closure response mediated by HXK, such that a high transpiration rate occurring under high VPD conditions may accelerate HXK-mediated stomatal closure and reduce transpiration.

DISCUSSION

The current study was motivated by a previous study in which we showed that HXK within guard cells mediates stomatal closure in *Arabidopsis* and tomato (Kelly et al., 2013). The results of the current study support our hypothesis that stomatal closure is mediated by HXK within guard cells and that this is the case not only in annual plants, but in perennial plants as well. HXK is the only enzyme that can phosphorylate glucose in plants (Granot et al., 2013) and, therefore, we would expect to find it in all plant species, including citrus plants. In fact, several citrus HXK mRNAs appear in databases (Supplementary Table S1) and HXK activity has been detected in citrus leaves (Lu et al., 2014).

The observed stomatal-closure effect was stronger in citrus plants expressing *AtHXK1* in their guard cells (GCHXK plants) and the transpiration rate of intact GCHXK plants was lower

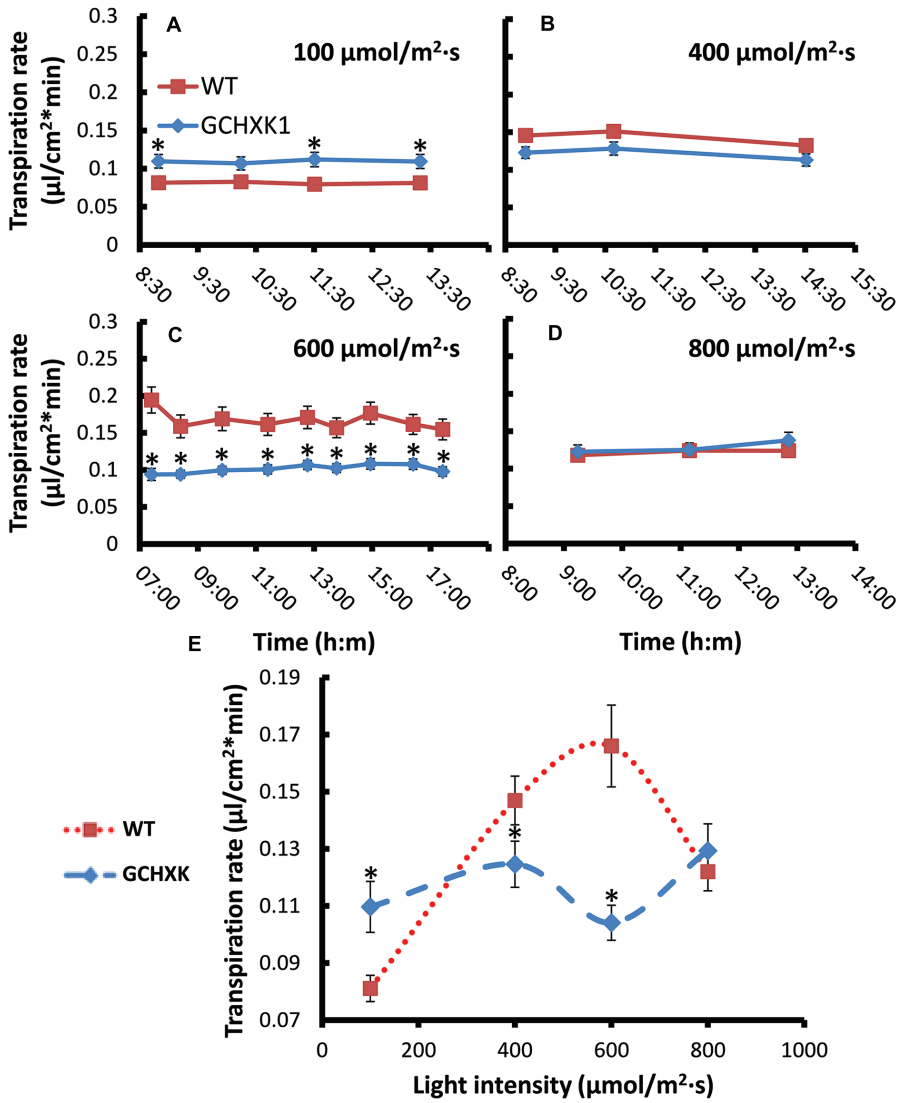

FIGURE 7 | Transpiration rates of WT and GCHXK leaves under different light intensities. (A–D) Transpiration rate per unit leaf area of rooted WT and rooted GCHXK leaves at different light intensities **(A)** 100 µmol/m^2 s. **(B)** 400 µmol/m^2 s. **(C)** 600 µmol/m^2 s **(D)** 800 µmol/m^2 s.measured throughout the day. The transpiration rate per unit leaf area was calculated based on the weight loss noted between two consecutive measurements divided by leaf area. Data are means ± SE from at least 11 and 13 repeats of WT and GCHXK, respectively. **(E)** A plot of the mean transpiration rates versus light intensity. Data are daily means ± SE from at least 11 and 13 repeats of WT and GCHXK, respectively. The asterisks denote significant differences relative to the WT (*t*-test; $P < 0.01$).

than that observed in WT plants primarily in the middle of the day when light intensity and sugar production are probably high (**Figure 4**). The transpiration rate of rooted GCHXK leaves was also lower than that of rooted WT leaves at optimal light intensity, indicating a link between sugar production, HXK and stomatal closure. The link between sugar, HXK and stomatal closure could be part of a natural mechanism to coordinate photosynthesis with transpiration. It has been shown that sucrose concentrations increase in leaves over the course of the photoperiod (Blasing et al., 2005; Comparot-Moss et al., 2010). It has also been shown that, in the apoplastic loader *Vicia faba*, some of the sugar produced over the course of the photoperiod is carried by the transpiration stream and accumulates in the vicinity of the guard cells (Lu et al., 1995; Outlaw and De Vlieghere-He, 2001). Citrus is an apoplastic loader in which sucrose produced in the mesophyll cells is exported to the apoplast prior to being loaded into the phloem (Lowell et al., 1989; Etxeberria et al., 2005; Hijaz and Killiny, 2014). The amount of apoplastic sucrose carried by the transpiration stream and arriving at the guard cells is probably increased when the rates of photosynthesis and transpiration are high.

GCHXK plants exhibit less transpiration than WT plants, especially in the middle of the day (between 9 a.m. and 3 p.m.; **Figure 4**) when transpiration rates are high and more sucrose is supposedly being carried toward the guard cells. Our

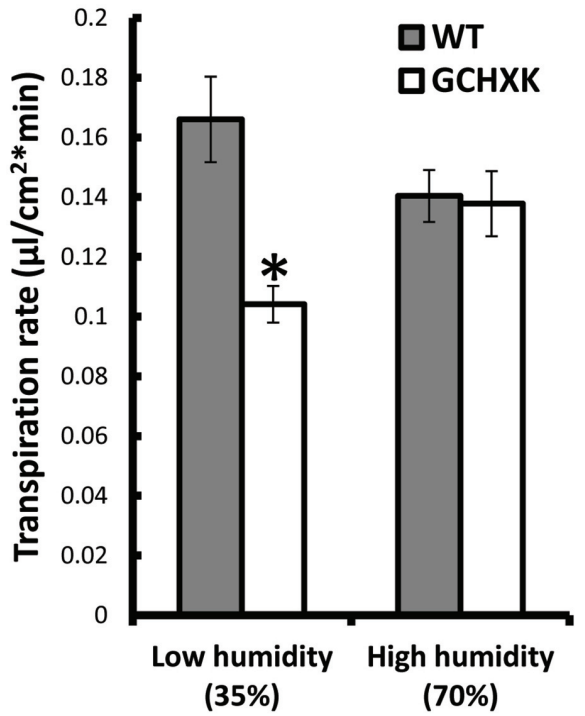

FIGURE 8 | The effect of humidity on the transpiration rate of GCHXK leaves. Mean daily transpiration rates of WT and GCHXK leaves were measured under low-humidity (35%) and high-humidity (70%) conditions. Data are means ± SE from 8 biological repeats of WT and GCHXK leaves. The asterisks denote significant differences relative to the WT at low-humidity (*t*-test; $P < 0.01$).

observation that low humidity also reduces the transpiration of GCHXK rooted leaves (**Figure 8**) further supports the hypothesis that sugars carried by the transpiration stream are sensed by HXK within guard cells and close stomata. At low humidity levels, the large difference in water potential between the outside and inside of the leaf (high VPD) is bound to accelerate transpiration, so that more sugar is carried toward the guard cells, which would eventually reduce the stomatal apertures and transpiration of GCHXK (Outlaw and De Vlieghere-He, 2001).

The effect of low humidity on stomatal closure and gene expression has been tested in *Arabidopsis* and, in that study, few sugar-cleaving enzymes and sugar transporters were up-regulated (Bauer et al., 2013). Based on the previous hypothesis of sugars as osmolytes that open stomata, the authors of that study suggested that low humidity accelerates the export of sugars from the guard cells, to reduce guard cell osmolarity and close stomata (Bauer et al., 2013). However, our results may suggest that sugars are imported into guard cells when the humidity level is low, which would stimulate stomatal closure.

In spite of its effects on stomatal closure, the expression of *AtHXK1* in guard cells had no negative effect on plant growth and development, but rather improved growth and increased *WUEi* (**Figures 2** and **3**; Kelly et al., 2013), indicating that the total amount of sugars produced over the course of the day was not impaired. Previous efforts to reduce water loss by manipulating the number of stomata or stomatal responses led to increased WUE, but frequently reduced growth and yield, probably due to an unbalanced reduction in CO_2 uptake, which lowered photosynthesis (Boyer, 1982; Condon et al., 2004; Blum, 2005; Dow et al., 2014; Kim et al., 2014; Roche, 2015). We assume that in the case of GCHXK, AtHXK1 within the guard cells accelerates stomatal closure only when sugar production exceeds the plant's phloem-loading and transport capacities (Nikinmaa et al., 2013), which would explain why no negative effect on plant growth was observed. We also suggest that a surplus of sugars may serve as a signal to close stomata, reduce CO_2 uptake and temporarily decrease the rate of photosynthesis. When transpiration decreases and sugar levels drops, stomata re-open and the rate of photosynthesis increases, thereby balancing sugar levels with the rate of transpiration.

Sucrose must be cleaved outside or inside the guard cells to be sensed by HXK. Only two groups of enzymes can cleave sucrose in plants, invertases (INV) and sucrose synthases (SUS). Apoplastic or intracellular INV cleave sucrose into glucose and fructose while intracellular SUS cleaves sucrose into UDP-glucose and fructose. The hexose monomers, glucose and fructose, are both substrates of HXK, but the affinity of HXK to glucose is two orders of magnitude higher than its affinity to fructose (Granot, 2007). Several studies have been conducted in recent years to explore the role of sugars in stomatal gene expression and movement (Antunes et al., 2012; Bates et al., 2012; Daloso et al., 2015a,b). A few studies have suggested that sucrose metabolism contributes to stomatal opening, perhaps through energy production rather than an osmotic effect (Antunes et al., 2012; Daloso et al., 2015a,b). The findings of those studies may seem to conflict with our observation of the closure effect of sugars, yet it is important to note that those studies focused on the opening stage of stomata. Furthermore, these studies show that expression of SUS in guard cells contributes to the opening (Daloso et al., 2015a,b). SUS cleaves sucrose to fructose and UDP-glucose and yields no glucose, the preferred substrate of HXK (Granot, 2007). Nevertheless, our work does not exclude and even support the possibility that sucrose metabolism may yield energy required for the opening of stomata (see below about the effect of low light intensity).

Another study explored the effect of trehalase, an enzyme that cleaves trehalose (a glucose–glucose disaccharide) on stomatal movement, and found that increased expression of trehalase reduces stomatal aperture (Van Houtte et al., 2013). The findings of that study are in line with our observation that HXK closes stomata, since trehalose cleavage yields glucose monomers, which are the primary substrate of HXK (Granot, 2008).

The rooted-leaves system provided an accurate, controlled and easy-to-manipulate biological setup for reproducible physiological experiments under uniform, stable conditions. Once harvested from the trees, the citrus leaves did not expand any further and their size remained constant throughout the rooting period and afterward. Accordingly, the rooted-leaves set-up proved to be a reliable source of experimental data.

Rooting of citrus leaves was done in the past to explore vegetative propagation following the cutting of the midribs of lemon leaves. In those cases, roots were regenerated from the cut and shoot regeneration was sometimes observed (Salomon and Mendel, 1964). However, to the best of our knowledge, no further use was made of those rooted leaves (Salomon and Mendel, 1964).

The consistent conditions in the growth chamber (temperature, humidity, and light intensity) are probably the reason for the fairly consistent transpiration rates of the WT and GCHXK over the course of the day. Yet, the rooted GCHXK leaves were less affected by light intensity than the WT leaves. While at low light intensity (100 $\mu mol/m^2 \cdot s$), the transpiration of GCHXK was higher than that of the WT rooted leaves, at the optimal light intensity of 600 $\mu mol/m^2 \cdot s$ (Pimentel et al., 2004), the transpiration rate of GCHXK was significantly lower than that of the WT (**Figure 7**). Stomatal opening is energy-dependent and requires the activation of proton ATPases (Shimazaki et al., 2007). We, therefore, assume that at a low light intensity, ATP generated from glucose metabolism following the phosphorylation of glucose by HXK may provide energy that accelerates the opening of stomata of GCHXK leaves, in line with other studies that have suggested that sucrose metabolism provides energy for the opening of stomata (Daloso et al., 2015a,b). At a high light intensity, on the other hand, the excess of sugar might be sensed by HXK and stimulate stomatal closure. Thus, HXK may have both opening and closing functions, which are dependent on the level of sugars.

Two different growth strategies have generally been associated with woody (perennial) and non-woody (annual) plants. Annual plants have higher rates of photosynthesis and higher stomatal conductance and *WUEi*, perhaps to accommodate their short life spans (Gago et al., 2014). In that regard, it could have been thought that perennials might be less sensitive to stomatal regulation by sugars and HXK. Yet, studies with robusta coffee (*Coffea canephora* – a perennial plant) have shown that shade (low light intensity) increases stomatal conductance (g_s) and high light intensity reduces (g_s), supporting our hypothesis that sugars might regulate and reduce stomatal aperture also in perennial species (Rodríguez-López et al., 2013). No studies dealing with direct effects of sugars on perennial stomata could be found and this is probably the first study to show that HXK stimulates stomatal closure in trees.

CONCLUSION

The scientific understanding of the role of sugars in the regulation of guard-cell behavior has been revised over the course of the last century. Originally, sugars were considered the major osmolytes that open stomata and recent studies suggest that sugar metabolism contributes to stomatal opening. Yet, our study and others have shown that sugar stimulates stomatal closure, thereby coordinating photosynthesis with transpiration (Kelly et al., 2013; Lawson et al., 2014; Li et al., 2015). A previous study demonstrated that HXK within guard cells mediates stomatal closure in the annual species *Arabidopsis* and tomato. The current study shows that HXK also mediates stomatal closure in citrus trees, suggesting that this might be a widespread mechanism for coordinating photosynthesis with transpiration.

AUTHOR CONTRIBUTIONS

NL, GK, ER, NC, and DG planned and designed the research. NL, GK, and DG wrote the manuscript. NL, GK, LF, YY, ZA, and AL performed experiments. NL, GK, AL, VA, MM, ER, NC and DG analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01114

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REVIEW
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MAPK Cascades in Guard Cell Signal Transduction

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Guard cells form stomata on the epidermis and continuously respond to endogenous and environmental stimuli to fine-tune the gas exchange and transpirational water loss, processes which involve mitogen-activated protein kinase (MAPK) cascades. MAPKs form three-tiered kinase cascades with MAPK kinases and MAPK kinase kinases, by which signals are transduced to the target proteins. MAPK cascade genes are highly conserved in all eukaryotes, and they play crucial roles in myriad developmental and physiological processes. MAPK cascades function during biotic and abiotic stress responses by linking extracellular signals received by receptors to cytosolic events and gene expression. In this review, we highlight recent findings and insights into MAPK-mediated guard cell signaling, including the specificity of MAPK cascades and the remaining questions.

Keywords: MAPK cascade, stomatal pore, guard cell development, stomatal movement, environment

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INTRODUCTION

During photosynthesis, the gas exchange and water transpiration between the plant and the atmosphere take place through the microscopic pores surrounded by guard cells in the epidermis, called stomata (Schroeder et al., 2001). Open stomata help in CO_2 absorption but also render plants vulnerable to dehydration and pathogen attacks (Schroeder et al., 2001; Nilson and Assmann, 2007; Gudesblat et al., 2009). Precise regulation of stomatal aperture in response to endogenous and environmental stimuli, such as light, CO_2, temperature, hormones, drought, and pathogens, is crucial for plant growth and survival. To achieve this, guard cells integrate developmental and environmental stimuli into the signaling networks, which include mitogen-activated protein kinase (MAPK) cascades.

Mitogen-activated protein kinase cascades are composed of at least three kinases: MAPK kinase kinases (MAP3Ks), MAPK kinases (MAP2Ks or MKKs), and MAPKs (for which we have adopted MPK). These enzymes are highly conserved in all eukaryotes, and they play crucial roles in diverse developmental and physiological processes (Mapk-Group, 2002; Rodriguez et al., 2010). In general, signals received by receptors are transduced to MAP3Ks, which evoke subsequent phosphorylation events. MAP3Ks are Ser/Thr kinases and phosphorylate MAP2Ks on two Ser/Thr residues (S/T-XXXXX-S/T, X denotes any amino acid). MAP2Ks are dual-specificity protein kinases that phosphorylate MPKs on Thr and/or Tyr residues in the TXY motif. Phosphorylated MPKs transduce the signals to various effector molecules regulating their functional activity, turnover, and localization.

In plants, MAPK pathways function in developmental processes, immune response, abiotic stress responses, and hormonal regulation (Liu et al., 2010; Rodriguez et al., 2010; Moustafa et al., 2014; Arnaud and Hwang, 2015). The multi-functionality of MAPK cascades in cellular signaling

is probably due to the fact that MPKs, MAP2Ks, and MAP3Ks are encoded by gene families with large numbers of genes (Mapk-Group, 2002). In the *Arabidopsis* genome, approximately 110 genes encode MAPK cascade components: 20 MPKs, 10 MAP2Ks, and 80 MAP3Ks (Jonak et al., 2002; Mapk-Group, 2002; Menges et al., 2008). MAP3Ks form the largest family of MAPK cascades with three clades: MEKKs (MAP3K1-21), RAF-like (RAF1-48), and ZIK-like (ZIK1-11). The members of these gene families could form different combinatorial MAPK cascades, allowing the cells to deal with diverse stimuli, possibly in a stimulus-specific manner. Identifying functional, stimulus-specific MAPK modules out of thousands of possible combinations has long been a goal, which has begun to be achieved, at least in part, by systematical approaches (Asai et al., 2002; Lee et al., 2008; Umezawa et al., 2013). In addition to the classical three-tiered kinase cascades, there are 10 genes in the *Arabidopsis* genome encoding MAP3K kinases, but their implication on MAPK cascades is still unclear.

