ARP2/3 complex-mediated actin dynamics is required for hydrogen peroxide-induced stomatal closure in Arabidopsis

Running title: \( H_2O_2 \) product and actin dynamics in ABA signaling

XIN LI\(^1\,2\)\(^\ddagger\), JIAN-HUA LI\(^1\)\(^\ddagger\), WEI WANG\(^1\)\(^\ddagger\), NAI-ZHI CHEN\(^2\), TONG-SUO MA\(^3\), YA-NAN XI\(^1\), XIAO-LU ZHANG\(^1\), HAI-FEI LIN\(^1\), YANG BAI\(^1\), SHAN-JIN HUANG\(^2\), YU-LING CHEN\(^1\)

\(^1\)Hebei Key Laboratory of Molecular and Cellular Biology, College of Life Science, Hebei Normal University, Shijiazhuang, 050024, China

\(^2\)Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing, 100093, China

\(^3\)College of Biological Science and Engineering, Hebei University of Economics & Business, Shijiazhuang, 050061, China

\(^\ddagger\)These authors contributed equally to this work.

Authors for correspondence:

SHAN-JIN HUANG, Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing, 100093, China; E-mail: sjhuang@ibcas.ac.cn

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YU-LING CHEN, College of Life Science, Hebei Normal University, No. 20 East Road of 2nd Ring South, Shijiazhuang, 050024, China; E-mail: yulingchen@mail.hebtu.edu.cn

ABSTRACT

Multiple cellular events like dynamic actin reorganization and hydrogen peroxide (H$_2$O$_2$) production were demonstrated to be involved in abscisic acid (ABA) -induced stomatal closure. However, the relationship between them as well as the underlying mechanisms remains poorly understood. Here, we showed that H$_2$O$_2$ generation is indispensable for ABA induction of actin reorganization in guard cells of Arabidopsis that requires the presence of ARP2/3 complex. H$_2$O$_2$-induced stomatal closure was delayed in the mutants of arpc4 and arpc5, and the rate of actin reorganization was slowed down in arpc4 and arpc5 in response to H$_2$O$_2$, suggesting that ARP2/3-mediated actin nucleation is required for H$_2$O$_2$-induced actin cytoskeleton remodeling. Furthermore, the expression of H$_2$O$_2$ biosynthetic related gene AtrbohD and the accumulation of H$_2$O$_2$ was delayed in response to ABA in arpc4 and arpc5, demonstrating that misregulated actin dynamics affects H$_2$O$_2$ production upon ABA treatment. These results support a possible causal relation between the production of H$_2$O$_2$ and actin dynamics in ABA-mediated guard cell signaling: ABA triggers H$_2$O$_2$ generation that causes the reorganization of the actin cytoskeleton partially mediated by ARP2/3 complex, and ARP2/3 complex-mediated actin dynamics may feedback regulate H$_2$O$_2$ production.
Key words: hydrogen peroxide; actin dynamics; ARPC4; ARPC5; ABA; guard cells
INTRODUCTION

Guard cells localized in the epidermis form stomatal pores, and stomatal aperture variation regulates both CO$_2$ influx from the atmosphere and transpirational water loss from plants. Various stimuli, including phytohormones, CO$_2$, light and darkness, regulate stomatal movements. Abscisic acid (ABA) is a widely studied phytohormone that enhances stomatal closure and inhibits stomatal opening through a complicated network. Although many molecules have been identified in this network, ABA guard cell signaling is not completely understood (Schroeder et al. 2001; Kim et al. 2010). Series of evidence showed that hydrogen peroxide (H$_2$O$_2$) is a rate-limiting second messenger in ABA guard cell signaling. H$_2$O$_2$ enhances stomatal closure and inhibits stomatal opening (Gudesblat et al. 2007), and is generated in guard cells in response to ABA (Pei et al. 2000; Zhang et al. 2001a), extracellular calmodulin (Chen et al. 2004; Li et al. 2009), pathogen elicitors (Lee et al. 1999), methyl jasmonate (Suhita et al. 2004), light/darkness (She et al. 2004), and ozone (Joo et al. 2005). H$_2$O$_2$ activates Ca$^{2+}$ channels in the plasma membrane (Hamilton et al. 2000; Pei et al. 2000) and inhibits the inward K$^+$ current in guard cells (Zhang et al. 2001b). Several protein kinases and protein phosphatases have been identified as playing an important role in H$_2$O$_2$ signal transduction, including NtMPK4 (Gomi et al. 2005), AtMPK3 (Gudesblat et al. 2007), AtMPK9/AtMPK12 (Jammes et al. 2009), members of the protein phosphatase 2C family (Murata et al. 2001), and protein tyrosine phosphatase (PTP) (MacRobbie, 2002). Nitric oxide (NO) is another pivotal member in ABA-induced stomatal closure (Neill et al. 2002a), and evidence shows that ABA-aroused NO generation is dependent on ABA-induced H$_2$O$_2$.
production in Arabidopsis guard cells (Bright et al. 2006). In addition, external application of 
H$_2$O$_2$ induces both cytosol alkalization and vacuolar acidification in guard cells of *Vicia faba* 
(Zhang et al. 2001c).

The actin cytoskeleton in plant cells forms a network that is involved in numerous cellular 
processes, such as participating in vesicle trafficking between endomembrane compartments 
(Kim et al. 2005) and serving as cellular motorways for the transport of various organelles 
(Sparkes et al. 2008; Gabrys 2004; Yokota et al. 2009). The actin array changes during plant 
development and in response to external (Hardham et al. 2007) and endogenous stimuli 
(Limichez et al. 2001). The cycle of actin filament assembly, or the actin dynamics, is 
regulated by diverse actin-related proteins, including profilin, ADF/Cofilin, CAP, capping 
protein, villin/gelsolin, formin, and Actin-Related Protein 2/3 (ARP2/3) complex (reviewed 
by Staiger & Blanchoin 2006). The ARP2/3 complex is implicated to play an essential role in 
actin filament nucleation and branching, therefore enhances formation of new actin filaments 
at a distinctive $70^\circ$ angle to the sides of pre-existing filaments (Kabsch & Holmes 1995; 
Cooper et al. 2001). The ARP2/3 complex was originally identified in Acanthamoeba 
(Machesky et al. 1994). Homologs for seven subunits (ARP2, ARP3, ARPC1-ARPC5) of the 
ARP2/3 complex have been revealed in Arabidopsis (Szymanski 2005), and the mutants 
arpc2/dis2, arpc3/dis1, arpc2/wrm, crooked/arpc5, and arpc4 show similar phenotypes (Li et al. 
2003; Mathur et al. 2003a, 2003b; Kotchoni et al. 2009). The ARP2/3 complex alone is 
inactive, and nucleation-promoting factors such as WAVE/SCAR (for WASP family
Verprolin homologous protein/Suppressor of cAMP Repressor) increase the efficiency of actin filament nucleation by the ARP2/3 complex (Welch & Mullins 2002).

