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

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ABSTRACT

Multiple cellular events like dynamic actin reorganization and hydrogen peroxide (H_2O_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_2O_2 generation is indispensable for ABA induction of actin reorganization in guard cells of Arabidopsis that requires the presence of ARP2/3 complex. H_2O_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_2O_2, suggesting that ARP2/3-mediated actin nucleation is required for H_2O_2-induced actin cytoskeleton remodeling. Furthermore, the expression of H_2O_2 biosynthetic related gene *AtrbohD* and the accumulation of H_2O_2 was delayed in response to ABA in *arpc4* and *arpc5*, demonstrating that misregulated actin dynamics affects H_2O_2 production upon ABA treatment. These results support a possible causal relation between the production of H_2O_2 and actin dynamics in ABA-mediated guard cell signaling: ABA triggers H_2O_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_2O_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_2O_2) is a rate-limiting second messenger in ABA guard cell signaling. H_2O_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_2O_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_2O_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_2O_2

production in Arabidopsis guard cells (Bright *et al.* 2006). In addition, external application of H_2O_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^{o} 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*, *arp3*/*dis1*, *arp2*/*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_2O_2-induced guard cell actin reorganization. Changes in the ABA-induced H_2O_2 levels were affected by aberrant actin dynamics in *arpc4* and *arpc5*. We hypothesize that a possible mutual regulation between H_2O_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_2O_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'-ATGGCAAACTCATTACGGCTGT-3'; ARPC4R, 5'-TTACATGAACTGTTTCAAGAAC-3'; ARPC5F, 5'-ATGGCAGAATTCGTTGAAGCTG-3'; ARPC5R 5'-TCAAACGGTGTTGATGGTATCA-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 $CaCl_2$, pH 6.1) for 90 min under light to open the stomata. To study the effects of ABA or H_2O_2 on stomatal closure, leaves with open stomata were transferred to MES buffer containing 10 μM ABA, 10^{-5} M or 10^{-4} M H_2O_2 for 5, 15, 30, 60 or 120 min. To investigate the effects of jasplakinolide or LatB on H_2O_2 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 H_2O_2 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_2O_2 level in guard cells

H_2O_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_2DCF-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_2O_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_2O_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 PrimeScriptTM 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'-TTTGTTCTTCTATATCCCTACCGT-3'; AtrbohD(R), 5'-CATGTTTACAACACCAAAGCTG-3'; AtrbohF(F),

5’-AGAGAGGTGAGGTTTGGTGAGGG-3’; AtrbohF(R), 5’-TTCCATCATTTATCTTCCCTGC-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’-CGGGATCCTACCGTTCTTCGTTCACCAT-3’; ARPC4R, 5’-CGGAATTCATACAGCCGTAATGAGTTTGC-3’; ARPC5F, *5’*-AACTGCAGCGCCGAGCAACTTGTGATA-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_{ARPC}*::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_{ARPC4}*::GUS* or P_{ARPC5}*::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_2O_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_2O_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_2O_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_2O_2 production, we tried the exogenous application of H_2O_2 to *atrbohD/F* leaves, and analyzed actin changes in guard cells. Exogenous H_2O_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_2O_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_2O_2 treatment (47% and 60% of the cell population at 5 min and 15 min, respectively); by 30 min of H_2O_2 treatment, *atrbohD/F* guard cells having type 3 actin increased to 44% of the total cell population. By 60 min of H_2O_2 treatment, guard cells containing type 3 actin increased to 52% of the cell population (Fig. 1d). These results imply that H_2O_2 generation is downstream and indispensable for the ABA induction of actin dynamic changes.

Both actin polymerization inhibitor and actin stabilizer inhibit H_2O_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_2O_2 induction of stomatal closure, we determined the effect of a treatment with H_2O_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_2O_2 failed to induce stomatal closure. Stomatal apertures treated with H_2O_2 and jasplakinolide were bigger than those due to H_2O_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_2O_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_2O_2-induced stomatal closure and implied that re-assembly of actin filaments is required for the progression of H_2O_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_2O_2

Direct visualization of the change in the actin cytoskeleton during H_2O_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_2O_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_2O_2 treatment. Meanwhile, to determine the role of the ARP2/3 complex during H_2O_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_2O_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_2O_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_2O_2 (Fig. 3b). These results suggested that ARP2/3 is involved in ABA-induced stomatal closure and acts

downstream of H_2O_2 production.