In this review, we highlight recent findings for MAPK-mediated guard cell signaling and discuss the specificity of MAPK cascades and questions that await answers.

MAPK CASCADES IN ABA-INDUCED STOMATAL CLOSURE

ABA regulates stomatal movements in response to biotic and abiotic stresses. ABA signaling received by the ABA receptor PYR/PYL/RCAR is transduced to activate SNF1-related protein kinase 2 (SnRK2) (Ma et al., 2009; Park et al., 2009; Soon et al., 2012). This initiates subsequent cellular events including the production of reactive oxygen species (ROS) and nitric oxide, elevation of cytosolic Ca^{2+} levels, cytosolic alkalization, activation of anion and calcium channels, and loss of guard cell turgor (Lim et al., 2015). In addition, a MAPK activity has been detected in guard cell protoplasts treated with ABA (Mori and Muto, 1997). A pharmacological study using the MAP2K inhibitor PD98059 in pea epidermal peels has shown inhibition of ABA-induced stomatal closure and ABA-inducible dehydrin gene expression (Burnett et al., 2000). These biochemical and pharmacological studies suggest that MAPK cascades are involved in ABA signaling in guard cells.

Spatial expression patterns of genes provide some hints to their roles where they are expressed. Gene regulation in response to certain stimuli indicates the role of these genes in dealing with the stimuli. A proteomics study of guard cell proteins has shown that MPK4, MPK9, MPK12, and MKK2 proteins are present in *Arabidopsis* guard cells (Zhao et al., 2008). ABA upregulates the expression of *MPK3*, *MPK5*, *MPK7*, *MPK18*, *MPK20*, *MKK9*, *MAP3K1*, *MAP3K10* (*MEKK3*), *MAP3K14*, *MAP3K15*, *MAP3K16*, *MAP3K17*, *MAP3K18*, and *MAP3K19* (Menges et al., 2008; Wang et al., 2011). A cell type-specific transcriptomics analysis has revealed that *MPK4*, *MPK5*, *MPK9*, *MPK11*, *MPK12*, *MPK17*, and *MPK19* are highly expressed relatively in guard cells (Leonhardt et al., 2004; Jammes et al., 2009). Eleven of the 80 MAP3K genes appear to be highly expressed relatively in *Arabidopsis* guard cells: *MAP3K11* (*MEKK4*), *RAF6*, *RAF15*, *RAF17*, *RAF19*, *RAF22*, *RAF29*, *RAF33*, *RAF34*, *RAF40*, and *ZIK4* (Leonhardt et al., 2004). In contrast, *MAP2K* genes seem to be expressed in these cells at very low levels (Leonhardt et al., 2004). Combined analysis of spatial expression patterns and ABA regulation of the MAPK cascade genes could contribute to deciphering specific MAPK cascades involved in ABA signaling in guard cells. Interestingly, antisense suppression of *MPK3* (in a guard cell-specific manner) results in impaired ABA inhibition of stomatal opening and H_2O_2-induced stomatal closure. However, ABA-induced stomatal closure and ABA-induced H_2O_2 production are not affected (Gudesblat et al., 2007). Lu et al. (2002) have reported that MPK3 is activated by ABA and H_2O_2, suggesting its role in ABA signaling. However, *MPK3* expression in guard cells has not been examined by Gudesblat et al. (2007). Thus, it is unclear whether the stomatal phenotype in the antisense of *MPK3* plants is because of the suppression of *MPK3* in guard cells or suppression of other member(s) of the MPK gene family expressed in guard cells which are closely related to *MPK3*.

A cell type-specific functional genomics approach has revealed a high and preferential expression of two MPK genes, *MPK9* and *MPK12* in guard cells (Jammes et al., 2009). *Arabidopsis* mutants with mutations in both *MPK9* and *MPK12* show reduced ABA promotion of stomatal closure, ABA inhibition of stomatal opening, impaired ABA and calcium activation of anion channels, and enhanced transpiration water loss in the leaves (Jammes et al., 2009). MPK12 kinase activity is enhanced by ABA and H_2O_2 treatment. MPK9 and MPK12 show functional overlap and act downstream of calcium and upstream of anion channels in ABA signaling (Jammes et al., 2009). Another study has reported that MPK9 and MPK12 function in ABA-induced stomatal closure, whereas MPK3 and MPK6 function in flg22-induced stomatal closure (Montillet et al., 2013). An independent quantitative trait locus analysis of natural accessions of *Arabidopsis* has shown that MPK12 carrying an amino acid substitution causes reduction in water use efficiency (Des Marais et al., 2014). The effect MPK12 has on water use efficiency is not shared by MPK9, unlike the overlapping functions of these enzymes in ROS-mediated stomatal closure in response to ABA. This result suggests that all functions of MPK12 are not redundant (Des Marais et al., 2014). It would be interesting to identify substrates of MPK9 and MPK12 in these cellular processes, which would provide further insights into MPK9- and MPK12-mediated guard cell signaling. Moreover, which specific MAP2K and MAP3K participate in the complete cascade involving MPK9 and MPK12 in ROS-mediated ABA signaling and in water use efficiency remains to be established. Further studies are required to determine the detailed mechanism by which these MAP kinases regulate anion channel activity and stomatal closure.

Besides MPK9 and MPK12, the MKK1–MPK6 module positively regulates *CATALASE1* expression and ABA-induced H_2O_2 production in guard cells (Xing et al., 2008). However, reduced ROS production in single *mpk6* mutants does not impair the stomatal closure in response to ABA (Montillet et al., 2013). Hydrogen peroxide activates the ANP1 (MAP3K1) activity, which subsequently leads to phosphorylation and activation of

MPK3 and MPK6 (Kovtun et al., 2000). However, it is not clear whether ANP1-initiated MAPK cascades act in ROS-mediated guard cell signaling.

In guard cells, the soluble PYR/PYL/RCAR ABA receptors bind ABA and interact with the inhibitory protein phosphatases 2C (PP2Cs), which results in release of the active form of SnRK2 protein kinases (Ma et al., 2009; Park et al., 2009; Soon et al., 2012). In addition to other SnRK2.6 substrates, Ser13 and Ser174 on NADPH oxidase RbohF are phosphorylated by SnRK2.6 (Sirichandra et al., 2009). Interestingly, a phosphoproteomics study has shown that MPK1 and MPK2, which are activated by ABA and H_2O_2 (Ortiz-Masia et al., 2007), are phosphorylated in a SnRK2-dependent manner (Umezawa et al., 2013). ABA dependent phosphorylation of MPK1 and MPK2 is reduced in *snrk2.2/2.3/2.6* triple mutants, suggesting that SnRK2 promotes the activation of MPK1 and MPK2 by ABA (Umezawa et al., 2013). In addition, MPK1 and MPK2 have been identified as a part of ABA-activated MAPK modules, MAP3K17/18-MAP2K3-MPK1/2/7/14 (Danquah et al., 2015). Interestingly, MAP3K18 is expressed in guard cells and has been recently shown to be regulated by the ABI1 protein phosphatase, suggesting its role in ABA signaling in guard cells. Indeed, *map3k18* mutants show an increase in stomatal aperture under normal growth conditions as well as in response to ABA treatment, compared with the wild-type (Mitula et al., 2015). However, *map3k18* mutants have significant reduction in the stomatal index, which could affect stomatal apertures (Bussis et al., 2006; Franks et al., 2015). Thus further studies are required to determine whether and how ABA activation of MAP3K18 leads to stomatal closure.

MAPK CASCADES IN IMMUNE RESPONSE IN GUARD CELLS

Open stomata make the plants vulnerable to microbial invasion and closing mechanisms have been evolved to prevent stomatal pores from being used as a gate for pathogens by rapidly closing the pores upon pathogen recognition (Melotto et al., 2006). Pathogen-induced stomatal closure is triggered by pathogen/microbe-associated molecular patterns (PAMPs or MAMPs). Various molecules are involved in this response: oligogalacturonic acid, chitosan, flg22 (a peptide derived from bacterial flagellin), and lipopolysaccharide (Lee et al., 1999; Klusener et al., 2002; Melotto et al., 2006; Gudesblat et al., 2009). PAMPs registered by the host pattern-recognition receptors initiate a variety of defense responses. These responses include the production of ROS and nitric oxide, elevation of cytosolic Ca^{2+} levels, activation of salicylic acid signaling pathway, synthesis of ethylene, and stomatal closure (Arnaud and Hwang, 2015).

Mitogen-activated protein kinase cascades confer another line of defense mechanism by regulating the activation of defense genes, synthesis of antimicrobial metabolites, and hypersensitive response-like cell death (Zhang and Klessig, 2001; Asai et al., 2002; Pedley and Martin, 2005). Recent studies have found that major regulators of innate immune response such as MPK3, MPK4, and MPK6 also participate in the stomatal defense. Antisense suppression of *MPK3* in a guard cell-specific manner causes impaired stomatal closure in response to bacteria, lipopolysaccharide, and stomatal inhibiting factor from bacterial phytopathogen *Xanthomonas campestris* pv *campestris* (Gudesblat et al., 2009). MPK3 and MPK6 activate guard cell-specific lipoxygenase, LOX1 that contributes to the synthesis of a large number of oxylipins thereby closing stomatal pores in response to both bacteria and flg22 (Montillet et al., 2013). MPK3- and MPK6-mediated stomatal closure in response to pathogen infection is independent of ABA, as *mpk3* and *mpk6* single mutants show normal stomatal closure in response to this compound (Montillet et al., 2013).

The role of MPK4 appears to be distinct from those of MPK3 and MPK6 in stomatal immune response. *mpk4* mutant plants display enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato (Pst)* (Petersen et al., 2000). Transgenic plants that harbor a constitutively active form of *MPK4* (*CA-MPK4*) have compromised disease resistance and are more susceptible to *Pst* infection than normal plants. However, the resistance of *CA-MPK4* plants is not weakened when bacteria are injected directly into the leaf apoplast. As *MPK4* is expressed in guard cells (Petersen et al., 2000), it has been suggested that it mediates stomata-based defense against bacterial entry (Berriri et al., 2012). However, the stomatal closure in response to *Pst* DC3000 and flg22 in *CA-MPK4* transgenic plants and in wild-type plants showed no difference. This implies that the defense function of MPK4 is not linked to the regulation of stomatal apertures (Berriri et al., 2012). Interestingly, *MPK4* homologs in *Nicotiana attenuata* and *N. tabacum* positively regulate stomatal closure in response to biotic and abiotic stresses (Gomi et al., 2005; Marten et al., 2008; Hettenhausen et al., 2012). These differences might be species specific, and further investigation is necessary to clarify the mechanism by which MPK4 enzymes regulate stomatal closure.

Two *Arabidopsis* guard cell MPK genes, *MPK9* and *MPK12*, regulate stomatal apertures in response to biotic stimuli. *mpk9mpk12* double mutants are highly susceptible to *Pst* when the pathogen is sprayed on the leaves (Jammes et al., 2011). Moreover, in *mpk9mpk12* double mutants, but not in *mpk9* or *mpk12* single mutants, the methyl jasmonate-induced stomatal closure is impaired. This indicates a functional redundancy of these two genes in the methyl jasmonate signaling in guard cells (Khokon et al., 2015). Anion channel activation by methyl jasmonate is impaired in *mpk9mpk12* double mutants, suggesting that MPK9 and MPK12 act upstream of anion channels during stomatal closure (Khokon et al., 2015). Two other studies have shown that yeast elicitor- and chitosan-induced stomatal closure are defective in *mpk9mpk12* double mutants (Salam et al., 2012, 2013). Interestingly, flg22-induced stomatal closure is normal in these double mutants (Montillet et al., 2013). Overall, these studies show that ABA, methyl jasmonate, and biotic stimuli converge on MPK9 and MPK12 in guard cells (**Figure 1**). Identification of target proteins of MPK9 and MPK12 would

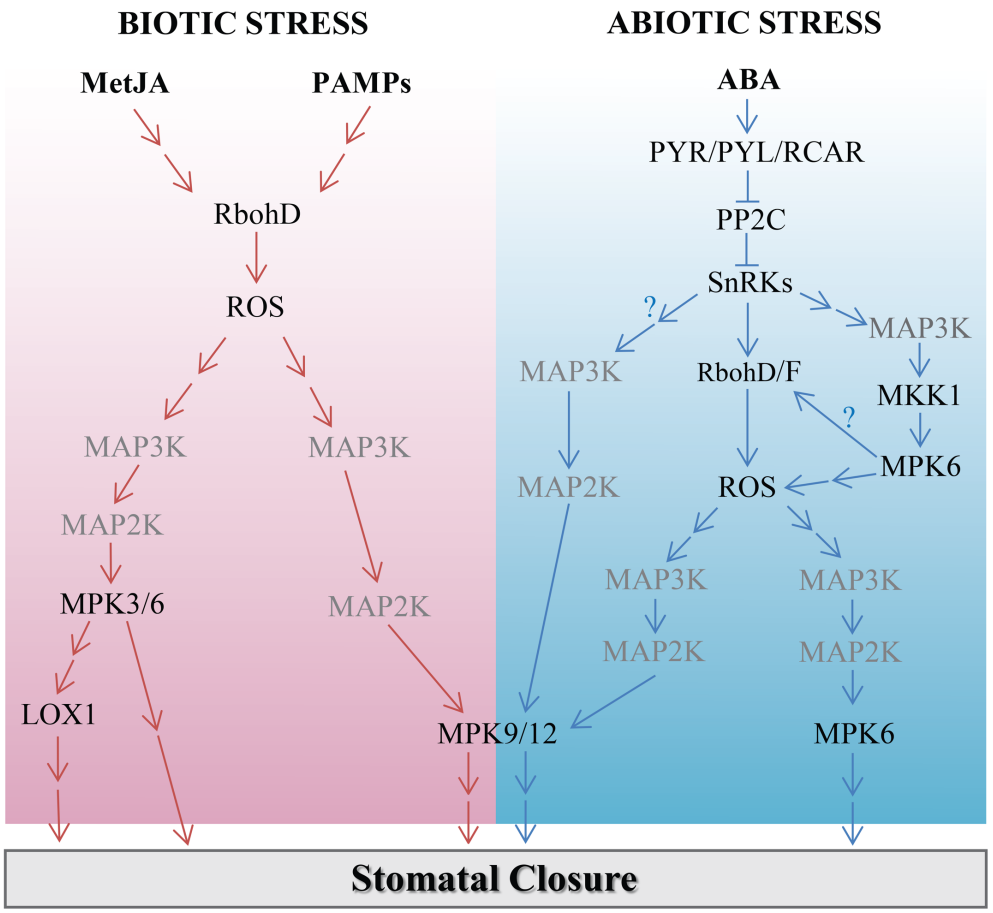

FIGURE 1 | Working model of mitogen-activated protein kinase (MAPK) cascades regulating stomatal movement in response to biotic and abiotic stimuli. Unidentified MAPK components are presented in gray color. MAPK cascades waiting to be revealed are indicated in arrows with a question mark.

broaden our understanding of MAPK-mediated signaling in guard cells.

MAPKS IN STOMATAL DEVELOPMENT

Not only guard cell signaling but also the development of stomata, including stomatal density (number of stomata per mm^2 of leaf) and stomatal index (ratio of stomata to epidermal cells), are tightly regulated in response to various developmental and environmental stimuli (Lake et al., 2001). For instance, high CO_2 concentration and low intensity light decrease both stomatal index and density, whereas high intensity light increases these values (Schoch et al., 1980; Lake et al., 2001; Chater et al., 2015).

Stomatal development is accomplished by a series of asymmetric cell divisions, differentiation, and then a symmetric division that generates a pair of guard cells. These processes require a ligand–receptor module, MAPK signaling cascades, and nuclear transcription factors (Lau and Bergmann, 2012). In these developmental processes, MAPK pathways integrate the intrinsic signal from the ligand–receptor module and transduce the signal to the transcriptional factors to allow stomatal development and patterning.

Involvement of MAPK signaling in stomatal development has been examined in a study of the MAP3K YODA (Bergmann et al., 2004). *yoda* mutants have a large number of clustered guard cells, whereas constitutive activation of YODA results in stomataless phenotypes. Stomatal clusters in *yoda* mutants, consisting of paired guard cells, are different from the clusters of unpaired guard cells formed by repeated divisions of the guard mother cells in *four lips (flp)* mutants (Yang and Sack, 1995). This suggests that increased density of guard cells in *yoda* mutants is due to the defective asymmetric cell division before the differentiation into guard mother cells.

The downstream cascades of YODA include MKK4/MKK5 and MPK3/MPK6 (Wang et al., 2007). MKK4/MKK5 and MPK3/MPK6 act as negative regulators of stomatal patterning by controlling asymmetric cell divisions and differentiation of stomatal cells. *mkk4mkk5* or *mpk3mpk6* knockdown mutants have clustered guard cells. Stomatal differentiation is suppressed in transgenic plants expressing constitutively active forms of MKK4 and MKK5 (Wang et al., 2007). In addition,

stress-associated MKK7 and MKK9 have functions overlapping with those of MKK4 and MKK5, thus activating MPK3/MPK6 and preventing meristemoids from entering the stomatal lineage (Lampard et al., 2009). At later stages of stomatal development, however, YODA–MKK7/9 modules promote stomatal development in guard mother cells in a cell-type specific manner. In addition to YODA, a recent study shows ABA-activated MAP3K18 acts as a positive regulator of stomatal development (Mitula et al., 2015). These results suggest that stomatal development regulated by MAPK cascades is not a simple, linear process. The complex regulatory mechanisms of this process are yet to be elucidated.

Recent studies have revealed a broad range of downstream targets of MAPK cascades in stomatal development. Breaking of Asymmetry in the Stomatal Lineage (BASL) is one of them, and phosphorylation of BASL by MPK3/MPK6 is required for its polarized localization (Zhang et al., 2015). *basl* mutants lack asymmetric divisions, resulting in daughter cells with similar size and identity (Dong et al., 2009). BASL localizes initially in the nucleus and begins to accumulate in a cortical crescent before the asymmetric cell division. This polarized localization pattern is inherited only by a larger daughter cell, which becomes a non-stomatal lineage cell. BASL has five serine residues phosphorylated by MPK3 and MPK6 (Zhang et al., 2015). When these residues are modified to Ala, BASL is sequestered in the nucleus and the stomatal defects of *basl* mutants do not rescued in complementation lines (Zhang et al., 2015). This observation suggests that MPK3/6 -mediated phosphorylation of BASL is required for the transfer of this protein to the cortical pool; this localization is critical for BASL function in a polarity module. Interestingly, phosphorylated BASL strongly interacts with YODA to recruit it into the cell cortex (Zhang et al., 2015). These results indicate that a biased spatial regulation of BASL by the MAPK cascades through the positive feedback loop determines the fate of daughter cells during cell division.