Guard cells undergo volume changes during stomatal movements. It has been shown that actin filaments participate in the regulation of guard cell volume during stomatal movements (Liu & Luan 1998). Several lines of evidence suggest that array and orientation of actin filaments are positively correlated with the stomatal aperture. Actin filaments are radially oriented in the guard cells of open stomata, whereas long actin filaments have been found in a longitudinal direction or random orientation in guard cells of closed stomata of Commelina communis, Vicia faba, Arabidopsis thaliana, and Nicotiana tabacum (Kim et al. 1995; Hwang & Lee 2001; Gao et al. 2008; Zhao et al. 2011). It has been shown that actin dynamic changes during stomatal movements are regulated by cytosolic calcium, protein kinase and phosphatase (Hwang & Lee 2001), phosphatidylinositol 3- and 4-phosphate and ROS (Choi et al. 2008), and AtRac1 (Lemichez et al. 2001). In addition, actin filament disruption and abnormal stomatal closure are co-induced by overexpression of Arabidopsis-depolymerization factor, AtADF1 (Dong et al. 2001). STOMATAL CLOSURE-RELATED ACTIN BINDING PROTEIN1 (SCAB1) has been shown to be involved in the precise regulation of actin remodeling during stomatal closure (Zhao et al. 2011). Recently, Jiang et al. (2012) reported that mutation in ARPC2, a subunit of the ARP2/3 complex, affects both actin disorganization and stomatal closure in response to ABA. Furthermore, the aberrant actin organization in guard cells of arp2 and arp3 mutants possibly impairs vascular fusion during stomatal
opening (Li et al. 2013). However, the regulatory mechanism of actin dynamics in guard cells is not completely understood. In this study, we provide evidence that ARPC4 and ARPC5, two other subunits of the ARP2/3 complex, were involved in ABA- and H$_2$O$_2$-induced guard cell actin reorganization. Changes in the ABA-induced H$_2$O$_2$ levels were affected by aberrant actin dynamics in arpc4 and arpc5. We hypothesize that a possible mutual regulation between H$_2$O$_2$ generation and ARP2/3 complex-regulated actin dynamic changes exists in ABA guard cell signaling.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild type and various mutants of Arabidopsis (*Arabidopsis thaliana*) plants in a Col-0 background were grown in a greenhouse under long day conditions (16-h-light/8-h-dark cycle) with a photon flux density of 0.30 mmol m$^{-2}$ s$^{-1}$ and a temperature of 18 – 22 °C. Fully expanded leaves from 3- to 4-week-old plants were used for the stomatal bioassay, the visualization of actin configurations, the measurement of H$_2$O$_2$ levels in guard cells and quantitative RT-PCR analysis.

Analysis of mutants

Arabidopsis ecotype Columbia (Col-0) was used as the wild type in this study. The previous reported T-DNA insertion mutants of *arpc4* (SALK_073297, Kotchoni et al. 2009) and *arpc5* (SALK_123936, Li et al. 2003) from Arabidopsis Biological Resource Center (ABRC,
http://abrc.osu.edu/) were confirmed by RT-PCR with the primers: ARPC4F, 5’-ATGGCAAAACTCATTACGGCTGT-3’; ARPC4R, 5’-TTACATGAACTGTTTCAAGAAC-3’; ARPC5F, 5’-ATGGCAGAATTCTGTTGAAGCTG-3’; ARPC5R 5’-TCAAACGTTGATGATGATCA-3’. The dSpm transposon insertion mutant of atrbohD/F was confirmed according to a previous report (Chen et al. 2004).

**Stomatal bioassay**

Stomatal assays were carried out essentially as described previously (Li et al. 2009). Briefly, 3- to 4-week-old rosette leaves were harvested and incubated in MES buffer (10 mM MES-Tris, 30 mM KCl and 0.1 mM CaCl₂, pH 6.1) for 90 min under light to open the stomata. To study the effects of ABA or H₂O₂ on stomatal closure, leaves with open stomata were transferred to MES buffer containing 10 μM ABA, 10⁻⁵ M or 10⁻⁴ M H₂O₂ for 5, 15, 30, 60 or 120 min. To investigate the effects of jasplakinolide or LatB on H₂O₂ induction of stomatal closure, leaves with open stomata were pretreated either with 1 μM jasplakinolide or 10 μM LatB in MES buffer for 30 min and then transferred to and incubated in 10⁻⁴ M H₂O₂ solution plus 1 μM jasplakinolide or 10 μM LatB for 5, 15, 30 or 60 min. All experiments were conducted under light. Subsequently, abaxial epidermal strips were peeled, and the stomatal apertures were determined with a microscope. Fifty stomata were randomly selected for three independent repeats at each indicated time point. The data are presented as the mean ± SE (n = 150).
Visualization of F-Actin by Confocal Laser Scanning Microscopy (CLSM)

$arpc4$, $arpc5$ and $atrbohD/F$ mutants expressing GFP-ABD2-GFP were obtained by crossing between these mutants and 35S::GFP-ABD2-GFP transgenic lines (Wang et al. 2008). The intact leaves were treated as described for stomatal assays to open the stomata, and then the distribution of actin filaments in guard cells on leaves with various treatments was observed using CLSM (Carl Zeiss, 510 LSM meta) with a setting of 488 nm excitation and 525 nm emission. Eighty to 200 guard cells were observed and classified into 3 types for each indicated time point.

Quantitative analyses of peak intensity, occupancy and skewness of the actin cytoskeleton

The peak intensity of bundles in type 1 actin was determined by measuring the continuous fluorescent intensity along the middle of the longitudinal direction of the guard cells (Fig. 5a) according to Eisinger et al. (2012), and the filament numbers were determined by the number of peaks with an intensity higher than 50. By Image J software described previously (Higaki et al. 2010), the density of actin filaments was estimated by defining the occupancy of the GFP signal in guard cells, and the actin bundling was determined by measuring the skewness of GFP fluorescence intensity distribution. Each parameter was the statistical result of more than 80 guard cells. All the analyses were carried out using the 8 bit raw scanning images.
Detection of the H$_2$O$_2$ level in guard cells

H$_2$O$_2$ detection in guard cells was performed as described previously (Chen et al. 2004). Leaves with open stomata were incubated in MES buffer containing 50 μM H$_2$DCF-DA (Molecular Probes; D399) in the dark for 15 min and then washed three times. The leaves were then transferred to MES buffer containing 10 μM ABA for 15, 30, 60 or 120 min. To study the effects of jasplakinolide or LatB on ABA-induced H$_2$O$_2$ generation, leaves with open stomata were transferred to and incubated in MES buffer containing 10 μM ABA with 1 μM jasplakinolide or 10 μM LatB for 15, 30, 60 or 120 min. At the indicated time points, abaxial epidermal strips were peeled from the leaves for H$_2$O$_2$ detection by CLSM with a setting of 488 nm excitation and 525 nm emission. The experiments were repeated at least three times with 80 cells for each time point.