The actin cytoskeleton became disorganized and the switch in actin arrays was delayed in *arpc4* and *arpc5* upon H_2O_2 and ABA treatment

We next sought to trace and analyze the actin dynamic changes in wild type, *arpc4* and *arpc5* upon treatment with 10^{-4} M H_2O_2. As shown in Figure 4, actin filaments in wild type showed similar changes to that of *atrbohD/F* in response to H_2O_2 (Fig. 4a), and *arpc4* and *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 *arpc4* and *arpc5* showed slower actin dynamic changes than wild type (Fig. 4c-e). For instance, by the stage of treatment with 10^{-4} M H_2O_2 for 5 min, major guard cells contained type 1 actin in *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 *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 *arpc5* compared with wild type. By 60 min, most guard cells had type 3 actin in both *arpc5* and wild type (64% of the cell population in *arpc5* and 77% of the cell population in wild type) (Fig. 4c,d). *arpc4* guard cells showed similar actin changes as *arpc5* in response to H_2O_2 (Fig. 4e). These results demonstrated that *arpc5* and *arpc4* were slower in both actin disorganization and remodeling in guard cells in response

to H_2O_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_2O_2 and ABA treatments.

Furthermore, the differences in the configurations of actin filaments in *arpc5* and wild type upon H_2O_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 gurad cells of open or closed stomata in *arpc5*, which was found in trichome of *arpc5* previously (Mathur *et al*. 2003b).

H_2O_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_2O_2, a lower concentration of H_2O_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_2O_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_2O_2 (Fig. 6b). H_2O_2 may enhance stomatal closure by inducing H_2O_2 generation by NADPH oxidases: H_2O_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_2O_2 generation (Sagi & Fluhr, 2001). 10^{-4} M H_2O_2 is adequate to induce stomatal closure of *atrbohD/F* mutant to the size as wild type, and the role of H_2O_2-induced H_2O_2 production by NADPH oxidases could be omitted (Kwak *et al.* 2003); whereas 10^{-5} M H_2O_2 is not sufficient to induce a full closure of stomata as 10^{-4} M H_2O_2 did in wild type (Fig. 3,6). With 10^{-5} M H_2O_2 treatment, H_2O_2-induced H_2O_2 production played a role in stomatal

closure in wild type. Because lacking the activities of AtrbohD and AtrbohF, the H_2O_2-induced H_2O_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_2O_2 treatment. This failure of the stomatal closure of *arpc4*, *arpc5* and *atrbohD/F* in response to 10^{-5} M H_2O_2 might imply that *arpc4* and *arpc5* guard cells have a deficiency in H_2O_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_2O_2 generation in response to ABA. The H_2O_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_2O_2 levels at all time points of ABA treatment, while the H_2O_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_2O_2 levels in ABA-treated *arpc4* and *arpc5* guard cells were much lower than in wild type guard cells for the first 30 min, and the peak of H_2O_2 level appeared by 60 min, which was later than in the wild type (Fig. 7a,b). This result clearly showed that H_2O_2 generation in *arpc4* and *arpc5* guard cells was delayed, suggesting that misregulated actin dynamic reorganization affects H_2O_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

AtrbohD and *AtrbohF* 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 *AtrbohD* expression both in wild type, *arpc4* and *arpc5*. However, the peak of *AtrbohD* 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 *AtrbohD* in *arpc4* and *arpc5* was consistent with the delayed peak of H_2O_2 content evoked by ABA in *arpc4* and *arpc5* guard cells (Fig. 7a,b). The expression of *AtrbohF* mRNA in wild type, *arpc4* and *arpc5* did not increase to the level as *AtrbohD* in response to ABA (Supporting Information Fig. S2b). These results indicated that delayed expression of *AtrbohD* may be one of the reasons for the delayed peak of H_2O_2 upon ABA treatment.