Transcription factors are other downstream targets of MAPK cascades in stomatal development. Three basic helix–loop–helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA, are necessary for three critical steps of stomatal development: initiation by SPCH, meristemoid differentiation by MUTE, and final guard cell differentiation by FAMA (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). *In vitro* phosphorylation analysis has shown that SPCH is a direct phosphorylation target of MPK3 and MPK6. Constitutive expression of the mutated *SPCH* without MAPK target domains induces the formation of excess stomata (Lampard et al., 2008). Genetic analyses have revealed that MPK3 and MPK6 negatively regulate SPCH function by altering persistence of SPCH (Lampard et al., 2008). Moreover, MUTE and the MYB transcription factor MYB88 are phosphorylated by MPK6 and MPK4 *in vitro*, respectively (Popescu et al., 2009). These findings indicate that MPK3, MPK4, and MPK6 have targets in both the nucleus and cytosol. Thus, it would be interesting to investigate how the localization of these MAPK proteins is controlled upon different stimuli.

MAPK PHOSPHATASES IN GUARD CELL SIGNALING

Mitogen-activated protein kinase cascades activated by sequential events of phosphorylation can be reversed by dephosphorylation. This process is mediated by protein phosphatases including Tyr-specific phosphatases, Ser/Thr phosphatases, and dual-specificity phosphatases (Brock et al., 2010). Regulation of the duration of MAPK activities has been suggested as one of the mechanisms to render specificity and fine-tune the MAPK-mediated signal transduction (Ebisuya et al., 2005; Murphy and Blenis, 2006).

Mitogen-activated protein kinase phosphatases (MKPs) belong to a group of specialized dual-specificity phosphatases, which dephosphorylate both Ser/Thr and Tyr residues, and act as negative regulators of MAPKs (Camps et al., 2000). The *Arabidopsis* genome encodes five potential MKPs (MKP1, MKP2, DsPTP1, PHS1, and IBR5; Kerk et al., 2002). They regulate the activities of components of MAPK cascades in various stress signaling pathways (Ulm et al., 2002; Lee and Ellis, 2007; Schweighofer et al., 2007; Bartels et al., 2009; Lee et al., 2009; Brock et al., 2010). Indole-3-Butyric acid-Response 5 (IBR5) dephosphorylates MPK12 through direct interaction and functions in auxin and ABA signaling (Lee et al., 2009). MKP1 and Protein Tyrosine Phosphatase 1 interact with MPK6 and negatively regulate MPK3/MPK6-mediated stress responses (Ulm et al., 2002; Bartels et al., 2009). MKP2 dephosphorylates *Arabidopsis* MPK3 and MPK6 *in vitro*, and *mkp2*-knockdown plants exhibit enhanced sensitivity to ozone stress (Lee and Ellis, 2007). However, it remains unclear whether those enzymes are also involved in stomatal movement.

A recent study of *OsIBR5*, the closest homolog of *AtIBR5*, has supplied a clue to the involvement of MKP in stomatal movements (Li et al., 2012). Tobacco plants overexpressing *OsIBR5* show impaired stomatal closure in response to drought and ABA and are hypersensitive to drought and oxidative stresses. These suggest that OsIBR5 has a role as a negative regulator in ABA- and drought-mediated response. Interestingly, *OsIBR5* is upregulated in response to ABA and H_2O_2 in rice seedlings, suggesting a regulatory feedback mechanism between MAPKs and MKPs. OsIBR5 interacts with two tobacco MAPKs: SIPK and WIPK, and the drought-inducible kinase activity of WIPK is suppressed in *OsIBR5*-overexpressing plants. Though further studies are required to obtain detailed physiological functions of the OsIBR5–MAPK complexes in rice, these results clearly show the involvement of MKP in the regulation of stomatal apertures.

Besides the MKPs, PP2Cs utilize phosphorylated MAPKs as substrates. PP2C-type phosphatase AP2C1 inactivates the stress-responsive MPKs, MPK4, and MPK6 (Schweighofer et al., 2007). PP2C5 directly interacts with MPK3, MPK4, and MPK6 and inhibits their kinase activities induced by ABA (Brock et al., 2010). Both *pp2c5* and *ap2c1* single mutants show increased stomatal apertures. This phenotype is clearly pronounced in the

pp2c5ap2c1 double mutants, suggesting functional redundancy of the two genes (Brock et al., 2010). Interestingly, these double mutants do not affect the response to *Pst* DC3000 infection, indicating that a different regulatory mechanism is involved in pathogen-induced stomatal closure.

PERSPECTIVES AND CONCLUDING REMARKS

It is clear that MAPK cascades play important roles in the fine-tuning of complex cellular signaling networks in response to biotic and abiotic stimuli in plants (**Figure 1**). Although the MPK, MAP2K, and MAP3K gene families contain a large number of genes, the functions of only a handful of these genes have been identified in cell signaling and/or development in plants (**Figure 1**). This is probably largely due to the high level of functional redundancy of these genes. Therefore, it would be advisable to use biochemical and genetic approaches that can address the issue, including artificial miRNAs that simultaneously knockdown homologous genes with potential overlapping functions (Hauser et al., 2013), use of constitutively active forms of MAPKs (Berriri et al., 2012), and simultaneous targeting of homologous genes using the CRISPR/Cas9 system (Woo et al., 2015). A cell type-specific phosphoproteomics approach using genetic mutants could help to complete MAPK cascades; it could also provide the means to the classification of components of MAPK cascades mediating stimulus-specific response in guard cells.

AUTHOR CONTRIBUTIONS

YL and JK conceived the manuscript, and all authors contributed to writing the manuscript. All authors approved the final manuscript.

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REVIEW
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Protein Phosphorylation and Redox Modification in Stomatal Guard Cells

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Post-translational modification (PTM) is recognized as a major process accounting for protein structural variation, functional diversity, and the dynamics and complexity of the proteome. Since PTMs can change the structure and function of proteins, they are essential to coordinate signaling networks and to regulate important physiological processes in eukaryotes. Plants are constantly challenged by both biotic and abiotic stresses that reduce productivity, causing economic losses in crops. The plant responses involve complex physiological, cellular, and molecular processes, with stomatal movement as one of the earliest responses. In order to activate such a rapid response, stomatal guard cells employ cellular PTMs of key protein players in the signaling pathways to regulate the opening and closure of the stomatal pores. Here we discuss two major types of PTMs, protein phosphorylation and redox modification that play essential roles in stomatal movement under stress conditions. We present an overview of PTMs that occur in stomatal guard cells, especially the methods and technologies, and their applications in PTM identification and quantification. Our focus is on PTMs that modify molecular components in guard cell signaling at the stages of signal perception, second messenger production, as well as downstream signaling events and output. Improved understanding of guard cell signaling will enable generation of crops with enhanced stress tolerance, and increased yield and bioenergy through biotechnology and molecular breeding.

Keywords: phosphorylation, redox, guard cell, signaling, abiotic and biotic stresses

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INTRODUCTION

Stomata are composed of a pair of specialized epidermal cells termed guard cells, which are responsible for regulating gas exchange and water loss through changing the size of the stomatal pores. The opening and closing of stomatal pores are affected by numerous factors, such as humidity, CO_2, temperature, light, hormones, and pathogens. Changes in the turgor and volume of guard cells accordingly are required for stomatal movement, which are controlled by complex signaling networks (Azoulay-Shemer et al., 2015).

Abscisic acid (ABA) plays important roles in a broad range of plant physiological processes (e.g., seed germination and seedling growth) and plant responses to abiotic and biotic stresses (Lee and Luan, 2012). Under high salinity and drought conditions, the increased levels of ABA are perceived by the guard cells to promote stomatal closure and to inhibit of stomatal opening (Assmann, 2003). The mechanisms underlying ABA signaling in guard cells have been extensively studied (Pei et al., 1997; Schroeder et al., 2001; Assmann, 2003; Acharya et al., 2013; Zhang et al., 2015), which involve the binding of ABA to the receptors, activation of protein kinases, production of second messengers

such as reactive oxygen species (ROS) and nitric oxide (NO), regulation of membrane ion channels, and eventually the decrease in turgor and stomatal closure (Schroeder et al., 2001; Zhang et al., 2015). In addition to abiotic stress, guard cells play an important role in limiting pathogen entrance to the plant body. The guard cell response to bacteria is triggered by the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the plasma membrane. Upon PAMP recognition, one of the earliest responses is the change in ion fluxes across the membrane, leading to a rapid and transient extracellular alkalization and increase of Ca^{2+} in the cytosol (Boller and Felix, 2009). Ca^{2+} functions as a second messenger, activating downstream signaling players such as calcium-dependent protein kinases (CDPKs) to promote stomatal immunity responses. In addition, the apoplastic production of ROS by NADPH oxidase (Boller and Felix, 2009) is a hallmark of successful recognition of plant pathogens. Subsequent plant immune responses include transcriptional reprogramming, which involves the regulation of ROS homeostasis and activation of other protein kinases such as mitogen-activated protein kinases (MAPKs) (Boudsocq et al., 2010).

Stomatal studies are technically challenging because guard cells are small and of low abundance in leaves (Tallman, 2006). Methods for isolating guard cell protoplasts with relatively high purity have been reported over the past 30 years (Outlaw et al., 1981; Gotow et al., 1982, 1984; Zhu et al., 2010, 2014; Obulareddy et al., 2013). They have contributed considerably to the understanding of guard cell signaling. However, these methods are usually laborious and the yield is relatively low. The general principle of guard cell isolation is to release the guard cells from epidermal peels in a two-step process. In the first step the pavement and mesophyll cells are removed, and in the second step the guard cell wall is digested to facilitate the release of the guard cell protoplasts. It is important to note that there are important variations of the procedures according to different plant species (Zhu et al., 2016).

Stomatal movement in response to abiotic and biotic stresses is a fast process, which requires an efficient molecular regulation mechanism to relay the signals. Phosphorylation and redox control of the key players during both the signal perception and transduction in plant responses to abiotic and biotic stresses have demonstrated the high efficiency of protein PTMs in cell signaling (Grennan, 2007; Waszczak et al., 2015; Zhang et al., 2015). As the relevance of PTMs in plant stress responses has been demonstrated by independent studies over the years (Kodama et al., 2009; Lindermayr et al., 2010; Stecker et al., 2014; Kim et al., 2015; Yang et al., 2015), there is a growing interest to understand how specific PTMs control various aspects of stomatal guard cell functions. In this review, the frequently used approaches and methods in identification and quantification of PTMs are described. The main objective is to focus on the phosphorylation and redox events, and the recently identified proteins that undergo PTMs in guard cells in response to phytohormone and stress signals. We also discuss the different types of PTMs in the regulation of stomatal movement, and the challenges and perspectives of PTM proteomics.

ADVANCES IN PROTEIN PTM TECHNOLOGIES

Significance of PTMs in Biological Processes

PTMs include chemical modifications of specific amino acid residues of a protein and/or cleavage of the translated sequence. They greatly increase the structural and functional diversity of proteins in a proteome. Currently, more than 300 different types of PTMs have been identified (Zhao and Jensen, 2009), including phosphorylation, glycosylation, acetylation, nitrosylation, ubiquitination, and proteolytic cleavage. These modifications affect the properties of the proteins (e.g., charge status and conformation), resulting in changes of activity, binding affinity, localization as well as stability. Most PTMs are highly controlled in the cells, and they often serve as rapid, specific, and reversible molecular switches to regulate biochemical and physiological processes. Different PTMs have also been shown to crosstalk in the modulation of molecular interactions between proteins or regulation within the same protein through multiple site modification, e.g., the histone code (Bannister and Kouzarides, 2011). Therefore, identification and functional characterization of PTMs are critical toward deciphering their roles in cellular processes in many different areas of biology and biomedical research.

Qualitative Analysis of PTMs

In the past, PTMs were often studied at a specific amino acid residue of a particular protein level using molecular and biochemical approaches (Zhu et al., 2000; Reimer et al., 2002). Nowadays, the advances in biological mass spectrometry (MS) have allowed accurate identification and quantification of PTMs at the proteome scale. Two-dimensional gel electrophoresis (2-DE) was widely used in the early years of proteomics to identify PTMs, such as phosphorylation, nitrosylation, acetylation, and glycosylation (Llop et al., 2007; Roux et al., 2008; Scheving et al., 2012). Because PTMs can alter the isoelectric point and/or molecular weight, they may be detected when a change of spot location on the gel is observed between different samples. Different PTM protein stains have been developed to reveal specific PTMs, such as ProQ diamond and ProQ emerald to detect phosphoproteins and glycoproteins in the gels, respectively (Steinberg et al., 2001; Schulenberg et al., 2003; Ge et al., 2004). A big challenge has been to identify the PTM peptides and map the sites of modifications due to the low abundance nature of the modified protein species.

To overcome the challenge of capturing the relatively low abundance of PTM proteins compared with unmodified proteins, fractionation, and/or enrichment strategies have been employed during sample preparation (Lenman et al., 2008; Guo et al., 2014a; Aryal et al., 2015). The MS-based proteomics coupled with PTM enrichment typically has four steps. First, samples containing the total protein of interest are digested by a protease, such as trypsin. Second, the resulting peptides are subject to enrichment, in order to separate the PTM peptides of interest from the often abundant non-modified peptides. Third, the isolated PTM-peptide is

analyzed by liquid chromatography (LC)-MS/MS for peptide identification and PTM site mapping. Finally, the MS spectra of the peptides are analyzed using different software algorithms and/or evaluated manually to ensure the accuracy and statistical significance of the data.

Among the different fractionation and enrichment strategies, affinity-based approaches are commonly used to enrich PTM proteins/peptides (Blagoev et al., 2004; Rush et al., 2005; Zhang et al., 2005; Fíla and Honys, 2012; Wang et al., 2015b). The affinity-based enrichment has the advantage of relatively high specificity and significant reduction of sample complexity for downstream LC-MS/MS analyses. For example, antiphosphotyrosine antibodies were successfully used to enrich for peptides with phosphotyrosines residues (Blagoev et al., 2004; Rush et al., 2005; Zhang et al., 2005). However, the antibody-based method is often limited by the availability and quality of the antibodies for the specific PTM of interest. Thus, in order to overcome this limitation, several non-antibody based strategies have been developed. For instance, immobilized metal affinity chromatography (IMAC) utilizes a metal chelating agent to bind trivalent metal cation, such as Fe^{3+} or Ga^{3+} (Thingholm and Jensen, 2009). The charged resin is used to bind phosphoproteins or phosphopeptides. Although this strategy is widely used, it has the following shortcomings: (1) If multiply phosphorylated peptides are present in high abundance, they may saturate the IMAC resin, resulting in retention of few singly and doubly phosphorylated species (Thingholm et al., 2008). (2) Acidic peptides will be enriched along with the phosphopeptides (Thingholm et al., 2008). In order to overcome this issue, the incubation buffer needs to be acidified to pH 2–2.5. At this pH, most acidic amino acids will be protonated, which will mask the negative charge of the carboxyl groups, preventing acidic peptides from binding onto the column. In contrast, at this pH most of the phosphate moieties are deprotonated and will bind to the column (Fíla and Honys, 2012). Another approach is to use titanium dioxide (TiO_2) as a substitute for the metal chelating resin. The use of TiO_2 resin under acidic conditions also prevents the retention of acidic peptides (Fíla and Honys, 2012). Interestingly, these two approaches are complementary in that IMAC has higher affinity for multiply phosphorylated peptides, while TiO_2 preferentially binds singly phosphopeptides (Silva-Sanchez et al., 2015). Therefore, application of both approaches in a single experiment leads to a high coverage of the phosphoproteome.

For cysteine redox modifications, such as S-nitrosylation, a classic biotin-switch method developed by Jaffrey et al. (2001) was often used. Free cysteines of proteins are firstly blocked by a thiol-reactive reagent through alkylation. The S-nitrosylated cysteines are then reduced using ascorbate, which is not a strong reducing reagent allowing specific reduction of the S-NO bonds. After chemical substitution with a biotin-containing affinity molecule, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP), the biotinylated proteins/peptides can be enriched by avidin chromatography. Although the classic biotin-switch method has been widely used, and over 300 proteins have been reported to be S-nitrosylated using this method (Lefièvre et al., 2007; Forrester et al., 2009), there are some technical issues inherent to this approach. The disulfide bonds in the proteins may decrease the efficiency of trypsin digestion and further peptide identification (Imai and Yau, 2013). Furthermore, the decomposition of biotin-HPDP may lead to a side reaction with free thiols, which can introduce false-positive signals through disulfide interchange (Forrester et al., 2007). Alternatively, a Thiopropyl Sepharose 6B (TPS6b) enrichment method was developed. The free thiols are alkylated during protein extraction. The proteins are then digested and further reduced prior to enrichment. TPS6b captures reduced thiols via disulfide exchange. TPS6b was initially used to increase the depth of proteome coverage for discovery experiments (Tambor et al., 2012). To date, it has been applied in several redox proteomics studies using cyanobacteria (Guo et al., 2014b), rat myocardium (Paulech et al., 2013), at enrichment efficiencies >95%. Recently, a six-plex iodoTMT technology has been developed to identify and quantify redox cysteines, including S-nitrosylation. Similar to the biotin-switch, free thiols are labeled with iodoTMT, and the TMT-labeled proteins or peptides can be enriched using an anti-TMT resin. This technology allows analysis of up to six samples simultaneously, thus increases throughput and reproducibility.

Quantitative Analysis of PTMs

Multiple proteomics tools are available to quantify the absolute or relative abundances of proteins and their specific PTMs. The quantification of PTMs is crucial, since simple identification of a modification may not provide adequate information for determining its functional importance. *In vivo* and *in vitro* labeling methods have been developed to couple with MS in order to identify, map, and quantify PTMs (Gygi et al., 1999; Goodlett et al., 2001; Ong et al., 2002; Ross et al., 2004; Balmant et al., 2015; Glibert et al., 2015; Parker et al., 2015). Stable isotopes can be used to label proteins *in vivo* via metabolic incorporation. In this approach, one set of sample is grown in a natural nitrogen source (N^{14}) and the other set is grown in a substituted isotopic nitrogen source (N^{15}) as either an amino acid (stable isotopic labeling of amino acids in cell culture, SILAC) or an inorganic nitrogen source ($K^{15}NO_3$) (Thelen and Peck, 2007; Stecker et al., 2014; Minkoff et al., 2015). In SILAC, since the isotopes are introduced as a specific amino acid, the mass differences between the heavy and light peptides in the MS scan can be predicted, making the quantification easy. However, this approach is challenging in plant studies, since plants can synthesize amino acids from inorganic nitrogen. For example, the labeling efficiency achieved using exogenous amino acid in *Arabidopsis* cell cultures has been reported to only 70–80% (Gruhler et al., 2005). In contrast, metabolic labeling with ^{15}N as a inorganic source has been shown to achieve 98% incorporation in both intact plants (Ippel et al., 2004) and cell cultures (Engelsberger et al., 2006). However, the mass difference between differentially labeled samples cannot be easily predicted. Sophisticated software is needed to perform quantitative analysis, which can be challenging when working with highly complex samples (Thelen and Peck, 2007).