Quantitative RT-PCR analysis

Leaves with open stomata of wild type, arpc4 and arpc5 were incubated to 10 μM ABA in MES buffer for 5, 10, 15, 30, or 60 min. Samples were frozen in liquid nitrogen. For each time point, 200 mg of leaves was used for total RNA isolation by TRIZOL Reagent (Invitrogen). After DNase treatment, 500 ng of total RNA was used for the first-strand cDNA synthesis using the PrimeScript(TM) RT Reagent Kit (TaKaRa, Dalian, China). Real time reverse transcription PCR was performed as described by Zhang et al. (2009). The specific primer pairs were as follows: AtrbohD(F), 5’-TTTGTTCTCTATATCCCCTACCCT-3’; AtrbohD(R), 5’-CATGTTTACAAACACCAAGCTG-3’; AtrbohF(F),...
5’-AGAGAGGTAGGTTTGATGAGGG-3’; AtrbohF(R),
5’-TTCCATCATATATCTGCTG-3’.

Construction of the ARPC4 and ARPC5 promoter-fused GUS gene and detection of GUS activity

The entire 1550-bp 5’-flanking region of ARPC4 and 1645-bp 5’-flanking region of ARPC5 were amplified from genomic DNA of Arabidopsis as the full-length promoters of the two genes. The two pairs of primers used in this study were: ARPC4F, 5’-CGGGATCTACCGTTCTTCACCAT-3’; ARPC4R, 5’-CGGAATTCATACAGCCGTAATGAGTTGC-3’; ARPC5F, 5’-AACTGCAGCGCCGTAATGAGTTG-3’; ARPC5R, 5’-CGGAATTCTTCTGCCGTTCTTCGATTC-3’. The ARPC4 and ARPC5 promoter regions were then inserted into the binary vectors pCambia 1391 and pCambia 1391z and fused to a β-glucuronidase (GUS) reporter gene, respectively. The recombinant P<sub>ARPC</sub>·GUS fusion constructs were introduced into Arabidopsis plant according to the flower-tip method (Clough and Bent, 1998). Hygromycin-resistant plants were transferred to soil for GUS activity assays.

P<sub>ARPC4</sub>·GUS or P<sub>ARPC5</sub>·GUS transgenic plants were grown for 3 to 4 weeks. Rosette leaves were harvested and the abaxial epidermis was peeled. Checking of GUS activity was carried out according to Jefferson et al. (1987).
RESULTS

H$_2$O$_2$ generation in guard cells is indispensable for ABA-induced actin dynamics during stomatal closure

To provide direct genetic evidence supporting the role of H$_2$O$_2$ generation in ABA-induced actin dynamics in Arabidopsis, we analyzed wild type and atrbohD/F mutant (the double mutant of D and F subunits of NADPH oxidases, Kwak et al. 2003) that express a vector with GFP fused to both the C- and N-termini of the actin-binding domain 2 (35S::GFP-ABD2-GFP, Wang et al. 2008). First, we visualized the actin cytoskeleton in living guard cells at 0, 15, 30, 60 and 120 min after 10 μM ABA treatment. The actin configurations were classified into three types according to Zhao et al. (2011): 1) radial arrangement of cortical actin bundles around the longitudinal axis of guard cells in the open stomata; 2) random distribution of actin filaments in the guard cells during stomatal closing; or 3) long actin cables along the longitudinal direction of guard cells in closed stomata (Fig. 1a). The statistical results showed that most guard cells had type 1 actin in open stomata of wild type at the beginning of ABA treatment (73% of the cell population at 0 min). During the progression of treatment, the proportion of guard cells with type 1 actin decreased gradually, and actin filaments in the majority of guard cells became randomly distributed. For example, by 15 and 30 min, the percentage of guard cells with type 1 actin decreased to 47% and 29%, and those with type 2 actin increased to 28% and 45% of the total cell population, respectively. From 60 to 120 min of ABA treatment, stomata were nearly closed, and most guard cells had type 3 actin (51% and 64% of the cell population by 60 min and 120 min, respectively) (Fig. 1b). Guard cells of
*atrbohD/F* also exhibited three actin types as wild type, but there was a great difference in the composition of cells with different actin types at each time point. At all time points of ABA treatment, the majority of the guard cells had type 1 actin in *atrbohD/F* (Fig. 1c), suggesting that H$_2$O$_2$ generation plays an important role in ABA-induced actin dynamic changes in guard cells. To confirm whether it was indeed caused by the defect in H$_2$O$_2$ production, we tried the exogenous application of H$_2$O$_2$ to *atrbohD/F* leaves, and analyzed actin changes in guard cells. Exogenous H$_2$O$_2$ at a concentration of $10^{-4}$ M is widely used in stomatal experiments (Bright *et al.* 2006; Hua *et al.* 2012) and has been demonstrated to induce stomatal closure of both wild type and *atrbohD/F* (Kwak *et al.* 2003). As shown in Fig. 1d, $10^{-4}$ M H$_2$O$_2$ was able to restore the actin change from type 1 to type 3 in *atrbohD/F*. For example, most guard cells in *atrbohD/F* plants contained type 2 actin from 5 min to 15 min of H$_2$O$_2$ treatment (47% and 60% of the cell population at 5 min and 15 min, respectively); by 30 min of H$_2$O$_2$ treatment, *atrbohD/F* guard cells having type 3 actin increased to 44% of the total cell population. By 60 min of H$_2$O$_2$ treatment, guard cells containing type 3 actin increased to 52% of the cell population (Fig. 1d). These results imply that H$_2$O$_2$ generation is downstream and indispensable for the ABA induction of actin dynamic changes.