Alternatively, isotope labeling can be done to extracted proteins/peptides *in vitro* through several different approaches, e.g., isotope-coded affinity tag (ICAT), isobaric tag for relative and absolute quantification (iTRAQ), tandem mass tag (TMT), and iodoTMT. Except for ICAT, the relative quantification of

peptides between samples is obtained by comparing the ion intensities of the different tags in the MS/MS spectra. The use of stable isotope labeling for absolute quantification requires internal standards, which are pre-selected synthetic peptides with isotope amino acids from a protein of interest. An absolute quantification of a PTM can be achieved by measuring the abundances of the modified and unmodified peptides and comparing them with the known amount of the isotope standard used (Xie et al., 2011). Recently, the use of label-free approaches to quantify PTMs has shown promise. Label-free analysis allows direct comparison of MS signals between any numbers of samples, which makes it applicable to any types of samples, avoiding isotope reagent costs. One label-free approach is spectral counting, where the levels of a modified form of a protein can be estimated by counting the number of the MS/MS spectra of the modified peptide from the protein. It has been noted that the number of assigned MS/MS spectra directly correlates with protein amount (Cooper et al., 2010; Olinares et al., 2011). Although spectral counting is fairly reliable in the measurement of large changes, its accuracy decreases considerably when measuring small changes of proteins (Jurisica et al., 2007) This is why peptide precursor peak alignment and peak area based label-free approach has been more popular in accuracy and robustness (Zhu et al., 2009; Zhang et al., 2010; Lin et al., 2014b).

It is important to note that although all the approaches mentioned above have found utility in the identification and quantification of PTMs, they do not often address the issue of protein turnover in the course of the experiment. Overlooking this important issue may lead to misleading results (Muthuramalingam et al., 2013; Go et al., 2014). In order to account for differences in global protein level change, which could lead to a false positive or false negative result, researches have started to acquire PTM proteomics results and total protein proteomics results from parallel or different studies (Rose et al., 2012; Zhu et al., 2014). However, the success of this strategy is often low because some proteins identified in the PTM proteomics experiments are either absent or not quantified with confidence in the total proteomics experiments (vice versa) due to experimental variation and MS2 stochastical sampling (Chong et al., 2006; Lee and Koh, 2011). To overcome this problem, Parker et al. (2015) developed a double-labeling strategy, called cysTMTRAQ, where the isobaric tags iTRAQ and cysTMT are employed in a single experiment for the simultaneous determination of quantifiable cysteine redox changes and protein level changes. This notion of normalizing against total protein turnover can certainly be applied in the studies of other PTMs. PTMs exist in many different forms, are highly dynamic and important in rapid adjustment of protein functions as molecular switches (Lothrop et al., 2013). The aforementioned approaches and the development of new tools are expected to advance the PTMs studies in many areas of biology.

PROTEIN PHOSPHORYLATION IN STOMATAL FUNCTIONS

Protein phosphorylation provides plants with a rapid and versatile mechanism to allow guard cells to respond rapidly to different environmental changes and adjust stomatal aperture accordingly (Zhang et al., 2015; Zou et al., 2015). Although the involvement of protein kinases and phosphorylation in stomatal movement has been known for decades, detailed molecular mechanisms connecting the key components have just emerged during the past 5 years. For instance, blue-light triggered stomatal opening is featured with phosphorylation and activation of the plasma membrane H^+-ATPase by Blue Light Signaling 1 (BLUS1) (Takemiya et al., 2013). Here we focus on recent progress on the functions of protein phosphorylation in stomatal movement under abiotic and biotic stresses.

Protein Phosphorylation in Guard Cells under Abiotic Stresses

Guard cells are responsive to a plethora of environmental factors. The drought stress induced ABA signaling pathway has been well studied. In guard cells, the central node in the core ABA network is the Sucrose non-fermenting Receptor Kinase 2.6 (SnRK2.6), also known as Open Stomata 1 (OST1). In the absence of ABA, type A Protein Phosphatase 2C (PP2C) inhibits the kinase activity of OST1. In the presence of ABA, ABA binds to its receptor PYRabactin resistance/PYrabactin-Like/Regulatory Components of ABA Receptor (PYR/PYL/RCAR). This hormone-receptor complex further binds and inhibits PP2C, thus releasing OST1 (Geiger et al., 2011; Lee et al., 2013; Zhang et al., 2015). Activated OST1 phosphorylates an array of substrates (**Figure 1**), including Respiratory Burst Oxidase Homolog (RBOH F) (Sirichandra et al., 2009; Acharya et al., 2013), SLow Anion Channel-associated 1 (SLAC1) (Vahisalu et al., 2008), QUickly-activating Anion Channel 1 (QUAC1) (Imes et al., 2013), K^+ inward rectifying channel (KAT1) (Sato et al., 2009; Takahashi et al., 2013), and membrane water channel Plasma membrane Intrinsic Protein 2;1 (PIP2;1) (Grondin et al., 2015, **Table 1**). Phosphorylation of the substrates leads to the ROS burst, the promotion of anion and water efflux, and the inhibition of K^+ influx. ROS can activate Ca^{2+} spikes in the cytosol, which can be further transduced by CDPK and CIPKs via phosphorylation of downstream target proteins (Drerup et al., 2013; Ye et al., 2013). Genetic and biochemical data indicated that MAPKs and some CDPKs such as CPK8 are activated by ABA downstream of ROS production in guard cells (Jammes et al., 2009; Wang et al., 2011; Marais et al., 2014; Zhang et al., 2015; Zou et al., 2015).

Evidence also indicates there are protein kinases that function in parallel to OST1. For example, CPK6 (Brandt et al., 2012b), CPK21/23 (Geiger et al., 2010), and Guard cell Hydrogen peroxide-Resistant 1 (GHR1) phosphorylate SLAC1 to activate the anion channels upon ABA treatment, forming a redundant signaling pathway (**Table 1**). However, detailed characterizations using a loss- and gain-of-function approach imply that OST1 is still the central node and limiting factor in ABA guard cell signaling (Acharya et al., 2013). In addition to drought stress, protein phosphorylation may also play a role in stomatal movement in response to other abiotic stresses. For example, mutants of *MPK9* and *MPK12* are partially impaired in cold-induced stomatal closure, suggesting that the two kinases may function in a cold signaling pathway (Jammes et al., 2009).

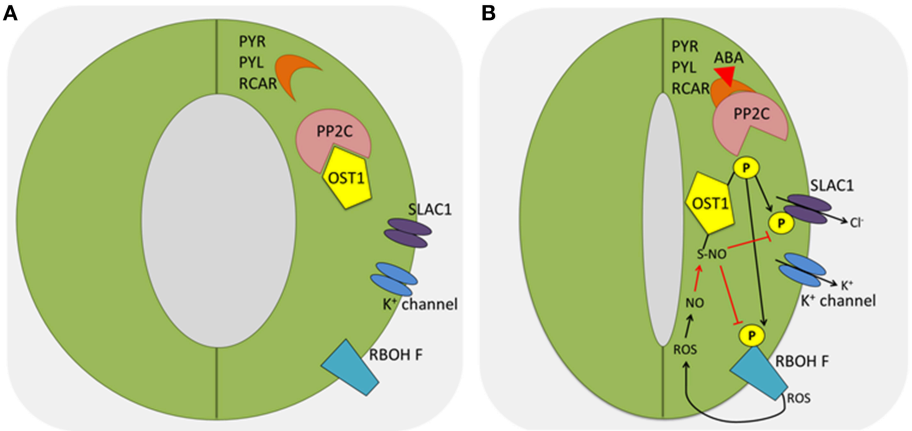

FIGURE 1 | Protein phosphorylation and redox modification in stomatal closure triggered by ABA. (A) In the absence of ABA, type A PP2C inhibits the kinase activity of OST1. **(B)** In the presence of ABA, ABA binds to its receptor PYR/PYL/RCAR, which further binds and inhibits PP2C, releasing and activating OST1. Activated OST1 phosphorylates an array of substrates, including RBOH F and SLAC1. Phosphorylated and active RBOH F promotes ROS burst. Later, ROS can activate Ca^{2+} spikes in the cytosol, which can be further transduced by CDPK and CIPKs via phosphorylation of downstream target proteins. In addition, ROS can modify OST1 and RBOH to inhibit their activities as a feedback mechanism to tune down ABA signaling (red arrows). ABA, abscisic acid; PP2C, protein phosphatase 2C; OST1, Open Stomata 1; PYR, pyrabactin resistance; PYL, PYR like; RCAR, regulatory components of ABA receptors; SLAC1, slow anion channel-associated 1; ROS, reactive oxygen species; NO, nitric oxide; RBOH F, respiratory burst oxidase protein F; CDPK, calcium dependent protein kinase; CIPK, CBL (Calcineurin B-like)-interacting protein kinase.

TABLE 1 | List of proteins undergoing PTMs in plant response to abiotic and biotic stresses.

Organism	PTM	Modifier	Target	Evidence in GC	References
A. thaliana	Phosphorylation	OST1 protein kinase	AtRBOH F	Yes	Sirichandra et al., 2009
A. thaliana	Phosphorylation	OST1 protein kinase	SLAC1	Yes	Maierhofer et al., 2014
A. thaliana	Phosphorylation	CPK6	SLAC1	Yes	Brandt et al., 2012
A. thaliana	Phosphorylation	CPK21/23	SLAC1	Yes	Geiger et al., 2010
A. thaliana	Phosphorylation	OST1 protein kinase	QUAC1 channel	Yes	Imes et al., 2013
A. thaliana	Phosphorylation	OST1 protein kinase	K^+ inward channel	Yes	Sato et al., 2009
A. thaliana	Phosphorylation	OST1 protein kinase	PIP 2;1 aquaporin	Yes	Grondin et al., 2015
A. thaliana	Phosphorylation	FLS2	BAK1	No	Schulze et al., 2010
A. thaliana	Phosphorylation	BAK1	FLS2	No	Schulze et al., 2010
A. thaliana	Phosphorylation	BAK1	BIK1	No	Lin et al., 2014a
A. thaliana	Phosphorylation	BIK1	RBOH D	No	Li et al., 2014
A. thaliana	Phosphorylation	CPK5	RBOH D	Yes	Dubiella et al., 2013
A. thaliana	Phosphorylation	BIK1	MPK3	Yes	Montillet et al., 2013
A. thaliana	Phosphorylation	BIK1	MPK6	Yes	Montillet et al., 2013
A. thaliana	Phosphorylation	RPM1-Induced kinase	RIN4	Yes	Lee et al., 2015
A. thaliana	Nitrosylation	NO	OST1	Yes	Wang et al., 2015a
B. napus	Redox	ROS	BnSnRK2.4	Yes	Zhu et al., 2014
B. napus	Redox	ROS	IPMDH1	Yes	Zhu et al., 2014
A. thaliana	Nitrosylation	NO	NPR1	No	Mou et al., 2003; Waszczak et al., 2015
A. thaliana	Nitrosylation	NO	TGA transcriptional factor	No	Lindermayr et al., 2010
A. thaliana	Nitrosylation	NO	RBOH D	No	Yun et al., 2011
A. thaliana	Nitrosylation	NO	SABP 3	No	Wang et al., 2009

Please refer to the text for abbreviations.

Further studies are needed to identify the MPK9 and MPK12 targets, and their roles in guard cell cold stress signaling cascade.

Protein Phosphorylation in Guard Cells under Biotic Stress

Stomatal pores, as the major gate of pathogen entry, constitute the first line of defense to prevent infection of the plant body by efficient stomatal closure. This process is initiated with the detection of the conserved PAMPs by various immune receptors. One of the best characterized interactions is the flagellin N-terminal 22 amino acid peptide (flg22) and the PRR Flagellin-Sensitive 2 (FLS2) and co-receptor Brassinosteroid insensitive 1-Associated Kinase 1 (BAK1) (Chinchilla et al., 2007; Sun et al., 2013). Using genetic and biochemical approaches, Schulze et al. (2010) showed that phosphorylation of FLS2 and BAK1 were detected within 15 s after flg22 treatment of *Arabidopsis* plants, and the kinase activity of BAK1 was required for flg22 perception (**Table 1**). Although there is no study specific for guard cells showing that FLS2 and BAK1 are phosphorylated after flg22 perception, the same events are likely to occur in the guard cells. It is known that FLS2 plays an important role in flg22-induced stomatal closure, since stomata in Arabidopsis *fls2* mutant are completely impaired by flg22 carrying pathogen *Pst*. DC3000 (Zeng and He, 2010). Genetics and biochemical approaches showed that activation of FLS2 and BAK1 in *Arabidopsis* plants promote formation of the receptor complex with the botrytis-induced kinase 1 (BIK1, **Table 1**). BIK1 phosphorylates RBOH D (**Table 1**), which directly modulates stomatal closure in response to flg22, as *rboh D* mutant and Arabidopsis carrying RBOH $D^{S39A,S343A,S347A}$ exhibited completely impaired stomatal closure under flg22 treatment (Li et al., 2014). Interestingly, *Arabidopsis* RBOH D was also shown to be phosphorylated by CPK5 upon flg22 treatment (Dubiella et al., 2013, **Table 1**). In addition to RBOH D activation, flg22-induced FLS2 receptor complex also activates MPK3 and MPK6 to induce stomatal closure (Montillet et al., 2013). Thus, phosphorylation is an essential and common mechanism in pattern triggered immunity (PTI) responses. Downstream of PTI signaling includes regulation of K^+ channels, turgor decrease in guard cells, and closure of the stomatal pores to prevent pathogen entry (Zhang et al., 2008; Zeng et al., 2010).

Successful pathogens deliver effector proteins into the plant cells to overcome PTI, and the effectors trigger the second layer of plant immunity called effector triggered immunity (ETI). For example, the bacterial effectors AvrB can be recognized by the plant immune receptor Resistance to *Pseudomonas syringae* pv Maculicola 1 (RPM1). Recognition of AvrB by RPM1 causes phosphorylation of RPM1-Interacting Protein4 (RIN4) by RPM1-Induced Protein Kinase (RIPK, **Table 1**). Recently, Lee et al. (2015) showed that $RIN4^{T21D/S160D/T166D}$, a mutant with three phosphorylation sites changed to phosphorylation mimic aspartate residues, rendered Arabidopsis plants to exhibit large stomatal apertures and decreased resistance to *P. syringae*. This exemplifies how an effector protein facilitates pathogen infection by modulating host cell protein phosphorylation events.

Current Questions in Guard Cell Protein Phosphorylation Research

As more aspects of phosphorylation in stomatal movement have been revealed, more questions have also been raised. The findings of OST1, as a central player in the core ABA pathway, open doors for questions such as how the activity of this key modulator is controlled. Is it activated by autophosphorylation or by an upstream kinase? How is OST1 dephosphorylated? Recently, Casein Kinase 2 (CK2) has been shown as a negative regulator of OST1 by increasing the binding of CK2-phosphorylated OST1 to PP2C (Vilela et al., 2015). With many key kinases identified in guard cells including SnRKs, CPKs, and MPKs, how are these kinase pathways crosstalk to minimize redundancy, and how is the signal specificity determined? What are the target proteins involved in stomatal movement? With the development of kinase substrates screening (Umezawa et al., 2013; Wang et al., 2013) and techniques in live-cell phosphorylation detection (Hayashi et al., 2011), more studies are forthcoming toward better understanding of the phosphorylation-mediated stomatal movement at high spatial and temporal resolution. In addition, since phosphorylation is essential in both ABA and flg22 triggered stomatal closure, what are the convergent nodes and edges? This question is still under debate. One study showed that the flg22 response was independent of ABA signaling (Montillet et al., 2013), while another study indicated that flg22 induced stomatal closure was impaired in the *ost1* mutant (Guzel Deger et al., 2015). It should be noted that in the first study 10 times more flg22 was used to cause stomatal movement in the *ost1* mutant. Therefore, it is likely that both ABA-dependent and independent pathways are functional. In addition, different protein kinases may be involved in different pathways. For example, MPK3 and MPK6 were shown to be important players in flg22 triggered stomatal closure (Montillet et al., 2013), while MPK9 and MPK12 play critical roles in the guard cell ABA and cold stress signaling (Jammes et al., 2009), as well as yeast elicitor signaling (Salam et al., 2013). Moreover, both protein kinases and phosphatases control the dynamics of protein phosphorylation in guard cell signaling. However, only a few phosphatases have been identified in guard cells (Tseng and Briggs, 2010; Sun et al., 2012; Takemiya et al., 2013), and their interactions with key signaling proteins remaining largely elusive.

REDOX-DEPENDENT PTMS IN STOMATAL FUNCTIONS

As with protein phosphorylation and other PTMs, redox-dependent PTMs may function as molecular switches to turn on or off signaling processes in plant response to abiotic and biotic stresses. Thiol is a nucleophile that when exposed to oxidative stress, undergoes reversible inter- and intra-molecular disulfide bond formation, nitrosylation, glutathionylation, sulfenic acid and sulfinic acid modification, and irreversible sulfonic acid modification. Additionally, the high pKa values of protein cysteines make these residues highly responsive to small redox perturbation (Spoel and Loake, 2011). The production of ROS and NO is a common event during stomatal closure (Xie et al.,

2014). The ROS and NO can serve as signaling molecules by modifying the reactive protein thiol groups. Here we focus on recent progress on the roles of redox-dependent cysteine PTMs in stomatal movement under abiotic and biotic stresses.

Redox PTMs in Guard Cells under Abiotic Stress

As described in the previous section, under drought stress ABA-induced stomatal closure is associated with an increase in NO and ROS production in guard cells (Zhang et al., 2001; Neill et al., 2008). The ROS production is catalyzed mainly by two types of enzymes, the plasma membrane NADPH oxidases, and the cell wall peroxidases (Sharma et al., 2012). Other ROS-generating enzymes, such as apoplastic amine oxidases and oxalate oxidases, may also be involved in ROS production leading to stomatal closure (Tripathy and Oelmüller, 2012). The NADPH oxidases are regulated by direct binding of Ca^{2+} (Kadota et al., 2015), phosphatidic acid (Zhang et al., 2009), Rac GTPases (Wong et al., 2007), and via phosphorylation by OST1 (Sirichandra et al., 2009), CDPKs (Kadota et al., 2015), and BIK1 (Kadota et al., 2014). Consequently, NADPH oxidase may integrate multiple upstream signaling events to promote stomatal closure. NO is produced by the nitrite-dependent nitrate reductase pathway (Desikan et al., 2002) and a nitric oxide associated 1 (NOA1) protein-dependent pathway (Lozano-Juste and León, 2010). It is important to note that the NOA1 is not a NO synthase (Moreau et al., 2008).