**Both actin polymerization inhibitor and actin stabilizer inhibit H$_2$O$_2$-induced stomatal closure**

Both actin polymerization inhibitors and actin stabilizers disturb stomatal movements caused by ABA and light, implying that actin dynamic changes are essential for stomatal opening and
closing in response to stimuli (Kim et al. 1995; MacRobbie & Kurup 2007; Gao et al. 2008). To investigate whether actin dynamic changes are essential for the H$_2$O$_2$ induction of stomatal closure, we determined the effect of a treatment with H$_2$O$_2$ and an actin polymerization inhibitor or an actin stabilizer on stomatal apertures. As shown in Fig. 2, when leaves were pretreated with jasplakinolide, H$_2$O$_2$ failed to induce stomatal closure. Stomatal apertures treated with H$_2$O$_2$ and jasplakinolide were bigger than those due to H$_2$O$_2$ treatment alone (Fig. 2a). In contrast, a quicker decrease in the stomatal aperture was observed at 5 min after the leaves were treated with LatB along with H$_2$O$_2$, but stomata failed to close by the final time point (Fig. 2b). These data suggested that rapid actin turnover is required for and promotes the initial stage of H$_2$O$_2$-induced stomatal closure and implied that re-assembly of actin filaments is required for the progression of H$_2$O$_2$-induced stomatal closure at late stage.

**Mutation in ARPC4 and ARPC5, two subunits of the ARP2/3 complex, leads to slower stomatal closure in response to ABA and H$_2$O$_2$**

Direct visualization of the change in the actin cytoskeleton during H$_2$O$_2$-induced stomatal closure as well as the results of actin drug treatments indicated that an actin nucleation factor could be involved in this process. The ARP2/3 complex is extremely relevant, because it has been shown that a mutation in ARPC2, a subunit of ARP2/3 complex, causes impeded actin disorganization and reduced sensitivity of stomatal closure in response to ABA (Jiang et al. 2012). To examine whether the ARP2/3 complex is involved in this process, we analyzed the response to H$_2$O$_2$ of guard cells from plants with a mutation in ARPC4 and ARPC5. We first
confirmed the previous reported arpc4 and arpc5 mutants by RT-PCR, and both were null mutants (Supporting Information Fig. S1a). We next determined whether ARPC4 and ARPC5 are expressed in guard cells. We detected a strong GUS signal in guard cells of plants expressing P_{ARPC4::GUS} and P_{ARPC5::GUS} (Supporting Information Fig. S1b,c), and the expression of ARPC4 and ARPC5 was also shown in a guard cell transcriptome published recently (Obulareddy et al. 2013), suggesting that ARPC4 and ARPC5 are indeed expressed in guard cells. We initially determined the response of stomatal closure to ABA in arpc4 and arpc5 and found that the apertures of stomata in arpc4 and arpc5 were larger than those in wild type at different time points after ABA treatment prior to 120 min of treatment, but the stomatal apertures in arpc4 and arpc5 reached a similar size to those of wild type at the final time point (Supporting Information Fig. S1d), suggesting that stomatal closure was delayed in arpc4 and arpc5 in response to ABA. We next determined the response of stomatal closure to H$_2$O$_2$ treatment. Meanwhile, to determine the role of the ARP2/3 complex during H$_2$O$_2$-induced stomatal closure, we generated arpc4 atrbohD/F and arpc5 atrbohD/F triple mutant. Consistent with the data presented above, the exogenous application of 10$^{-4}$ M H$_2$O$_2$ was sufficient to induce the closure of atrbohD/F stomata (Fig. 3). However, the closure of both arpc5 and arpc5 atrbohD/F stomata was delayed compared to that of wild type and atrbohD/F, and arpc5 and arpc5 atrbohD/F exhibited similar stomatal closure behavior in response to H$_2$O$_2$ (Fig. 3a). The responses of stomata in arpc4 and arpc4 atrbohD/F were greatly consistent with arpc5 and arpc5 atrbohD/F when treated with 10$^{-4}$ M H$_2$O$_2$ (Fig. 3b). These results suggested that ARP2/3 is involved in ABA-induced stomatal closure and acts
downstream of \( \text{H}_2\text{O}_2 \) production.

The actin cytoskeleton became disorganized and the switch in actin arrays was delayed in \textit{arpc4} and \textit{arpc5} upon \( \text{H}_2\text{O}_2 \) and ABA treatment

We next sought to trace and analyze the actin dynamic changes in wild type, \textit{arpc4} and \textit{arpc5} upon treatment with \( 10^{-4} \) M \( \text{H}_2\text{O}_2 \). As shown in Figure 4, actin filaments in wild type showed similar changes to that of \textit{atrbohD/F} in response to \( \text{H}_2\text{O}_2 \) (Fig. 4a), and \textit{arpc4} and \textit{arpc5} guard cells exhibited similar actin configurations in each type, whereas the actin filaments looked sparser than in wild type guard cells (Fig. 4b). However, both \textit{arpc4} and \textit{arpc5} showed slower actin dynamic changes than wild type (Fig. 4c-e). For instance, by the stage of treatment with \( 10^{-4} \) M \( \text{H}_2\text{O}_2 \) for 5 min, major guard cells contained type 1 actin in \textit{arpc5} (61% of the cell population), whereas most guard cells in wild type had type 2 actin (51% of the cell population). By 30 min, most \textit{arpc5} guard cells contained type 2 actin (60% of the cell population), and only 20% of the guard cells had type 3 actin; however, by the same treating time, majority of wild type had type 3 actin (55% of the cell population), and 34% of the guard cells had type 2 actin. These results showed that both the switches from type 1 to type 2, and type 2 to type 3 actins were delayed in \textit{arpc5} compared with wild type. By 60 min, most guard cells had type 3 actin in both \textit{arpc5} and wild type (64% of the cell population in \textit{arpc5} and 77% of the cell population in wild type) (Fig. 4c,d). \textit{arpc4} guard cells showed similar actin changes as \textit{arpc5} in response to \( \text{H}_2\text{O}_2 \) (Fig. 4e). These results demonstrated that \textit{arpc5} and \textit{arpc4} were slower in both actin disorganization and remodeling in guard cells in response
to H$_2$O$_2$. We also visualized the actin configurations in arpc5 and arpc4 after treatment with ABA. As shown in Supporting Information Fig. S1, actin filaments in arpc5 and arpc4 guard cells also exhibited type 1, 2 and 3 actin configurations with ABA treatment. Additionally, it took longer for the transition between two consecutive actin types in arpc5 than in wild type when treated with ABA. For example, after 30 min of ABA treatment, the majority of the guard cells (about 64% of the cell population) exhibited type 1 actin in arpc5, whereas major guard cells had type 2 actin in wild type (45% of cell population); after 60 min ABA treatment, approximately 43% of guard cells had type 2 actin in arpc5, while most wild type guard cells had type 3 actin (51% of cell population). The time required for most arpc5 guard cells to contain type 3 actin was delayed to 120 min (Supporting Information Fig. S1f & Fig. 1b). arpc4 guard cells also exhibited slower actin type transition than wild type (Supporting Information Fig. S1g). Taken together, our findings suggested that the transition of the actin cytoskeleton between two consecutive actin types in most arpc5 and arpc4 guard cells took longer than in wild type, and the actin cytoskeleton appeared sparse, indicating that actin reorganization had defect in arpc5 and arpc4 during stomatal closure upon H$_2$O$_2$ and ABA treatments.

Furthermore, the differences in the configurations of actin filaments in arpc5 and wild type upon H$_2$O$_2$ treatment were analyzed. Most of the bundles of type 1 actin radiated from the ventral side to the dorsal side of the guard cells; therefore, if we draw a line along the middle of the cells in the longitudinal direction, the majority of the actin bundles will be included,
and the number of actin bundles with fluorescent intensity > 50 in each guard cell can be counted (Fig. 5a). These results showed that the wild type contained an average of 13 actin bundles with a fluorescent intensity > 50 per guard cell, whereas arpc5 had a mean of 9 actin bundles per guard cell, which was substantially lower than that of wild type (Fig. 5b). These results implicated that a deficiency in the ARP2/3 complex may be what led to the dramatically reduced number of actin bundles.

We next evaluated the actin density in GFP-ABD2-GFP-labeled guard cells of wild type and arpc5 by defining GFP signal occupancy using Image J software. The results showed that the density increased with the disorganization of actin and decreased with the remodeling of actin in wild type. For example, type 1 and type 3 actins had relatively lower density values, and type 2 actin had a higher density. Furthermore, the occupancy of all three actin types was lower in arpc5 guard cells than in wild type guard cells (Fig. 5c), demonstrating that the new filament generation ability was considerably reduced in the plant lacking ARPC5.

Skewness of GFP fluorescence intensity distribution is an indicator of actin bundling. We also calculated the skewness values of type 1, 2 and 3 actins in both wild type and arpc5. Type 1 and 3 actin had relatively higher skewness values, whereas skewness of fluorescence intensity in type 2 actin was lower, suggesting that when stomata are in a stable open or closed state, actin filaments form bundles; when the stomata are undergoing closing, actin filaments are likely to be in randomly distributed thin arrays. Compared the skewness values between wild
type and arpc5, we found that arpc5 had a higher value in type 1 and 3 actin than that of wild type, whereas the difference between wild type and arpc5 in type 2 actin was not obvious (Fig. 5d). These results exhibited thicker actin bundles in guard cells of open or closed stomata in arpc5, which was found in trichome of arpc5 previously (Mathur et al. 2003b).

**H$_2$O$_2$ generation is delayed in arpc4 and arpc5 guard cells in response to ABA**

We also checked the stomatal response of arpc4 and arpc5 to 10$^{-5}$ M H$_2$O$_2$, a lower concentration of H$_2$O$_2$ that has been found to reduce the stomatal apertures of *Vicia faba* (Zhang et al. 2001c). Our results showed that 10$^{-5}$ M H$_2$O$_2$ reduced stomatal apertures in wild type, whereas failed to decrease the stomatal apertures in *atrbohD/F* and arpc5 mutants to the level of the wild type, and the apertures in *atrbohD/F* and arpc5 were significantly different from those of wild type at 15, 30 and 60 min of treatment (Fig. 6a). arpc4 had a similar response of stomata with arpc5 when treated with 10$^{-5}$ M H$_2$O$_2$ (Fig. 6b). H$_2$O$_2$ may enhance stomatal closure by inducing H$_2$O$_2$ generation by NADPH oxidases: H$_2$O$_2$ opens the Ca$^{2+}$ channel in plasma membrane which causes cytosolic Ca$^{2+}$ increase (Neill et al. 2002b; Hamilton et al. 2000), and Ca$^{2+}$ directly activates the activities of NADPH oxidases, which are responsible for H$_2$O$_2$ generation (Sagi & Fluhr, 2001). 10$^{-4}$ M H$_2$O$_2$ is adequate to induce stomatal closure of *atrbohD/F* mutant to the size as wild type, and the role of H$_2$O$_2$-induced H$_2$O$_2$ production by NADPH oxidases could be omitted (Kwak et al. 2003); whereas 10$^{-5}$ M H$_2$O$_2$ is not sufficient to induce a full closure of stomata as 10$^{-4}$ M H$_2$O$_2$ did in wild type (Fig. 3,6). With 10$^{-5}$ M H$_2$O$_2$ treatment, H$_2$O$_2$-induced H$_2$O$_2$ production played a role in stomatal
closure in wild type. Because lacking the activities of AtrbohD and AtrbohF, the 
H$_2$O$_2$-induced H$_2$O$_2$ production by NADPH oxidases was missing in atrbohD/F mutant. 
Therefore, stomatal aperture of atrbohD/F did not reduce to the size as wild type upon 10$^{-5}$ M 
H$_2$O$_2$ treatment. This failure of the stomatal closure of arpc4, arpc5 and atrbohD/F in 
response to 10$^{-5}$ M H$_2$O$_2$ might imply that arpc4 and arpc5 guard cells have a deficiency in 
H$_2$O$_2$ generation. Furthermore, the stomata of arpc4 and arpc5 plants closed slower than those 
of wild type upon ABA treatment. Based on these results, we speculated that the mutation in 
ARPC4 or ARPC5 caused an alteration in H$_2$O$_2$ generation in response to ABA. The H$_2$O$_2$ 
levels in guard cells of wild type, atrbohD/F, arpc4 and arpc5 were recorded with CLSM 
(Confocal Laser Scanning Microscopy). As a negative control, atrbohD/F guard cells had 
very low H$_2$O$_2$ levels at all time points of ABA treatment, while the H$_2$O$_2$ level in wild type 
guard cells increased gradually and reached a peak by 30 min, followed by a decrease to the 
basal level by 120 min ABA treatment. However, the H$_2$O$_2$ levels in ABA-treated arpc4 and 
arp5 guard cells were much lower than in wild type guard cells for the first 30 min, and the 
peak of H$_2$O$_2$ level appeared by 60 min, which was later than in the wild type (Fig. 7a,b). This 
result clearly showed that H$_2$O$_2$ generation in arpc4 and arpc5 guard cells was delayed, 
suggesting that misregulated actin dynamic reorganization affects H$_2$O$_2$ generation.