Although the essential function of ROS and NO in stomatal closure has been widely accepted, little is known about the underlying molecular mechanisms, by which they achieve the PTM regulation in guard cells. Thus, direct evidence for thiol-based redox regulation under stress conditions and a link between protein redox regulation and stomatal movement need to be established. A recent study showed that NO resulting from the ABA signaling caused S-nitrosylation of OST1 at the cysteine residue (Cys137) close to the kinase catalytic site (**Table 1**), and the PTM abolished the kinase activity (**Figure 1B**). This represents an interesting negative feedback mechanism by which ABA-induced NO helps to desensitize ABA signaling. Additionally, the authors showed that the Cys137 is evolutionarily conserved in some AMPK/SNF1-related kinases and glycogen synthase kinase 3/SHAGGY-like kinases (SKs) in plants, yeast and mammals, and the S-nitrosylation-mediated inhibition may be a general regulatory mechanism (Wang et al., 2015a). This example also highlighted how redox changes regulate protein kinase phosphorylation and signaling cascade in stomatal movement.

In a redox-proteomics study, Zhu et al. (2014) identified 65 and 118 potential redox responsive proteins in ABA and MeJA treated *Brassica napus* guard cells, respectively. The authors demonstrated that most of the proteins belong to functional groups such as energy, stress and defense, and metabolism. In addition, osmotic stress-activated protein kinase (BnSnRK2) and isopropylmalate dehydrogenase (IPMDH) were confirmed to be redox regulated and involved in stomatal movement (**Table 1**). These findings demonstrate the utility of redox-proteomics in discovering uncharacterized redox proteins and their roles in stomatal movement. Although some proteins have been identified to be redox regulated, their functions in regulating stomatal movement are still to be fully characterized.

Redox PTMs in Guard Cells under Biotic Stress

Pathogen perception initiates a signal transduction cascade including ROS and NO production, increase in Ca^{2+} influx, alkalization of the extracellular space, activation of MAPK, CDPK, salicylic acid (SA) pathway, and synthesis of ethylene (Arnaud and Hwang, 2015). The ROS and NO generated under biotic stresses are known to act as antimicrobial compounds. ROS are also known to be involved in cell wall cross-linking and blockage of pathogen infection (Torres et al., 2006). Furthermore, they play important signaling roles, e.g., in redox PTM of essential proteins in plant defense (Agurla et al., 2014). Methionine and cysteine residues of certain proteins are sensitive to H_2O_2 and NO (Hoshi and Heinemann, 2001). The sensitivity of the residues depends on the protein structure, neighboring residues, and solvent accessibility (Roos et al., 2013). H_2O_2 can react with a cysteine thiolate forming intra- or inter- disulfide bonds, sulfenic acid (-SOH), sulfinic acid ($-SO_2H$), and sulfonic ($-SO_3H$) acid (Dalle-Donne et al., 2006). NO can covalent bind to a cysteine thiol through S-nitrosylation.

Although redox-dependent PTMs in biotic stresses is an emerging field, there are some examples showing the redox regulation of proteins in guard cells. In plant defense, Nonexpresser of PR gene 1 (NPR1) is one of a limited number of examples of protein redox regulation. NPR1 was detected primarily in the cytoplasm and nuclei of guard cells (Kinkema et al., 2000). Under normal conditions, NPR1 is retained in the cytoplasm as inactive disulfide-bonded oligomers, which is promoted by the S-nitrosylation at cysteine 156 (**Table 1**). In the presence of pathogen, an increase in SA mediates cellular redox changes, leading to thioredoxin-mediated reduction of the NPR1 oligomer to monomeric forms, which are then transported into the nucleus to activate plant immune processes (Mou et al., 2003; Waszczak et al., 2015). In the nucleus, SA mediated redox change causes de-nitrosylation and reduction of disulfide bonds in TGA transcriptional factors (**Table 1**) so that they can form an active transcriptional complex with NPR1 to turn on pathogenesis related (PR) genes (Lindermayr et al., 2010, **Figure 2**), and NPR1 is then phosphorylated and ubiquitinylated for degradation (Waszczak et al., 2015). Although protein redox regulation is not well studied in plant innate immunity, it is clear from the above example that modification of cysteine thiols can alter protein activity, function, and redox crosstalk with other modifications.

Yun et al. (2011) demonstrated a NO biphasic control in pathogen triggered cell death. At the initial stage of pathogen infection, S-nitrosothiol (SNO) accumulation leads to accelerated cell death. Conversely, constitutively high SNO levels promote decreased cell death through S-nitrosylation of RBOH D (**Table 1**), leading to reduction in its activity and oxidative stress. This differential regulation seems important in fine tuning the extent of cell death under conditions of abiotic and biotic stresses, since both cause increases of NO

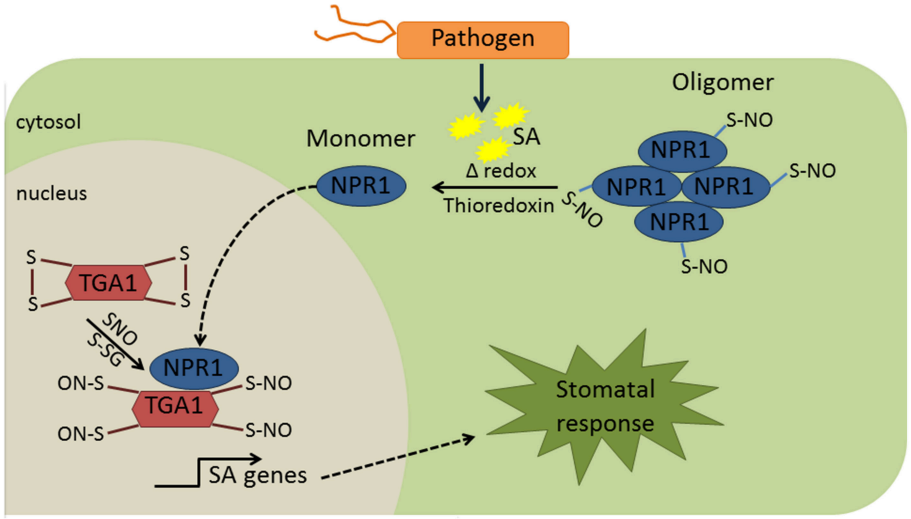

FIGURE 2 | Redox regulation of NPR1 and TGA1. Under normal conditions, NPR1 is retained in the cytosol as an oligomer. S-nitrosylation of NPR1 is known to promote NPR1 oligomerization. In the presence of pathogen, production of SA promotes cellular redox changes, which will contribute to reduction of the NPR1 oligomer to monomeric form. Monomeric form of NPR1 moves to the nucleus and binds to TGA1 that was nitrosylated due to cellular redox changes mediated by SA. The complex NPR1-TGA1 turns on the transcription of PR genes. Although this mechanism was not directly elucidated in the guard cells, it is likely to be the case since NPR1 was primarily in the cytosol and nucleus of guard cells (Kinkema et al., 2000). SA, salicylic acid; NPR1, nonexpresser of PR gene 1; TGA1, teosine glume architecture 1; SNO, S-nitrosylation; S-GS, S-glutathionylation.

levels. At a certain level of NO concentration, the signaling components of stomatal movement and plant response may be unresponsive or irreversibly regulated with detrimental effects on stress acclimation. During the NO burst, NO also promotes S-nitrosylation of an Arabidopsis SA-binding protein 3 (AtSABP3) at Cys280 (**Table 1**). The S-nitrosylation suppresses both SA binding, and its chloroplast carbonic anhydrase activity (Wang et al., 2009). Interestingly, in tobacco SABP3 showed antioxidant activity and plays a role in the hypersensitive defense response (Slaymaker et al., 2002). Although the role of SABP3 nitrosylation in stomatal closure in response to biotic stresses has not been studied, it may play a role in stomatal movement signaling as SA is known to promote stomatal closure (Khokon et al., 2011). The examples here demonstrated the great potential of redox regulation in stomatal movement in response to biotic stresses. The development of redox proteomics technologies such as the cysTMTRAQ (Parker et al., 2015) and application of genetics, biochemistry, metabolism, and bioinformatics tools would accelerate the discovery and characterization of redox-dependent PTMs of proteins and their roles in stomatal signaling and plant immunity.

CONCLUDING REMARKS

Regulation of the size of the stomatal aperture is an essential mechanism in plants for optimizing the efficiency of water usage and photosynthesis. Stomatal movement through dynamic changes of the turgor of guard cells represents the output of integration of environmental signals with cellular signal transduction networks. Perception of abiotic and/or biotic stress signals triggers activation of signal transduction cascade, leading to rapid guard cell responses, which are known to be regulated by PTMs (e.g., protein phosphorylation and redox modification) of key players in the complex guard cell signaling networks. Over the past years, improvement and development of new tools in proteomics and MS have enabled the identification of PTMs of proteins involved in stomatal movement. In fact, LC-MS/MS based PTMomics technologies have become indispensable in identification and mapping of novel protein phosphorylation and redox modification sites. Additional sample preparation techniques, such as PTM enrichment and specific isotope labeling have greatly helped the detection and quantification of protein phosphorylation and redox changes, and thereby the understanding of PTM-controlled signaling pathways. The past decade has seen exciting discoveries in ABA and bacterial pathogen-triggered PTMs, especially phosphorylation and redox modification. Despite of current progress, guard cell PTMomics is still in its infancy and many aspects of protein level regulations remain elusive. For example, the crosstalk among different PTMs, and PTMs involved in regulating stomatal movement in response to other environmental factors are largely unknown. The fast advancement of proteomics technologies, together with genetics, molecular biology, biochemistry, and bioinformatics tools will accelerate the discovery and characterization of novel PTMs, and provide new insights into the complex protein phosphorylation and redox regulatory networks in guard cell signal transduction.

AUTHOR CONTRIBUTIONS

KB drafted the manuscript with assistance from TZ. KB drew the figures. TZ focused on phosphorylation sections. SC provided guidance, edited and finalized the manuscript.

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MINI REVIEW
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14-3-3 Proteins in Guard Cell Signaling

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Guard cells are specialized cells located at the leaf surface delimiting pores which control gas exchanges between the plant and the atmosphere. To optimize the CO_2 uptake necessary for photosynthesis while minimizing water loss, guard cells integrate environmental signals to adjust stomatal aperture. The size of the stomatal pore is regulated by movements of the guard cells driven by variations in their volume and turgor. As guard cells perceive and transduce a wide array of environmental cues, they provide an ideal system to elucidate early events of plant signaling. Reversible protein phosphorylation events are known to play a crucial role in the regulation of stomatal movements. However, in some cases, phosphorylation alone is not sufficient to achieve complete protein regulation, but is necessary to mediate the binding of interactors that modulate protein function. Among the phosphopeptide-binding proteins, the 14-3-3 proteins are the best characterized in plants. The 14-3-3s are found as multiple isoforms in eukaryotes and have been shown to be involved in the regulation of stomatal movements. In this review, we describe the current knowledge about 14-3-3 roles in the regulation of their binding partners in guard cells: receptors, ion pumps, channels, protein kinases, and some of their substrates. Regulation of these targets by 14-3-3 proteins is discussed and related to their function in guard cells during stomatal movements in response to abiotic or biotic stresses.

Keywords: 14-3-3 proteins, guard cell, H^+-ATPases, ion channels, phototropins, protein kinases, protein phosphorylation, signal transduction

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INTRODUCTION

Reversible protein phosphorylation is recognized as one of the most important post-translational modifications in eukaryotes, playing major roles in the regulation of cellular processes (Cohen, 2002). However, in many cases, phosphorylation alone is not sufficient to achieve complete protein regulation, but is required to induce the binding of interactors that modulate protein function. Among the phosphopeptide-binding proteins, the 14-3-3 proteins are the best characterized in plants (Chevalier et al., 2009). The 14-3-3s form a family of highly conserved proteins found in all eukaryotes that bind to phosphoserine/phosphothreonine-containing motifs. 14-3-3 proteins have been found to be expressed in all eukaryotic organisms, in which they generally exist as multiple isoforms. While yeast expresses two 14-3-3 isoforms and mammals possess seven, plants have a varying number of isoforms, with e.g., thirteen identified in *Arabidopsis* and eight in rice (Van Heusden et al., 1995; DeLille et al., 2001; Aitken, 2006; Yao et al., 2007). *Arabidopsis* 14-3-3 proteins are designated by Greek letters (χ, ω, ψ, φ, υ, λ, ν, κ, μ, ε, ο, ι, π)

and are encoded by genes called *General Regulatory Factors* (*GRF1-13*) (Ferl et al., 2002; Chevalier et al., 2009). Both of these designations are currently used for *Arabidopsis* 14-3-3s in the literature. The 14-3-3s are small acidic proteins (∼30 kDa) that are highly conserved both within and across species (Ferl et al., 2002). These proteins form homo- and heterodimers (Paul et al., 2012) that bind, in most cases, to phosphorylated target proteins. To date, three consensus 14-3-3-binding phosphopeptide motifs have been described: mode I (R/K)XX(pS/pT)XP, mode II (R/K)XXX(pS/pT)XP (Muslin et al., 1996; Yaffe et al., 1997) and C-terminal mode III (pS/pT)X_{1-2}-COOH (Coblitz et al., 2005; Ganguly et al., 2005), where X is any amino acid and pS/pT represents a phosphoserine or phosphothreonine. However, many phosphorylated target proteins contain 14-3-3-binding sites that do not conform to these consensus motifs and 14-3-3-binding can also occur through non-phosphorylated sequences (Bridges and Moorhead, 2005; Taoka et al., 2011). Through these interactions, 14-3-3s regulate target activity, subcellular localization, proteolysis, or association with other proteins (Cotelle et al., 2000; Taoka et al., 2011; Paul et al., 2012). Plant 14-3-3s interact with a wide range of proteins, thereby playing prominent role in diverse aspects of plant physiology, including primary metabolism, development, abiotic and biotic stress responses, and regulation of stomatal movements (reviewed in Denison et al., 2011; Jaspert et al., 2011; de Boer et al., 2013; Lozano-Durán and Robatzek, 2015).

In plants, the majority of water loss occurs through pores on the leaf surface, which are called stomata. The size of the stomatal pores is variable and controls the rate of diffusion of water vapor out of the plant. In addition to controlling water loss, stomata allow CO_2 to diffuse into the leaf for photosynthesis. Thus, the primary role of stomata is to optimize the exchange of CO_2 and water vapor between the intracellular spaces in leaves and the atmosphere according to environmental conditions. Under favorable conditions, stomatal opening requires activation of plasma membrane H^+-ATPases, resulting in plasma membrane hyperpolarization (Assmann et al., 1985; Shimazaki et al., 1986) to drive K^+ uptake into guard cells via inward-rectifying K^+ channels (Schroeder et al., 1984), including K^+ channels *Arabidopsis thaliana* 1 and 2 (KAT1, KAT2), *Arabidopsis* K^+ transporter 1 and 2 (AKT1, AKT2) and K^+ rectifying channel (KC1) (Schachtman et al., 1992; Nakamura et al., 1995; Pilot et al., 2001; Szyroki et al., 2001). Uptake of K^+ ions, in combination with the accumulation of anions, increases the osmotic potential of the guard cells resulting in guard cell swelling, driving opening of the stomatal pore. In contrast, stomatal closure is triggered by transition from light to darkness, high CO_2 concentrations and abscisic acid (ABA), a hormone synthesized in response to drought stress. All these signals have been shown to induce an alkalinisation of the apoplastic space, which is correlated with the concomitant decrease of the plasma membrane H^+-ATPase activity (Hedrich et al., 2001; Jia and Davies, 2006). Moreover, the activation of rapid-type (R-type) anion channels, the aluminum-activated anion channel 12 (ALMT12), and slow-type (S-type) anion channels including slow anion channel-associated 1 (SLAC1) and SLAC1 homolog 3 (SLAH3), facilitate the efflux of anions such as malate^{2-}, Cl^-, and NO_3^- (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Roelfsema et al., 2004; Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010; Sasaki et al., 2010; Geiger et al., 2011). An elevation of cytoplasmic Ca^{2+} concentration due to the activation of plasma membrane and vacuolar channels is also observed during stomatal closure (Schroeder and Hagiwara, 1989; Ward and Schroeder, 1994). Altogether, inhibition of H^+-ATPases, activation of anion and Ca^{2+} channels induce plasma membrane depolarization. This plasma membrane depolarization activates guard cell outward-rectifying K^+ (GORK; Hosy et al., 2003). The efflux of solutes from the guard cells leads to a reduced turgor and stomatal closure.

In the past decades, guard cell research has revealed many new signal transduction components including channels mediating movement of ions. However, mechanisms by which the environmental cues are transduced to activate or deactivate the channels are still not completely understood. Using several approaches including genetics and biochemistry, the key role of protein phosphorylation involving binding of 14-3-3 proteins has been demonstrated in guard cell signal transduction. Moreover, several studies report expression of 14-3-3 isoforms in guard cells (**Table 1**). In this mini-review, we highlight the functions of 14-3-3 proteins in guard cell signaling, which are summarized in **Figure 1**.

REGULATION OF MEMBRANE PROTEINS BY 14-3-3 PROTEINS IN GUARD CELLS

Phototropins and H^+-ATPases in Response to Blue Light

Light stimulates stomatal opening via two signaling pathways. One depends specifically on blue light and is perceived by two phototropins, PHOT1 and PHOT2 and cryptochromes, while the other is stimulated by photosynthetically active radiations (Kinoshita et al., 2001; Shimazaki et al., 2007). Phototropins are serine/threonine protein kinases with two LOV (light, oxygen and voltage) domains (Briggs and Christie, 2002). The activated phototropins undergo autophosphorylation and bind 14-3-3 proteins, and ultimately activate the plasma membrane H^+-ATPase in guard cells (Kinoshita et al., 2001, 2003; Ueno et al., 2005). It is still unknown whether phototropin excitation induces direct phosphorylation of the H^+-ATPase via a direct association of the two proteins, or whether there are one or more signaling cascade elements. In *Vicia faba* and *A. thaliana* guard cells, blue light has been shown to induce phosphorylation-dependent binding of a non-epsilon 14-3-3 to PHOT1 (Kinoshita et al., 2003; Ueno et al., 2005). Using yeast two-hybrid and *in vitro* assays, PHOT1 was found to interact with 14-3-3λ with the strongest affinity followed by 14-3-3κ, 14-3-3φ, and 14-3-3υ (Sullivan et al., 2009). However, characterization of *Arabidopsis* mutants lacking both

TABLE 1 | Expression and subcellular localization of *Arabidopsis thaliana* 14-3-3 proteins.