AtrbohD and AtrbohF are the two catalytic subunit genes of NADPH oxidases that are 
responsible for ROS generation in guard cells, and the expression of the two genes is 
up-regulated by ABA (Kwak et al. 2003). Therefore, we determined the expression of
AtbohD and AtbohF in leaves of wild type, arpc4 and arpc5 following ABA treatment by quantitative RT-PCR. The results showed that ABA induced over 3-fold increase in AtbohD expression both in wild type, arpc4 and arpc5. However, the peak of AtbohD expression in wild type appeared at 10 min of ABA treatment, whereas it appeared at 15 min of ABA treatment in arpc4 and arpc5 (Supporting Information Fig. S2a). The delayed expression of AtbohD in arpc4 and arpc5 was consistent with the delayed peak of H$_2$O$_2$ content evoked by ABA in arpc4 and arpc5 guard cells (Fig. 7a,b). The expression of AtbohF mRNA in wild type, arpc4 and arpc5 did not increase to the level as AtbohD in response to ABA (Supporting Information Fig. S2b). These results indicated that delayed expression of AtbohD may be one of the reasons for the delayed peak of H$_2$O$_2$ upon ABA treatment.

**Alteration of actin reorganization affects ABA-evoked H$_2$O$_2$ generation in guard cells**

The delayed generation of H$_2$O$_2$ in arpc4 and arpc5 upon ABA treatment suggested that alteration of actin reorganization affects H$_2$O$_2$ generation in response to ABA. To test this possibility, we traced changes in H$_2$O$_2$ levels in wild-type, arpc4 and arpc5 guard cells under ABA treatment together with actin depolymerizing or stabilizing agents. First, we determined the effect of LatB on ABA-induced H$_2$O$_2$ production in guard cells. LatB treatment greatly accelerated ABA-triggered H$_2$O$_2$ generation in guard cells of three genotype plants, especially in arpc4 and arpc5. By 15 min, the H$_2$O$_2$ level in LatB and ABA treated guard cells of wild type was higher than that of the ABA-only treated plants (Fig. 8a). Expectedly, the H$_2$O$_2$ level in LatB and ABA treated guard cells of arpc4 and arpc5 increased to similar levels as wild
type treated with ABA by 15 min, and this high level was sustained until 60 min of treatment (Fig. 8b), indicating that slower actin depolymerization in arpc4 and arpc5 is possibly responsible for the slower H$_2$O$_2$ generation after ABA treatment. Furthermore, the actin stabilizer jasplakinolide blocked ABA-induced H$_2$O$_2$ generation. At all time points, the H$_2$O$_2$ level in jasplakinolide and ABA treated guard cells was lower than in ABA-only treated guard cells (Fig. 8c). These results demonstrated that the delayed H$_2$O$_2$ generation caused by slower actin dynamics in arpc4 and arpc5 is likely to be the reason for the slower stomatal closure upon ABA treatment.

**DISCUSSION**

**H$_2$O$_2$ generation is indispensable for the ABA induction of actin dynamic changes in guard cells**

A line of evidence showed that H$_2$O$_2$ generation plays an essential role in stimuli-induced actin reorganization in mammalian (Kim *et al.* 2009), yeast (Rinnerthaler *et al.* 2012), and plant cells (Wilkins *et al.* 2011). Plant stomata are gates for the exchange of water and gas with the atmosphere and therefore open or close rapidly in response to multiple physiological or environmental signals. ABA-induced stomatal closure is accompanied by actin dynamic changes (Gao *et al.* 2008; Zhao *et al.* 2011). ROS has been revealed to be an important molecule in phosphatidylinositol 3-phosphate (PtdIns3P)-regulated actin dynamics in *Commelina communis* (Choi *et al.* 2008). The role of H$_2$O$_2$ in ABA-induced actin changes in guard cells was also supported by the actin changes in the *abi1-1* mutant upon ABA treatment.
The *abi1-1* mutant carries a mutation in PP2C and fails to generate ROS in response to ABA (Murata *et al.* 2001). The completely abrogated ABA-induced actin disassembly in *abi1-1* provided evidence supporting not only the role of PP2C in regulating actin dynamics, but also the function of H$_2$O$_2$ generation in this process (Eun *et al.* 2001). However, the role of H$_2$O$_2$ in regulating actin changes in guard cells lacks genetic evidence. In this report, we obtained direct genetic evidence from the guard cells of the H$_2$O$_2$-deficient mutant *atrbohD/F* in Arabidopsis. ABA induced a change in the actin filaments, including type 1 actin in the guard cells of open stomata, disorganized type 2 actin during stomatal closing, and remodeled type 3 actin in the guard cells of closed stomata in wild type. However, ABA failed to arouse a complete actin dynamic change in the *atrbohD/F* mutant, whereas the external application of H$_2$O$_2$ completed the actin change from type 1 to 3 in guard cells of *atrbohD/F* (Fig. 1).

Similarly, H$_2$O$_2$ induced actin changes in guard cells of wild type similar to the changes caused by ABA (Fig. 4a,c). These results imply that H$_2$O$_2$ generation is crucial for ABA induction of actin reorganization. The time required for the actin changes due to H$_2$O$_2$ treatment was shorter than those due to ABA treatment because H$_2$O$_2$ is a second messenger in ABA signaling, and the accumulation of the highest amount of H$_2$O$_2$ required 30 min of ABA treatment (Fig. 7a,b). These results supported the hypothesis that H$_2$O$_2$ generation is indispensable for the ABA induction of actin changes in guard cells.

*arpc4* and *arpc5* exhibit slower actin rearrangement and stomatal closure in response to both ABA and H$_2$O$_2$.
It has been shown that the actin filaments change with the alteration of stomatal size (Gao et al. 2008). Both an actin polymerization inhibitor and a stabilizer blocked stomatal closure in response to H₂O₂ (Fig. 2). Therefore, it is interesting to know the actin-related protein that is involved in H₂O₂-regulated actin changes in guard cells. So far, only SCAB1 (Zhao et al. 2011), ARPC2 (Jiang et al. 2012) have been found to directly regulate the actin rearrangement that is induced by ABA. Our results support the hypothesis that ARPC4 and ARPC5, the other two subunits of the ARP2/3 complex, are involved in ABA-regulated actin dynamic changes in guard cells. ARPC4 and ARPC5 express in guard cells (Supporting Information Fig. S1b,c; Obulareddy et al. 2013), and arpc4 and arpc5 exhibit slow changes in actin reorganization and stomatal closure in response to ABA (Supporting Information Fig. S1d-g). In this regard, the phenotype of arpc4 and arpc5 is similar to that of arpc2 upon ABA treatment (Jiang et al. 2012). Interestingly, arpc4 and arpc5 also displayed slower actin changes (Fig. 4) and stomatal closure (Fig. 3) in response to H₂O₂. Meanwhile, the fewer actin bundles in type 1 actin and even a lower density of all three actin types demonstrated that the new actin filament generation activity in arpc5 might be reduced, thereby the bundles formed easily (Fig. 5a-c). Thick actin bundles were found in type 1 and 3 actins of arpc5 mutant (Fig. 5d), which may be the reason for the slower actin disorganization and remodeling of arpc5 in response to H₂O₂.