Gene name	Protein Name	Gene ID	Gene expression	Protein localization	References
GRF1	Chi	At4g09000	Seedling; root; root hair; bud; guard cell; flower; anther; stigma; pollen; silique	Cytoplasm; nucleus	[1]Daugherty et al., 1996; [1,2]Ferl et al., 2002; [1]Wang et al., 2008; [1]Zhao et al., 2008; [1,2]Paul et al., 2012; [2]Swatek et al., 2014; [1]Van Kleeff et al., 2014
GRF2	Omega	At1g78300	Seedling; root; leaf; stem; flower; pollen; silique; seed	Cytoplasm; nucleus	[2]Cutler et al., 2000; [1]Sorrell et al., 2003; [2]Paul et al., 2005; [1]Schmid et al., 2005; [1]Wang et al., 2006, 2008; [1]Hajduch et al., 2010; [1]Paul et al., 2012; [2]Yoon and Kieber, 2013
GRF3	Psi	At5g38480	Seedling; root; leaf; guard cell; stem; flower; pollen; silique; seed	Cytoplasm; nucleus	[1]Ferl et al., 2002; [1]Leonhardt et al., 2004; [1]Schmid et al., 2005; [1]Wang et al., 2006, 2008; [1]Rajjou et al., 2008; [1]Paul et al., 2012; [1,2]Catalá et al., 2014
GRF4	Phi	At1g35160	Root; leaf; guard cell	Plasma membrane; cytoplasm; nucleus; nuclear membrane	[2]Ferl et al., 2002; [1]Gepstein et al., 2003; [2]Paul et al., 2005; [1]Zhao et al., 2008; [1,2]Paul et al., 2012; [1]Van Kleeff et al., 2014
GRF5	Upsilon	At5g16050	Root; leaf; flower; pollen; silique; seed	Plasma membrane; cytoplasm; nucleus; nuclear membrane; chloroplast	[1,2]Sehnke et al., 2000; [2]Ferl et al., 2002; [1]Schmid et al., 2005; [1]Mayfield et al., 2007; [1]Wang et al., 2008; [1]Hajduch et al., 2010; [2]Pignocchi and Doonan, 2011; [1]Paul et al., 2012; [1]Van Kleeff et al., 2014
GRF6	Lambda	At5g10450	Seedling; root; leaf; guard cell; stem; flower; silique; seed	Plasma membrane; cytoplasm; nucleus; vacuole	[1,2]Ferl et al., 2002; [1]Sorrell et al., 2003; [1]Leonhardt et al., 2004; [2]Paul et al., 2005; [1]Schmid et al., 2005; [2]Latz et al., 2007; [1]Sullivan et al., 2009; [1]Hajduch et al., 2010; [1,2]Paul et al., 2012; [1,2]Carrasco et al., 2014; [1]Van Kleeff et al., 2014; [1,2]Zhou et al., 2014
GRF7	Nu	At3g02520	Root; leaf; flower; pollen; silique	Plasma membrane; cytoplasm; nuclear membrane; chloroplast	[1,2]Sehnke et al., 2000; [2]Ferl et al., 2002; [1]Schmid et al., 2005; [1]Wang et al., 2008; [1]Paul et al., 2012; [1]Van Kleeff et al., 2014
GRF8	Kappa	At5g65430	Seedling; root; leaf; stem; flower; silique; seed	Cell wall; plasma membrane; cytoplasm; nucleus	[2]Ferl et al., 2002; [1]Sorrell et al., 2003; [2]Paul et al., 2005; [1]Schmid et al., 2005; [1]Hajduch et al., 2010; [1,2]Paul et al., 2012; [1]Van Kleeff et al., 2014
GRF9	Mu	At2g42590	Seedling; root; leaf; guard cell; stem; flower; silique; seed	Plasma membrane; cytoplasm; nucleus; chloroplast	[1]Kuromori and Yamamoto, 2000; [1,2]Sehnke et al., 2000; [1,2]Ferl et al., 2002; [1]Leonhardt et al., 2004; [2]Koroleva et al., 2005; [1]Schmid et al., 2005; [1]Mayfield et al., 2007; [1]Hajduch et al., 2010; [1]Paul et al., 2012; [2]He et al., 2015
GRF10	Epsilon	At1g22300	Root; leaf; flower; pollen; silique; seed	Plasma membrane; cytoplasm; nucleus; nuclear envelope; chloroplast	[1,2]Sehnke et al., 2000; [1,2]Ferl et al., 2002; [1]Schmid et al., 2005; [1]Wang et al., 2008; [1]Hajduch et al., 2010; [1,2]Paul et al., 2012; [2]Swatek et al., 2014
GRF11	Omicron	At1g34760	Root; root hair; leaf; guard cell; stem; flower	nd	[1]Rosenquist et al., 2001; [1]Ferl et al., 2002; [1]Leonhardt et al., 2004; [1]Won et al., 2009; [1]Paul et al., 2012
GRF12	Iota	At1g26480	Leaf; flower; pollen	nd	[1]Rosenquist et al., 2001; [1]Ferl et al., 2002; [1]Schmid et al., 2005; [1]Wang et al., 2008; [1]Paul et al., 2012
GRF13	Pi	At1g78220	nd	nd	[1]Paul et al., 2012

nd: not determined.
[1]*Reference related to gene expression.*
[2]*Reference related to protein localization.*

14-3-3λ and 14-3-3κ has been unsuccessful in identifying a physiological role for 14-3-3-binding to PHOT1 (Sullivan et al., 2009). More recently, Tseng et al. (2012) demonstrate that 14-3-3λ interacts also with PHOT2 and plays a role in PHOT2-mediated blue light response. This interaction is dramatically reduced when the PHOT2 S747 in a putative mode I 14-3-3 recognition site was replaced by a non phosphorylatable residue. In addition, blue light-induced stomatal opening is dramatically impaired in *phot1-5 14-3-3λ Arabidopsis* double mutant. In contrast, *phot2-1 14-3-3λ* double mutant and *phot1-5 14-3-3κ* double mutant do not exhibit defects in stomatal opening in response to blue light. Altogether, these observations demonstrate that the closely related 14-3-3 isoforms λ and κ differentially affect PHOT2 signaling in guard cell and reveal the existence of remarkable functional specificity of 14-3-3 proteins.

Furthermore, blue light activates the plasma membrane H^+-ATPase (Shimazaki et al., 1986; Kinoshita and Shimazaki, 1999; Hayashi et al., 2011). The pump activation requires the phosphorylation of its penultimate threonine residue at the C-terminus end. However, this phosphorylation alone is not enough to activate H^+ pumping, as subsequent binding of 14-3-3 proteins is also needed (Palmgren, 2001; Bækgaard et al., 2005). In *Vicia faba* guard cells, a 32 kDa 14-3-3 protein has been shown to bind to the phosphorylated C-terminus of the H^+-ATPase, but not to the non phosphorylated one (Emi

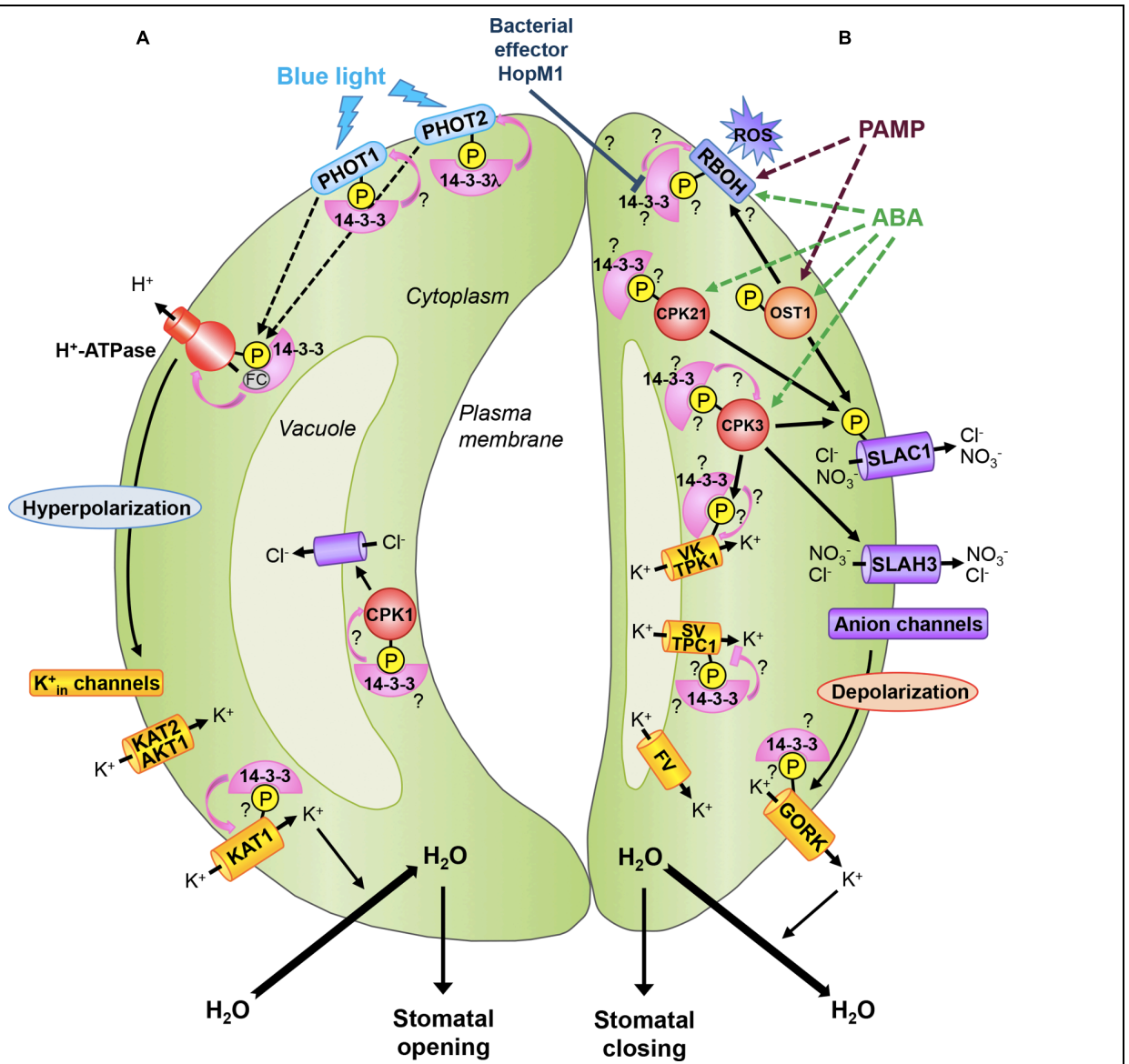

FIGURE 1 | 14-3-3 proteins during stomatal movements. (A) Stomatal opening: the perception of blue light by phototropins PHOT1 and PHOT2 leads to their autophosphorylation and subsequent 14-3-3 binding. In *Arabidopsis*, the 14-3-3λ isoform is required for PHOT2-mediated stomatal opening. Blue light stimulates the plasma membrane H^+-ATPase by phosphorylation at its C-terminus end and subsequent 14-3-3 binding. The fungal toxin FC stabilizes the 14-3-3/H^+-ATPase interaction leading to constant activation of the proton pump and thus irreversible stomatal opening. Activation of H^+-ATPases leads to hyperpolarization of the plasma membrane and uptake of K^+ via inward-rectifying K^+ channels (K_{in}) including K^+ channel *A. thaliana* 1 and 2 (KAT1, KAT2) and *Arabidopsis* K^+ transporter 1 (AKT1). KAT1 is activated by 14-3-3 binding. K^+ influx induces inward water movement leading to guard cell swelling and stomatal opening. At the tonoplast, a Cl^- channel providing a pathway for anion uptake into the vacuole is activated by the calcium-dependent protein kinase (CPK) CPK1, whose activity might be directly stimulated by 14-3-3s. **(B)** Stomatal closing: ABA induces activation of protein kinase open stomata 1 (OST1) as well as CPKs. Among the CPKs involved in guard cell ABA signaling, CPK21 could bind to 14-3-3 proteins and CPK3 might be stabilized by its interaction with 14-3-3s. OST1 and CPKs can activate the guard cell plasma membrane S-type anion channel SLAC1 (slow anion channel-associated 1) by phosphorylation. SLAC1 homolog 3 (SLAH3), another guard cell S-type anion channel, is also activated by CPK3. Activation of anion channels leads to plasma membrane depolarization and activation of the guard cell outward-rectifying K^+ (GORK) channel which is a putative 14-3-3 client protein. The efflux of ions leads to water loss and guard cell shrinkage, thus closure of the stomatal pore. During stomatal closure, K^+ release from vacuoles occurs via vacuolar K^+-selective (VK) channels, slow vacuolar (SV) channels and fast vacuolar (FV) channels. The tandem-pore K^+ channel 1 (TPK1) represents the guard cell VK channel which might be activated by 14-3-3 binding to a N-terminal site phosphorylated by CPK3. In contrast, SV channels, represented by the two-pore channel 1 (TPC1) in *Arabidopsis*, might be inactivated by 14-3-3 binding. Stomatal closure induced by pathogen-associated molecular patterns (PAMPs) or ABA involves plasma membrane respiratory burst oxidase homologs (RBOHs) which are targets of OST1. RBOHs are NADPH oxidases producing reactive oxygen species (ROS) in the apoplast and might be activated by interaction with 14-3-3 proteins. The *Pseudomonas syringae* effector HopM1 could suppress PAMP-triggered ROS production and stomatal closure by destabilization of 14-3-3 proteins. Pink lines show 14-3-3 regulation on target proteins. Arrowheads designate activation, bars indicate inhibition. Dashed lines denote more than one step, solid lines show direct interaction. Question marks denote signaling events that require further investigation in guard cells. The P in the yellow-colored disks indicates a phosphorylated protein. See the text for details.

et al., 2001; Kinoshita and Shimazaki, 2001). Moreover, blue light increases the amount of bound 14-3-3 protein which is proportional to H^+-ATPase activity (Kinoshita and Shimazaki, 2002). The binding of 14-3-3 proteins to the autoinhibitory C-terminal domain of the H^+-ATPase prevents its interaction with the catalytic domain leading to a high-activity state of the pump. The H^+-ATPase/14-3-3 complex is stabilized by fusicoccin (FC), a fungal phytotoxin (Palmgren, 2001). FC binds to 14-3-3 proteins, thereby increasing the affinity of 14-3-3 proteins for the autoinhibitory C-terminal end of the plasma membrane H^+-ATPase, which causes irreversible opening of stomata (Assmann and Schwartz, 1992; Kinoshita and Shimazaki, 2001). Interestingly, Pallucca et al. (2014) show that H^+-ATPase preferentially interacts with non-ε 14-3-3 isoforms. However, further studies will be needed to identify which 14-3-3 isoforms interact with guard cell-expressed proton pumps. Finally, overexpression of 14-3-3λ in cotton results in an increase in stomatal conductance suggesting that 14-3-3λ may interact with the plasma membrane H^+-ATPase or phototropins to regulate stomatal movements (Yan et al., 2004).

Ion Channels at the Plasma Membrane

Plasma membrane K^+ channels play a major role in K^+ fluxes that modulate guard cell turgor. The main plasma membrane K^+ channels identified in guard cell are from shaker superfamily (Véry et al., 2014). In *Arabidopsis* guard cells, the expression of six shaker-type K^+ channels can be detected including KAT1, KAT2, AKT1, AKT2, GORK, and KC1 (Schachtman et al., 1992; Nakamura et al., 1995; Pilot et al., 2001; Szyroki et al., 2001). KAT1, the first cloned plant K^+ channel, was demonstrated to be endowed with functional properties compatible with a role in mediating K^+ influx (Schachtman et al., 1992). KAT1 is the main inward-rectifying K^+ channel in guard cell since its disruption leads to more than 50% reduction of the inward K^+ currents in *Arabidopsis* guard cell (Szyroki et al., 2001). Moreover, dominant negative repressive mutants of KAT1 and KAT2 suppress light- and low-CO_2-induced stomatal opening (Kwak et al., 2001; Lebaudy et al., 2008). These data provide genetic evidences demonstrating the important role of inward K^+ channels for stomatal opening. Other mechanisms are also involved in the regulation of these channel activities. Notably, KAT1 is sensitive to internal and external pH (Blatt, 1992; Hoth et al., 2001). Cytosolic 14-3-3 proteins also regulate KAT1 activity. The binding of the maize GF14-6 isoform to KAT1 enhances channel activity by increasing channel open probability and also by controlling the number of channels at the plasma membrane (Sottocornola et al., 2006, 2008).

In contrast to inward K^+ channels, only one outward rectifying K^+ channel, GORK, is expressed in *Arabidopsis* guard cell. GORK disruption completely abolishes outward K^+ channel currents in guard cells and impairs dark- and ABA-induced stomatal closure (Hosy et al., 2003). GORK currents are regulated by external K^+ concentration and also by internal and external pH (Blatt, 1992; Ache et al., 2000). Interestingly, by mass spectrometry-based proteomic analysis of tag affinity-purified 14-3-3ω complexes, GORK was identified as a putative 14-3-3 client (Chang et al., 2009). However, further studies will be required to determine the physiological function of GORK regulation by 14-3-3s in guard cells.