The slower actin disassembly and remodeling upon ABA or H₂O₂ treatment in arpc4 and arpc5 guard cells is similar to the slower actin re-organization observed in guard cells of scab1 (Zhao et al. 2011) and arpc2 (Jiang et al. 2012). These results demonstrate that ARPC4 and ARPC5 play an essential role in tuning the precise actin changes of guard cells in
response to both ABA and H$_2$O$_2$.

**Actin dynamic changes affect H$_2$O$_2$ generation in guard cells with ABA treatment**

The measurement of H$_2$O$_2$ levels revealed that the changes of H$_2$O$_2$ production in $arpc4$ and $arpc5$ were different from wild type, implying that misregulated actin dynamics affect H$_2$O$_2$ generation in guard cells after ABA treatment. Several lines of evidence have shown that actin dynamic changes affect multiple physiological processes, including K$^+$ channels in rat CRI-G1 insulinoma cells (Harvey et al. 2000), Cl$^-$ channels in neocortical astrocytes (Lascola et al. 1998), and ROS generation in yeast (Thevissen et al. 2007). In plants, Hwang et al. (1997) reported that the disruption of actin filaments activated an inward K$^+$ current, whereas the stabilization of actin filaments inhibited the K$^+$ current in guard cells of *Vicia faba*. A report by Zhang et al. (2007) showed that actin dynamics also regulated stretch-activated calcium channels in *Vicia faba* guard cells. It has been discussed that ARP2/3 complex-regulated actin dynamics act as a hub in the signaling network of guard cells (Jiang et al. 2012). Therefore, it is possible that $ARPC4$ and $ARPC5$ mutation affects H$_2$O$_2$ generation upon ABA treatment. Our results supported this speculation. After ABA treatment, H$_2$O$_2$ levels in wild type guard cells increased gradually, reached a peak at 30 min, and then decreased to the basal level. However, the H$_2$O$_2$ levels in $arpc4$ and $arpc5$ guard cells were substantially lower than that of wild type by 30 min of ABA treatment, and the peak of the H$_2$O$_2$ content appeared by 60 min of ABA treatment (Fig. 7a,b). To make certain that delayed H$_2$O$_2$ generation in $arpc4$ and $arpc5$ is due to the slower actin disorganization, the H$_2$O$_2$ levels
in guard cells were measured after treatment with both LatB and ABA, and the results showed that the H$_2$O$_2$ content peaked earlier in both wild type, arpc4 and arpc5, supporting the hypothesis that actin disassembly greatly accelerated H$_2$O$_2$ generation (Fig. 8a,b). In contrast, H$_2$O$_2$ generation was greatly inhibited when wild type leaves were co-treated with the actin polymerization promoter and stabilizer jasplakinolide and ABA (Fig. 8c). Increased expression of *AtrbohD* is likely to be one of the reasons for H$_2$O$_2$ production induced by actin reorganization. In addition, considering that the NADPH oxidases can be directly activated by Ca$^{2+}$ (Sagi & Fluhr, 2001), and Ca$^{2+}$-permeable channels in plasma membrane were reported to be activated by the depolymerization of actin filaments (Zhang et al., 2007). Therefore, in this case, it could be possible that reorganization of actin filaments triggers the elevation of cytosolic Ca$^{2+}$ to consequently activate the activities of the two guard cell-expressed NADPH oxidases, AtrbohD and AtrbohF, to produce H$_2$O$_2$. From these results, we propose that slower actin disorganization and the correspondingly delayed H$_2$O$_2$ generation were the reason for the slower stomatal closure in arpc4 and arpc5 in response to ABA.

**Regulation between H$_2$O$_2$ generation and ARP2/3 complex-mediated actin dynamic changes possibly exists in ABA guard cell signaling**

Data in this research showed that H$_2$O$_2$ induced actin changes partially through the ARP2/3 complex and that ABA-induced H$_2$O$_2$ accumulation was delayed in arpc4 and arpc5. A mutual regulation between NADPH oxidase-mediated H$_2$O$_2$ generation and ARP2/3-mediated actin dynamic changes is likely to exist in ABA guard cell signaling. In fact, a positive
regulatory relationship between actin changes and calcium has been reported previously. ABA-induced actin changes in guard cells of *Commelina communis* were regulated by calcium (Hwang & Lee, 2001), while stretch-activated calcium channels were reported to be regulated by actin changes in *Vicia faba* guard cells (Zhang et al. 2007). Evidence from this research supports a possible regulatory relationship between actin dynamics and H$_2$O$_2$ generation in ABA-induced stomatal closure as shown in Fig. 9: ABA triggers H$_2$O$_2$ generation, and elevated H$_2$O$_2$ induces actin filaments in open stomata to disorganize through the ARP2/3 complex; actin disorganization enhances further H$_2$O$_2$ generation; when the actin cytoskeleton in the majority of the guard cells is remodeled to type 3, H$_2$O$_2$ levels decrease gradually to the basal level.

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FIGURE LEGENDS

Figure 1. H$_2$O$_2$ is essential for ABA-induced actin dynamic changes in guard cells. (a) Actin configurations in the three types induced by ABA. Bar = 10 μm. (b,c) Statistical results of 10 μM ABA-induced actin types at the indicated time points in wild type (b) and atrbohD/F (c). (d) Statistical result showing the composition of actin types at the indicated time points induced by 10$^{-4}$ M H$_2$O$_2$ in atrbohD/F. Actin filaments in guard cells of wild type or atrbohD/F expressing 35S::GFP-ABD2-GFP treated with ABA or H$_2$O$_2$ were observed under CLSM at the indicated time points, and the percentage of each actin type was calculated. At least 80 guard cells were analyzed for each time point.

Figure 2. Actin disorganization and remodeling are essential for H$_2$O$_2$ induction of stomatal closure. (a) Jasplakinolide, an actin stabilizer, inhibited H$_2$O$_2$-induced stomatal closure. (b) LatB, an actin polymerization inhibitor, blocked H$_2$O$_2$-induced stomatal closure. The leaves with open stomata of wild type were pretreated with 1 μM jasplakinolide or 10 μM LatB for 30 min and then moved to MES buffer containing 10$^{-4}$ M H$_2$O$_2$ and 1 μM jasplakinolide or 10 μM LatB. Stomatal apertures were measured at the indicated time points. Each experiment was repeated three times. The data are presented as the mean ± SE (n = 150). The P values (* <0.05, ** <0.01) were relatively to the control in the same time points.