Vacuolar Ion Channels

During stomatal closure, K^+ release from vacuoles into the cytosol occurs via channels. Three cation channel activities have been characterized in guard cell tonoplast: fast vacuolar (FV) channels, vacuolar K^+-selective (VK) channels, and slow vacuolar (SV) channels (Pandey et al., 2007). FV channels are instantaneously activated in response to voltage and inhibited by cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) (Hedrich and Neher, 1987; Allen and Sanders, 1996). However, the molecular identity of these channels still remains unknown. VK channels are voltage-independent, K^+-selective, and activated by increases in $[Ca^{2+}]_{cyt}$ (Ward and Schroeder, 1994; Allen and Sanders, 1996). In *Arabidopsis*, the voltage-independent K^+-channels of the TPK/KCO family consists of five "tandem-pore" channels (TPK1-TPK5) and one K_{ir}-like channel (KCO3) (Voelker et al., 2010). Except for TPK4, these channels are located in the tonoplast and contain 14-3-3-binding sites and Ca^{2+}-binding EF-hands in their N- and C-termini, respectively (Voelker et al., 2006). Their conserved 14-3-3-binding site conforms to the consensus mode I binding motif with a serine or threonine residue as potential phosphorylation site (Latz et al., 2007). Using this phosphorylated conserved motif in surface plasmon resonance (SPR) experiments, it was demonstrated that HvKCO1/HvTPK1, a barley homologue of *Arabidopsis* TPK1, interacts with three out of the five barley 14-3-3 isoforms (Sinnige et al., 2005). Moreover, *Arabidopsis* TPKs (TPK1, 3 and 5) also bind to 14-3-3s both *in vitro* and *in vivo* (Latz et al., 2007; Voelker et al., 2010; Shin et al., 2011). Phosphorylation of the 14-3-3-binding motif in TPK1 and TPK5 appears to be a prerequisite for their interaction with 14-3-3s. Indeed, in yeast two-hybrid assays, mutating serine or threonine residue to alanine in the 14-3-3-binding sites (TPK1 : S42A; TPK5 : T83A) abolishes the interactions between channel N-terminal segments and 14-3-3s (Voelker et al., 2010). In plant cells, TPK1, but not the TPK1-S42A mutant, co-localizes with 14-3-3λ at the tonoplast (Latz et al., 2007). In the same study, pull-down assays and surface plasmon resonance measurements show high affinity interaction of 14-3-3λ with phosphorylated TPK1. After TPK1 expression in yeast and isolation of vacuoles, 14-3-3λ when applied to the cytosolic side of the membrane, strongly increases TPK1 currents in patch-clamp experiments. TPK1 channel activity in yeast exihibits all the hallmarks of the VK channel, i.e., K^+ selectivity, activation by cytosolic Ca^{2+}, and voltage independence (Bihler et al., 2005; Latz et al., 2007). Furthermore, instantaneous VK channel currents are absent in *tpk1* knockout mutants (Gobert et al., 2007). Based on these results, it is assumed that TPK1 represents the VK channel characterized in guard cells (Ward and Schroeder, 1994; Allen and Sanders, 1996). In accordance, TPK1 loss-of-function mutants display slower ABA-induced stomatal closure, thus providing evidence that VK channels can mediate vacuolar K^+ efflux for stomatal closing (Gobert et al., 2007). SV channels are cation permeable, voltage-regulated and slowly activated at elevated $[Ca^{2+}]_{cyt}$

(Hedrich and Neher, 1987; Ward and Schroeder, 1994; Allen and Sanders, 1996). The SV channels are ubiquitous in plants and encoded by the single *TPC1* (*two-pore channel 1*) gene in *Arabidopsis* (Peiter et al., 2005; Ranf et al., 2008). In *tpc1* knockout mutants, inhibition of stomatal opening by extracellular Ca^{2+} is impaired, whereas ABA-promoted stomatal closure is not affected (Peiter et al., 2005). Besides Ca^{2+}, SV channels have also been reported to be regulated by 14-3-3 proteins. Indeed, in mesophyll cell vacuoles, the barley SV channel is strongly inhibited by the barley 14-3-3B isoform and 14-3-3λ suppresses SV channel currents in *Arabidopsis* (Van den Wijngaard et al., 2001; Latz et al., 2007). Interestingly, TPC1 has the C-terminal sequence STSDT, which is a potential 14-3-3 type III binding site (Furuichi et al., 2001). However, although SV and VK channels have been both shown to be regulated by 14-3-3 proteins, further studies are required to address the physiological role of these regulations in stomatal movements.

REGULATION OF PROTEIN KINASES AND THEIR SUBTRATES BY 14-3-3 PROTEINS IN GUARD CELLS

Protein phosphorylation plays key roles in regulation of stomatal movements (Zhang et al., 2014). Among the kinases involved in guard cell signaling, calcium-dependent protein kinases (CDPKs) can act as Ca^{2+} sensors able to translate Ca^{2+} transients into specific phosphorylation events (Boudsocq and Sheen, 2013; Liese and Romeis, 2013). In *Arabidopsis*, the CDPK gene family encompasses 34 members (Cheng et al., 2002). Two *Arabidopsis* CDPK isoforms (also named CPKs), CPK1 and CPK3, regulate ion channels in guard cells and have been identified as 14-3-3 targets. Indeed, three *Arabidopsis* 14-3-3 isoforms, ω, ψ, and φ, stimulate autophosphorylated CPK1 *in vitro* by direct binding and CPK1 interacts with endogenous 14-3-3ω (Camoni et al., 1998; Chang et al., 2009). In guard cells, CPK1 activates a vacuolar Cl^- channel which may provide a pathway for anion uptake into the vacuole required for stomatal opening (Pei et al., 1996). Recently, CPK3, previously identified as a 14-3-3-binding protein *in vitro* (Moorhead et al., 1999; Cotelle et al., 2000), was found associated with 14-3-3 proteins in *Arabidopsis* (Lachaud et al., 2013). CPK3 is not activated *in vitro* by 14-3-3 proteins (Moorhead et al., 1999), but its interaction with 14-3-3s protects CPK3 from proteolysis (Cotelle et al., 2000; Lachaud et al., 2013). CPK3 directly interacts with the VK channel TPK1 (see above) at the tonoplast and is able to phosphorylate the 14-3-3-binding motif (S42) in the N-terminus of TPK1 (Latz et al., 2013). Moreover, CPK3 does not only phosphorylate sites mediating 14-3-3 binding and interact with 14-3-3s, but this kinase is also able to phosphorylate 14-3-3 proteins themselves, suggesting a cross-regulation between CPK3 and 14-3-3s (Lachaud et al., 2013; Swatek et al., 2014). CPK3 interaction with 14-3-3 proteins has not been described in guard cells, but CPK3 is one of the CDPKs involved in the activation of anion channels at the plasma membrane of guard cells, which is a critical step in stomatal closure (Mori et al., 2006; Geiger et al., 2010, 2011; Brandt et al., 2012; Scherzer et al., 2012; Brandt et al., 2015). Guard cells of double *cpk3 cpk6* knockout mutants show impaired ABA and Ca^{2+} activation of S-type anion channels, and ABA- and Ca^{2+}-induced stomatal closing are also partially inhibited in these mutants (Mori et al., 2006). Furthermore, CPK3 and CPK6 activate guard cell slow anion channels SLAC1 and SLAH3 in *Xenopus* oocytes, and are able to phosphorylate SLAC1 *in vitro* (Brandt et al., 2012; Scherzer et al., 2012). Interestingly, SLAC1 is also activated by CPK21 (Geiger et al., 2010) whose closest homologue in tobacco (*Nicotiana tabacum*), NtCDPK1, is a 14-3-3-binding protein. NtCDPK1 acts as a scaffold transferring 14-3-3 to its substrate, the transcription factor REPRESSION OF SHOOT GROWTH (RSG) after its phosphorylation, thus promoting RSG interaction with 14-3-3 proteins which negatively regulate RSG by sequestering it in the cytoplasm (Ito et al., 2014).

Besides CDPKs, the Ca^{2+}-independent protein kinase OST1 (open stomata 1), which plays a central role in stomatal closure (Kollist et al., 2014), could also mediate 14-3-3 binding to partners in guard cells. Indeed, targets of *Arabidopsis* OST1 include KAT1 (Sato et al., 2009), the bZIP transcription factor ABA-responsive-element binding factor 3 (ABF3) (Sirichandra et al., 2010) and plasma membrane respiratory burst oxidase homologs (RBOHs) (Sirichandra et al., 2009; Acharya et al., 2013). RBOHs are NADPH oxidases generating reactive oxygen species (ROS) which are important secondary messengers in stomatal closure induced by ABA or pathogen-associated molecular patterns (PAMPs) (Kwak et al., 2003; Mersmann et al., 2010; Macho et al., 2012). The tobacco 14-3-3 isoform Nt14-3-3h binds the C-terminus of the tobacco NADPH oxidase NtrbohD in yeast (Elmayan et al., 2007) and it has been speculated that the *Pseudomonas syringae* effector HopM1, which significantly contributes to bacterial pathogenesis, suppresses PAMP-triggered ROS production and stomatal closure through degradation of 14-3-3κ in *Arabidopsis* (Lozano-Durán et al., 2014). Moreover, in *Vicia faba*, an ortholog of OST1, AAPK (ABA-activated protein kinase), is able to phosphorylate a 61 kDa protein whose binding to a 14-3-3 protein is induced by ABA in guard cells (Takahashi et al., 2007).

CONCLUSION AND FUTURE PERSPECTIVES

Although many indirect indications point out that 14-3-3 proteins play important roles in stomatal movements, regulation of target proteins by 14-3-3s has been characterized in only a few cases in guard cells, as described in this mini-review. Therefore, many questions remain to be addressed in guard cells. What is the extent of the 14-3-3 interactome? What is the functional consequence of 14-3-3 binding to targets and how are these interactions regulated? What is the specificity of 14-3-3 isoforms towards their targets and in the regulation of stomatal movements? Combining protein biochemistry, cell biology and genetics approaches, future work addressing these questions will

further our knowledge with regard to the role of 14-3-3 proteins in guard cell signaling.

AUTHOR CONTRIBUTIONS

VC and NL contributed equally to the writing and editing of the manuscript.

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METHODS
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Measuring stress signaling responses of stomata in isolated epidermis of graminaceous species

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Our current understanding of guard cell signaling pathways is derived from studies in a small number of model species. The ability to study stomatal responses in isolated epidermis has been an important factor in elucidating the mechanisms by which the stomata of these species respond to environmental stresses. However, such approaches have rarely been applied to study guard cell signaling in the stomata of graminaceous species (including many of the world's major crops), in which the guard cells have a markedly different morphology to those in other plants. Our understanding of guard cell signaling in these important species is therefore much more limited. Here, we describe a procedure for the isolation of abaxial epidermal peels from barley, wheat and *Brachypodium distachyon*. We show that isolated epidermis from these species contains viable guard cells that exhibit typical responses to abscisic acid (ABA) and CO_2, as determined by measurements of stomatal apertures. We use the epidermal peel assay technique to investigate in more detail interactions between different environmental factors in barley guard cells, and demonstrate that stomatal closure in response to external CO_2 is inhibited at higher temperatures, whilst sensitivity to ABA is enhanced at 30°C compared to 20 and 40°C.

Keywords: stomata, guard cells, isolated epidermis, cereal, Gramineae, abscisic acid, carbon dioxide, temperature

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Introduction

The maintenance of global food is one of the greatest challenges currently facing plant scientists. Water availability is a major constraint on crop yield (Sinclair and Rufty, 2012) and is the single most important factor limiting food production, with significant yield losses reported under water deficit (Boyer, 1982; Mueller et al., 2012; van Ittersum et al., 2013). Stomata play a key role in determining crop water use efficiency (biomass production or yield per unit of water used), through the regulation of the exchange of water vapor and CO_2 between plant tissues and the atmosphere (Mansfield et al., 1990; Hetherington and Woodward, 2003; Yoo et al., 2009). This gaseous exchange is controlled by the size of the stomatal pore, which is determined by changes in the turgor of the pair of specialized guard cells that surround the pore and which in turn are driven by fluxes of anions and cations (Pandey et al., 2007; Kim et al., 2010; Hedrich, 2012; Kollist et al., 2014). Guard cells integrate information from a variety of internal and external environmental signals in order to formulate the optimal pore size for a given set of environmental conditions (Mansfield et al., 1990; Hetherington and Woodward, 2003; Kim et al., 2010). For example, stomata close in response to abscisic acid

(ABA), produced under conditions of limited water availability, and to elevated CO_2 (Mansfield et al., 1990; Hetherington and Woodward, 2003; Kim et al., 2010). In contrast, stomata open at low CO_2 concentrations (Bunce, 2007), in high light (Shimazaki et al., 2007) and in response to auxin (Acharya and Assmann, 2009). Guard cells also respond to other environmental signals, such as the atmospheric pollutant ozone (Vainonen and Kangasjarvi, 2015) and pathogenic microbes (Sawinski et al., 2013), resulting in stomatal closure and thereby preventing entry to the leaf of damaging chemical and biological agents.

The diversity of stimuli to which stomata respond, together with the ease with which the response can be quantified (i.e., changes in stomatal aperture or conductance), have meant that guard cells have been extensively used as a model system for studying signaling pathways in plant cells (Mansfield et al., 1990; Hetherington and Brownlee, 2004; Kim et al., 2010). This has resulted in the elucidation of a complex signaling network controlling the molecular machinery integrating the different signals to which guard cells are exposed in order to regulate guard cell turgor (Mansfield et al., 1990; Hetherington and Woodward, 2003; Kim et al., 2010). The ability to measure changes in stomatal aperture in isolated epidermis, in response to externally-applied signals, and to manipulate these responses both pharmacologically and genetically, has been central to the advances in understanding of guard cell signaling that have been made in the last 20 years.

To date, studies of guard cell signaling have focused on a small number of model species, notably *Vicia faba*, *Commelina communis* and latterly, *Arabidopsis thaliana* (for reviews, see Hetherington and Brownlee, 2004; Kim et al., 2010). The stomata of all of these species possess kidney bean-shaped guard cells, which are typical of the large majority of plant families, including the mosses, ferns, gymnosperms and most angiosperms (Willmer and Fricker, 1996). However, the stomata of the monocotyledonous family, Gramineae (Poaceae; the true grasses), which includes the world's major cereal crops, have a different morphology, possessing characteristic dumb-bell shape guard cells and a pair of specialized subsidiary cells. The different morphology of graminaceous stomata provide them with different mechanical properties, which likely allow them to open and close more rapidly in response to environmental signals (Franks and Farquhar, 2007). It is therefore critical to understand fully the molecular mechanisms by which the stomata of the graminaceous species respond to environmental stresses, particularly in relation to the protection of global food security and the challenge of producing "more crop per drop" (Kijne et al., 2003) posed by future environmental changes in global temperature, CO_2 levels and water availability.

Although assays of stomatal responses have been performed using isolated epidermis from maize and wild grasses (Pallaghy, 1971; Incoll and Whitelam, 1977; Jewer and Incoll, 1980; Rodriguez and Davies, 1982), in general, graminaceous species are commonly regarded as poorly tractable systems for epidermal peel isolation. Other authors have isolated epidermal tissue for other purposes, such as microscopy (Zou et al., 2011) or metabolite analysis (Falter et al., 2015), but in these cases, tissues were not demonstrated to be suitable for stomatal assays. Here, we demonstrate that the epidermal peel assay used so extensively in other model systems is also applicable to model grass species, and we use it to identify interactions between temperature and signals stimulating stomatal closure in barley.

Materials and Methods

Plant Material

Seeds of *Brachypodium distachyon* (line *Bd21*, Vogel et al., 2006) were sown in a 1:1 mix of Sinclair multipurpose compost and silver sand (Sinclair Horticultural, UK) and grown under a 16-h photoperiod at 22°C ± 2°C, 70% relative humidity and 120 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) in a Microclima growth cabinet (CEC, Glasgow, UK). Wheat (cultivar Cadenza) and barley (cultivars Golden Promise and Optic) were grown in Levington M3 peat-based compost in a heated, passively ventilated glasshouse (minimum temperature 15°C, mean daytime temperature 25°C) with 14 h of supplementary lighting supplied by 125 W 50/60 Hz High output, Correct Spectrum Class 11 energy saving bulbs (wheat) or Osram Greenpower 600 W high pressure sodium lamps (barley). 2 days prior to peeling, plants were moved to controlled environment chambers set at 22°C, with a photoperiod of 14 h (wheat) or 16 h (barley) with Osram fluora lamps delivering 70–100 μmol m^{-2} s^{-1} light.

Preparation of Isolated Epidermis

Isolated epidermis was obtained from the abaxial surface of the first true leaf of 8–14 day old wheat and barley plants, when the first true leaf had stopped expanding (5–8 cm long). For *Brachypodium distachyon*, the youngest fully expanded leaves of 3–4 week old plants were used. Leaves were cut from the plant and bent over the forefinger with the adaxial surface facing upward. A shallow cut was made with a sharp razor blade horizontally across the leaf and a flap of leaf tissue lifted with a razor, leaving the lower epidermis intact (**Figure 1**). The leaf tissue was removed from the epidermis with forceps. Once a section of epidermis approximately 1 cm long was exposed, it was cut from the leaf and floated cuticle-side-up in 10 mM MES/KOH (pH 6.2), 50 mM KCl.

Viability Staining

Isolated epidermis was floated cuticle-side-up in 10 mM MES/KOH (pH 6.2), 50 mM KCl and incubated in a water bath at 22°C at a PPFD of 50–100 μmol m^{-2} s^{-1} provided by an array of five fluorescent tube lights (Sylvania White F13W) underneath the tank. Pieces of epidermis were transferred at intervals to a 0.001% (w/v) solution of fluorescein diacetate (FDA) in 10 mM MES/KOH (pH 6.2), 50 mM KCl and incubated for 30 min prior to observation under the fluorescence microscope (McAinsh et al., 1996). The percentage of viable guard cells was determined by comparing the fluorescent images to bright-field images.

Promotion of Stomatal Closure Assays

Isolated epidermis was floated cuticle-side-up in Petri dishes containing 10 ml of 10 mM MES/KOH (pH 6.2), 50 mM

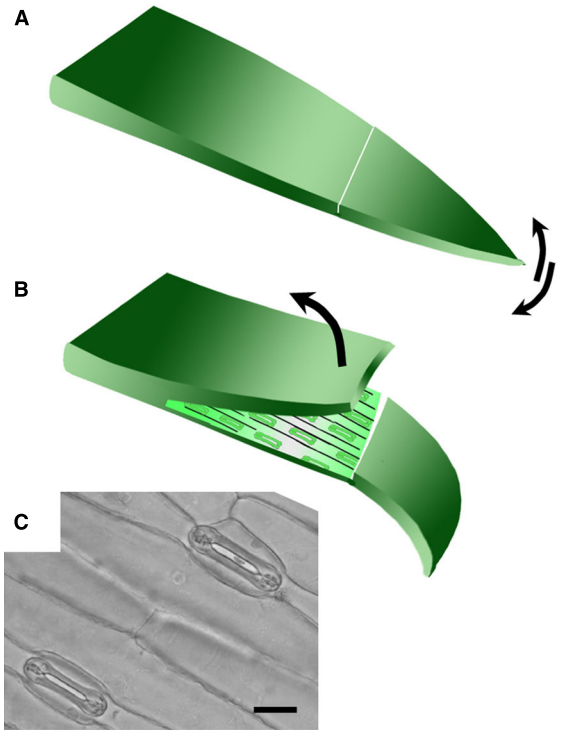

FIGURE 1 | Technique for removing abaxial epidermis. (A) The first true leaf is removed from the plant, bent over the finger adaxial side up, and a cut made across the lamina using a scalpel blade. The tip of the leaf blade is bent back and forth to detach a small section of the mesophyll from the lower epidermis. **(B)** The upper layer was then peeled back using forceps to leave the lower epidermis attached to the leaf tip. The size of the epidermal peel obtained typically varies from around 1–3 cm. **(C)** Bright field micrograph illustrating open stomata in a typical abaxial epidermal peel from wheat (20 μm scale bar).

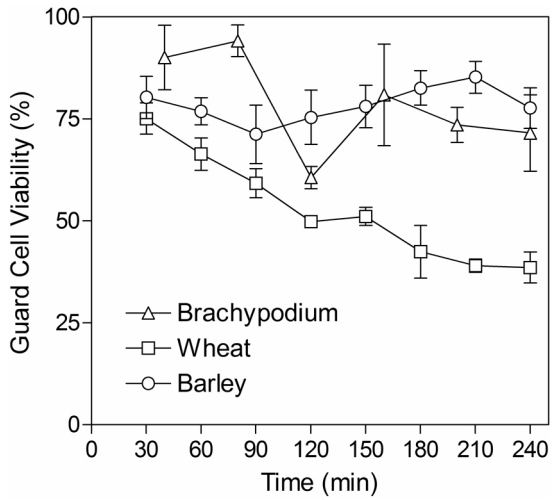

FIGURE 2 | Guard cell viability in epidermal peels. Peels were incubated in CO_2-free MES-KCl buffer at 22°C and then stained for 30 min in FDA. The x-axis indicates the time from the start of the initial incubation period until microscopic observation of FDA staining. Data represent counts from three areas of approximately 1 mm^2 from each piece of epidermis and three independent biological replicates.

KCl (a standard buffer to promote stomatal opening; McAinsh et al., 1991) under the conditions described above. Microlances, inserted through small holes in the lids, were used to deliver CO_2-free air to the Petri dishes. Pieces of epidermis were incubated for 2 h to promote stomatal opening, following which, they were transferred to fresh buffer containing the appropriate concentrations of ABA and incubated for a further one (barley) or two (wheat, *Brachypodium*) hours. ABA was diluted from a 10 mM stock of (±)-*cis*, *trans*-ABA dissolved in ethanol. Control solutions lacking ABA always contained ethanol equivalent to the concentration of the highest ABA concentration used. For experiments where CO_2 concentration was varied, gas from a balanced CO_2-air cylinder (BOC Industrial Gases, UK) at the appropriate concentration was bubbled through the incubation medium.

Measurement of Stomatal Apertures

Stomatal apertures were measured at the end of the incubation period by mounting pieces of isolated epidermis onto a microscope slide in a drop of assay buffer with a coverslip. Measurements of stomatal apertures were made using an inverted microscope connected to a sideport-mounted video monitor. A calibrated scale was used to make measurements of the width of the stomatal pore directly from the screen. Stomata containing non-viable guard cells (identifiable by FDA staining) were typically fully closed. Fully closed stomata were therefore not used for measurements. Where comparisons of multiple variables measured in experiments conducted at different times were required, stomatal apertures were expressed as relative values. Relative stomatal aperture was defined as the ratio of stomatal aperture measured under a treatment variable to that measured from the relevant control group.

Results

Guard Cells from Wheat, Barley, and *Brachypodium* Remain Viable in Epidermal Peels

Although grasses are less amenable than current model species used for stomatal research, we developed an approach that could be used routinely to generate intact abaxial epidermis isolated from leaves of wheat, barley and *Brachypodium* seedlings (see "Materials and Methods"). Peels were free of mesophyll cells and contained viable guard cells, subsidiary cells and pavement cells, as determined by FDA staining. Stomata were significantly larger in barley and wheat (typically around 25–50 μm in length) compared to those in the smaller *Brachypodium* plants (guard cells typically 6–9 μm in length). In order to be useful for measuring guard cell-mediated stomatal responses via an *in vitro* assay, it is essential that guard cell viability is maintained in isolated epidermis. We monitored viability over the period when assays are typically performed by performing FDA staining at regular intervals for up to 4 h following isolation. **Figure 2** illustrates that guard cell viability was around 80% 30 min after isolation for all species. This level was maintained throughout the test period for

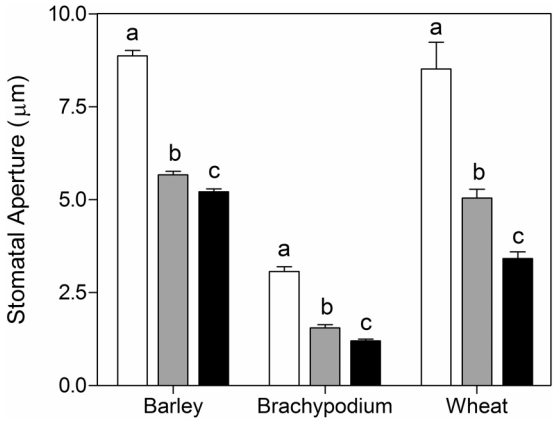

FIGURE 3 | Promotion of closure of cereal stomata by ABA. Following incubation under opening conditions, epidermal peels were exposed to zero (white bars), 10^{-7} M (gray bars), or 10^{-6} M (black bars) ABA at 20°C. Values shown are mean stomatal apertures ± SE from $n = 240$ (barley), $n = 90$ (wheat), and $n = 120$ (*Brachypodium*) measurements. Letters indicate statistically different means within species, determined using one-way ANOVA and a Tukey post-test.

both barley and *Brachypodium*, whilst viability gradually declined for wheat.

Cereal Leaf Epidermis Demonstrates Stomatal Closure in Response to ABA and CO_2

To test the validity of the stomatal assay in isolated epidermis of graminaceous plants, we examined the well-known response of guard cells to ABA in a promotion of closure assay. As expected, we observed that wheat, barley and *Brachypodium* all exhibit a characteristic dose-dependent response to ABA in the epidermal peel assay (**Figure 3**). We also measured responses to external CO_2. In comparison with CO_2-free air, stomatal closure was promoted by ambient CO_2 (360 ppm) in barley, but we observed no further response at elevated CO_2 (800 ppm; **Figure 4A**). *Brachypodium* stomatal apertures were also reduced by CO_2 (**Figure 4A**). We next examined the interaction between ABA and CO_2 signaling in barley guard cells. We observed a clear additive effect of ABA and CO_2, with lower stomatal apertures at all concentrations of ABA in the presence of either ambient or elevated CO_2 relative to CO_2-free controls (**Figure 4B**).

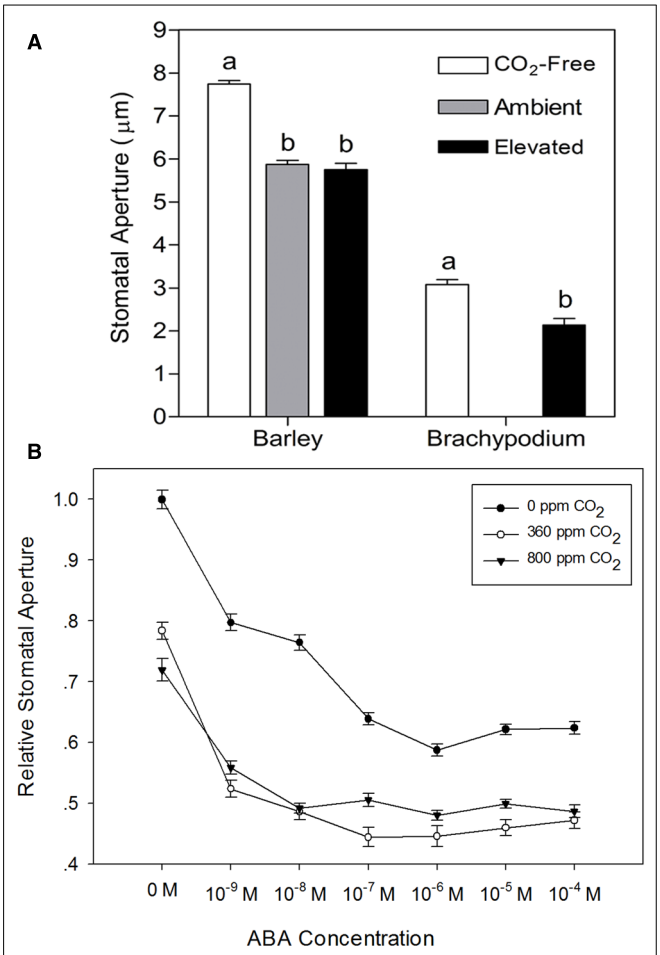

FIGURE 4 | Responses of cereal stomata to external CO_2. (A) Following incubation under opening conditions, epidermal peels were exposed to CO_2-free air (white bars), or air with ambient CO_2 (gray bar; 360 ppm, barley), or elevated CO_2 (black bars; 800 ppm, barley; 700 ppm, *Brachypodium*) at 20°C. Values shown are mean stomatal apertures ± SE ($n = 120$). Letters indicate statistically different means within species, determined using one-way ANOVA with Tukey post-test and a Student's *t*-test for barley and *Brachypodium* data respectively. **(B)** Interaction between CO_2 and ABA. Following incubation under opening conditions, barley epidermal peels were exposed to ABA at the concentrations shown on the x-axis under either CO_2-free air (filled circles), or air with ambient (open circles) or elevated CO_2 (filled triangles) at 20°C. Values shown are mean stomatal apertures ± SE ($n = 240$).

Barley Responses to ABA and CO_2 are Modified by Elevated Temperature

Stomatal responses to ABA and CO_2 have been shown to be temperature-dependent (e.g., Raschke, 1970; Rodriguez and Davies, 1982; Spence et al., 1984; Honour et al., 1995). We therefore used the epidermal peel assay to examine the temperature-dependence of ABA- and CO_2-induced stomatal closure in isolated epidermis of barley. First, we generated dose-response curves for ABA in isolated epidermis incubated at 20, 30, or 40°C. The results presented in **Figure 5A**, show that in comparison with the response at 20°C, incubation at 30°C significantly increased the sensitivity of guard cells to ABA. Increasing the temperature to 30°C had no effect on apertures in the absence of ABA, but apertures were reduced for all concentrations of ABA tested. By contrast, incubation at 40°C caused a significant increase in apertures in the absence of ABA and with 10^{-9} M ABA. At higher ABA concentrations, apertures were similar to, or slightly larger than, those observed at 20°C, suggesting a degree of inhibition of ABA sensitivity at 40°C. We also examined the response of barley guard cells to CO_2 at these three temperatures. Guard cell responses were again temperature-dependent. However, unlike the response to ABA, we observed maximum CO_2-induced stomatal closure at 20°C, with increasing temperatures causing an increasing degree of inhibition of the CO_2 response (**Figure 5B**).

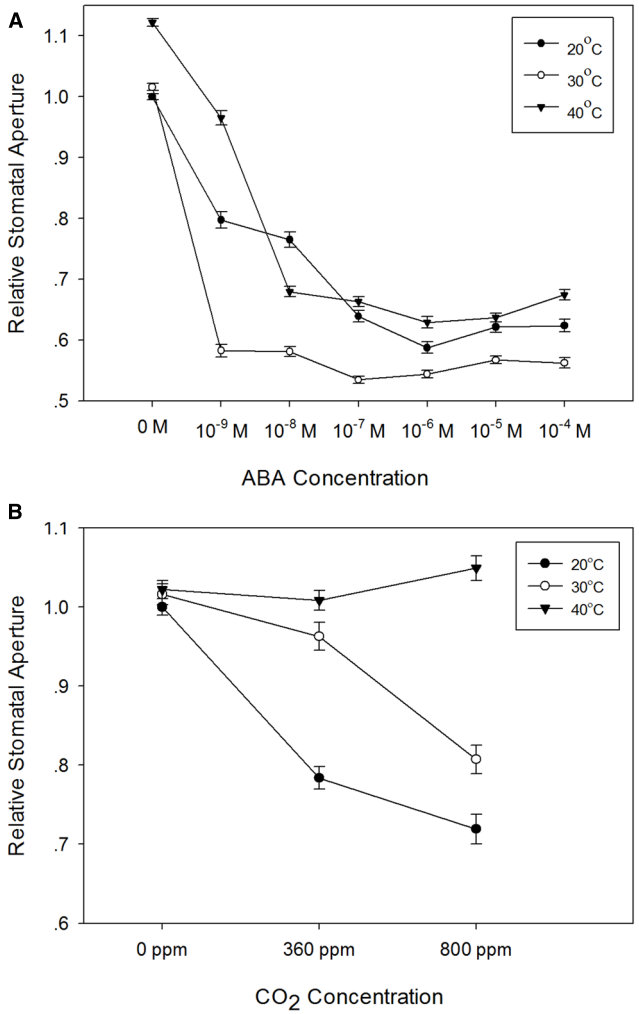

FIGURE 5 | Temperature-dependence of the responses of barley stomata to ABA and CO_2. (A) Interaction between temperature and ABA. Promotion of closure assays were performed at a range of concentrations of ABA in CO_2-free air at either 20°C (filled circles), 30°C (open circles), or 40°C (filled triangles). To normalize for variation between different experiments, stomatal apertures are expressed relative to that of the control (stomatal aperture at 20°C, no ABA). Data from at least 3 sets of independent experiments were pooled and values are means of at least 120 measurements ± SE. **(B)** Interaction between temperature and CO_2. Promotion of closure assays were performed in CO_2-free air or at ambient or elevated CO_2 at either 20°C (filled circles), 30°C (open circles), or 40°C (filled triangles). To normalize for variation between different experiments, stomatal apertures are expressed relative to that of the control (stomatal aperture at 20°C, no CO_2). Data from at least three sets of independent experiments were pooled and values are means of at least 120 measurements ± SE.

Discussion

With few exceptions, all of the components of the guard cell signaling network have to date been identified in model species with kidney bean-shaped guard cells, and similar responses have been assumed for graminaceous species containing dumb-bell shaped guard cells. However, there is increasing evidence for species-specific responses to common regulatory cues, driven by different environmental conditions (Prokic et al., 2006; Mori and Murata, 2011; Merilo et al., 2014). It is therefore important to consider signaling in key crop species as well as laboratory models. Given the current concerns over our ability to increase food production in the face of environmental change and to maintain global food security (van Ittersum et al., 2013), it is desirable to establish a robust experimental system for investigating guard cell signaling responses to the multiple environmental stresses currently faced by cereal crops.

The epidermal peel assay has been used to measure stomatal guard cell responses to external stimuli for several decades (Mansfield et al., 1990; Kim et al., 2010; Kollist et al., 2014). Although it has been suggested that the removal of the stomatal complex from the biochemical and physical influences of the mesophyll tissues means that the epidermal peel assay cannot always accurately reflect stomatal responses in intact leaves (Lee and Bowling, 1992; Roelfsema and Hedrich, 2002), it has nevertheless served as an important tool in the elucidation of the complex signaling network within guard cells (Mansfield et al., 1990; Hetherington and Brownlee, 2004; Kim et al., 2010). Despite this fact, few studies have examined the molecular mechanisms by which the dumb-bell shaped guard cells of the Gramineae respond to environmental stimuli and whether these reflect our current understanding of the signaling network in the kidney bean-shaped guard cells of the model species studied to date. This has been due largely to graminaceous species being considered intractable to the necessary cell physiological techniques. We have demonstrated here that the isolation of epidermis containing viable guard cells, whilst technically more demanding than in other model species, can be established as a routine technique to permit such investigations.

Epidermis was most easily obtained from young plants and guard cell viability was capable of being maintained at levels suitable for collection of aperture data from large numbers of stomata. While in all three species tested there was significant variability between individual peels, overall guard cell viability was maintained at around 70–80% for 4 h in barley and *Brachypodium*, although it declined to around 40% in wheat. This compares to between 85% (*Pisum sativum*) and 100% (*C. communis* and *V. faba*), respectively (Weyers and Travis, 1981). All three species exhibited stomatal closure in response to ABA and CO_2, consistent with previous reports of ABA- and CO_2-induced stomatal closure in isolated epidermis of model species with kidney bean-shaped guard cells (for reviews, see Hetherington and Brownlee, 2004; Kim et al., 2010; Mori and Murata, 2011). These responses highlight the epidermal peel assay as a useful tool for dissecting guard cell signaling pathways in grasses.

We used the epidermal peel assay to measure barley guard cell responses to ABA, CO_2 and temperature alone and in combination. In experiments where we simultaneously applied two closing signals, ABA and CO_2, we observed a simple additive response at 20°C, whereby apertures were smaller in the presence of both ABA and CO_2 than for the individual stimuli. Interactions between either ABA or CO_2 and temperature, however, were more complex. Incubation of epidermal strips under opening conditions (light, CO_2-free air, no ABA) at different temperatures

resulted in similar apertures, but with a small but statistically significant increase in aperture at 40°C. Upon addition of ABA, apertures were reduced much more markedly at 30°C than at 20°C or at 40°C. The enhanced closure at 30°C could result either from altered biophysical properties of the stomatal complexes, or from increased sensitivity to ABA. Since the effect was only apparent at 30°C, and not at 40°C, a simple biophysical effect of temperature seems less likely, and we therefore suggest that the temperature-dependency of ABA-induced stomatal closure in barley reflects interactions between temperature and ABA signaling pathways. Similar increases in sensitivity to ABA at elevated temperatures have previously been observed in some dicotyledonous species (Honour et al., 1995; Cousson, 2003). Interestingly, the response of the Arabidopsis *RESPONSIVE TO DESSICATION 29A* (*RD29A*) promoter to exogenous ABA was also enhanced at elevated temperature (Xiong et al., 1999), suggesting the possibility of a more general increase in ABA sensitivity at elevated temperature.

Temperature had a different and very pronounced effect on the response of barley guard cells to external CO_2. At 20°C, both ambient and elevated CO_2 treatments resulted in a substantial reduction in stomatal aperture. At 30°C, the effect of ambient CO_2 was strongly diminished, and whilst guard cells still responded to elevated CO_2 at 30°C, responses to both ambient and elevated CO_2 concentrations were lost at 40°C. These observations are consistent with previous work in maize (Raschke, 1970) and bean (Spence et al., 1984), where a loss of stomatal responses to CO_2 at higher temperatures was found for both species. Since barley guard cells are able to close in response to ABA at 40°C (**Figure 4A**), an interaction between temperature and CO_2 signaling pathways again provides the simplest explanation of our data.

Together, our results clearly demonstrate the suitability of the epidermal peel assay for studying guard cell signaling networks in the dumbell-shaped guard cells of the Gramineae and that these studies can provide important insights into the mechanisms by which the stomata of the world's major cereal crops respond to the multiple stresses resulting from predicted future changes in global temperature, CO_2 levels and water availability (Stocker et al., 2013). Such studies will help to inform future strategies for improving the water use efficiency of cereal crops and for mitigating the adverse effects of climate change on cereal crop production.

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