Figure 3. Stomata of arpc4, arpc5, arpc4 atrbohD/F and arpc5 atrbohD/F close slower than wild type (WT) and atrbohD/F in response to 10$^{-4}$ M H$_2$O$_2$. The leaves with open stomata
from wild type, arpc4, arpc5, atrbohD/F, arpc4 atrbohD/F and arpc5 atrbohD/F plants were incubated in MES buffer containing H$_2$O$_2$, and stomatal apertures were measured at the indicated time points. Each experiment was repeated three times. The data are presented as the mean ± SE (n = 150). The P values (** <0.01) were relative to that of wild type in the same time points.

Figure 4. Actin filaments in guard cells of arpc4 and arpc5 change slower than those of wild type in response to 10$^{-4}$ M H$_2$O$_2$. (a, b) Images of the three actin types in wild type (a), arpc4 or arpc5 (b) induced by H$_2$O$_2$. (c, d, e) Statistical results showing H$_2$O$_2$-induced actin types in wild type (c), arpc5 (d) and arpc4 (e) at the indicated time points. Actin filaments in guard cells of wild type, arpc4 or arpc5 expressing 35S::GFP-ABD2-GFP treated with 10$^{-4}$ M H$_2$O$_2$ were observed under CLSM at the indicated time points, and the percentage of each actin type was calculated. At least 80 guard cells were analyzed for each time point. Bar = 10 μm.

Figure 5. Actin bundle numbers in type 1 actin, the occupancy of actin filaments and skewness of fluorescence intensity distribution are different in wild type (WT) and arpc5 with 10$^{-4}$ M H$_2$O$_2$ treatment. (a) Continuous fluorescent intensity of the GFP signal along the line in guard cells with type 1 actin was measured in wild type and arpc5. (b) Filament numbers in type 1 actin were determined by the number of fluorescent peaks with an intensity higher than 50. (c) Occupancy of actin filaments of the three actin types in wild type (WT) and arpc5. (d) Skewness of fluorescence intensity distribution in wild type (WT) and arpc5. The data are
presented as the mean ± SE. At least 80 guard cells were analyzed for each time point. The P values (* <0.05, ** <0.01) were relative to that of wild type in the same actin types.

**Figure 6.** $10^{-5}$ M H$_2$O$_2$ fails to induce stomatal closure in *arpc4* and *arpc5*. Leaves with open stomata from wild type (WT), *atrbohD/F, arpc5* (a) and *arpc4* (b) were incubated in MES buffer containing H$_2$O$_2$, and stomatal apertures were measured at the indicated time points. Each experiment was repeated three times. The data are presented as the mean ± SE (n = 150). The P values (** <0.01) were relative to that of *atrbohD/F* in the same time points.

**Figure 7.** H$_2$O$_2$ accumulations were slower in *arpc4* and *arpc5* than in wild type in response to ABA. (a, b) Fluorescence images (a) and intensities (b) representing H$_2$O$_2$ levels in guard cells of wild type (WT), *atrbohD/F, arpc4* and *arpc5* with ABA treatment. Leaves with open stomata of wild type, *atrbohD/F, arpc4* and *arpc5* were preloaded with 50 µM H$_2$DCF-DA and then incubated in MES buffer containing 10 µM ABA. H$_2$O$_2$ levels in guard cells were measured by CLSM at the indicated time points. Fluorescent intensities from at least 80 guard cells were analyzed at each time point. Bar = 10 µm. The data are presented as the mean ± SE. The P values (** <0.01) were relative to that of wild type in the same time points.

**Figure 8.** The actin polymerization inhibitor LatB accelerated ABA-induced H$_2$O$_2$ generation and actin stabilizer jasplakinolide inhibited ABA-triggered H$_2$O$_2$ production. Leaves with open stomata of wild type (a,c) preloaded with 50 µM H$_2$DCF-DA were treated with 10 µM
ABA, ABA plus 10 μM LatB (a), or ABA plus 1 μM jasplakinolide (Jasp) (c); arpc4 and arpc5 guard cells preloaded with 50 μM H₂DCF-DA were treated with 10 μM ABA, or ABA plus 10 μM LatB (b). H₂O₂ levels in guard cells were measured by CLSM at the indicated time points. The fluorescent intensities of at least 80 guard cells were analyzed at each time point. The data are presented as the mean ± SE. The P values (**) <0.01 were relative to the control in the same time points.

Figure 9. A schematic draw showing the regulatory relationship between H₂O₂ generation and actin dynamics in ABA-induced stomatal closure. ABA triggers H₂O₂ generation, and elevated H₂O₂ induces actin reorganization mediated by the ARP2/3 complex; ARP2/3-mediated actin dynamics in turn affect H₂O₂ production.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. arpc4 and arpc5 have defect both in actin dynamic changes in guard cells and stomatal closure in response to ABA. (a) RT-PCR confirmation of arpc4 and arpc5 mutants. (b,c) ARPC4 (b) and ARPC5 (c) were expressed in guard cells. (d) Stomatal closure of wild type (WT), arpc4 and arpc5 following ABA treatment. Leaves from 3- to 4-week old wild type, arpc4 and arpc5 were harvested and incubated in MES buffer under light to open the stomata, and then the leaves were moved to MES buffer containing 10 μM ABA. Stomatal apertures were measured at the indicated time points. Each experiment was repeated three
times. The data were presented as mean ± SE (n = 150). The P values (** <0.01) were relative to that of wild type in the same time points. (e) Actin types in arpc4 or arpc5 with ABA treatment. Bar = 10 μm. (f, g) Statistical analysis of actin types in arpc5 (f) or arpc4 (g) guard cells at the indicated time points after ABA treatment. Leaves from 3- to 4-week old arpc4 or arpc5 expressing 35S::GFP-ABD2-GFP lines with open stomata were moved to MES buffer containing 10 μM ABA. Actin filaments in guard cells on leaves were observed under CLSM, and percentage of each actin type at the indicated time points were calculated. At least 80 guard cells were analyzed at one time point.

**Figure S2.** Expression of AtrbohD was delayed in arpc4 and arpc5 upon ABA treatment. Expression of AtrbohD (a) and AtrbohF (b) in leaves of wild type (WT), arpc4 and arpc5 at the indicated time points with 10 μM ABA treatment. Leaves with open stomata of wild type, arpc4 and arpc5 were transferred to 10 μM ABA in MES buffer for 5, 10, 15, 30, 60 min. Expression of AtrbohD and AtrbohF were analyzed by quantitative RT-PCR. The data are presented as the mean ± SE. The P values (** <0.01) were relative to that of wild type in the same time points.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
ABA

$\text{H}_2\text{O}_2$

ARP2/3 complex

Actin dynamics

Stomatal closure

Figure 9