Alteration of actin reorganization affects ABA-evoked H_2O_2 generation in guard cells

The delayed generation of H_2O_2 in *arpc4* and *arpc5* upon ABA treatment suggested that alteration of actin reorganization affects H_2O_2 generation in response to ABA. To test this possibility, we traced changes in H_2O_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_2O_2 production in guard cells. LatB treatment greatly accelerated ABA-triggered H_2O_2 generation in guard cells of three genotype plants, especially in *arpc4* and *arpc5*. By 15 min, the H_2O_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_2O_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_2O_2 generation after ABA treatment. Furthermore, the actin stabilizer jasplakinolide blocked ABA-induced H_2O_2 generation. At all time points, the H_2O_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_2O_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_2O_2 generation is indispensable for the ABA induction of actin dynamic changes in guard cells

A line of evidence showed that H_2O_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_2O_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_2O_2 generation in this process (Eun *et al.* 2001). However, the role of H_2O_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_2O_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_2O_2 completed the actin change from type 1 to 3 in guard cells of *atrbohD/F* (Fig. 1). Similarly, H_2O_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_2O_2 generation is crucial for ABA induction of actin reorganization. The time required for the actin changes due to H_2O_2 treatment was shorter than those due to ABA treatment because H_2O_2 is a second messenger in ABA signaling, and the accumulation of the highest amount of H_2O_2 required 30 min of ABA treatment (Fig. 7a,b). These results supported the hypothesis that H_2O_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_2O_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 H_2O_2 (Fig. 2). Therefore, it is interesting to know the actin-related protein that is involved in H_2O_2-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 H_2O_2. 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 type1 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 H_2O_2. The slower actin disassembly and remodeling upon ABA or H_2O_2 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_2O_2.

Actin dynamic changes affect H_2O_2 generation in guard cells with ABA treatment

The measurement of H_2O_2 levels revealed that the changes of H_2O_2 production in *arpc4* and *arpc5* were different from wild type, implying that misregulated actin dynamics affect H_2O_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_2O_2 generation upon ABA treatment. Our results supported this speculation. After ABA treatment, H_2O_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_2O_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_2O_2 content appeared by 60 min of ABA treatment (Fig. 7a,b). To make certain that delayed H_2O_2 generation in *arpc4* and *arpc5* is due to the slower actin disorganization, the H_2O_2 levels

in guard cells were measured after treatment with both LatB and ABA, and the results showed that the H_2O_2 content peaked earlier in both wild type, *arpc4* and *arpc5*, supporting the hypothesis that actin disassembly greatly accelerated H_2O_2 generation (Fig. 8a,b). In contrast, H_2O_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_2O_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_2O_2. From these results, we propose that slower actin disorganization and the correspondingly delayed H_2O_2 generation were the reason for the slower stomatal closure in *arpc4* and *arpc5* in response to ABA.

Regulation between H_2O_2 generation and ARP2/3 complex-mediated actin dynamic changes possibly exists in ABA guard cell signaling

Data in this research showed that H_2O_2 induced actin changes partially through the ARP2/3 complex and that ABA-induced H_2O_2 accumulation was delayed in *arpc4* and *arpc5*. A mutual regulation between NADPH oxidase-mediated H_2O_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_2O_2 generation in ABA-induced stomatal closure as shown in Fig. 9: ABA triggers H_2O_2 generation, and elevated H_2O_2 induces actin filaments in open stomata to disorganize through the ARP2/3 complex; actin disorganization enhances further H_2O_2 generation; when the actin cytoskeleton in the majority of the guard cells is remodeled to type 3, H_2O_2 levels decrease gradually to the basal level.

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FIGURE LEGENDS

Figure 1. H_2O_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_2O_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_2O_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_2O_2 induction of stomatal closure. (a) Jasplakinolide, an actin stabilizer, inhibited H_2O_2-induced stomatal closure. (b) LatB, an actin polymerization inhibitor, blocked H_2O_2-induced stomatal closure. The leaves with open stomata of wild type were pretreated with 1 μM jasplakinode or 10 μM LatB for 30 min and then moved to MES buffer containing 10^{-4} M H_2O_2 and 1 μM jasplakinode 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_2O_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_2O_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_2O_2. (a, b) Images of the three actin types in wild type (a), *arpc4* or *arpc5* (b) induced by H_2O_2. (c, d, e) Statistical results showing H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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_2DCF-DA and then incubated in MES buffer containing 10 μM ABA. H_2O_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_2O_2 generation and actin stabilizer jasplakinolide inhibited ABA-triggered H_2O_2 production. Leaves with open stomata of wild type (a,c) preloaded with 50 μM H_2DCF-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 H_2DCF-DA were treated with 10 μM ABA, or ABA plus 10 μM LatB (b). H_2O_2 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 H_2O_2 generation and actin dynamics in ABA-induced stomatal closure. ABA triggers H_2O_2 generation, and elevated H_2O_2 induces actin reorganization mediated by the ARP2/3 complex; ARP2/3-mediated actin dynamics in turn affect H_2O_2 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.

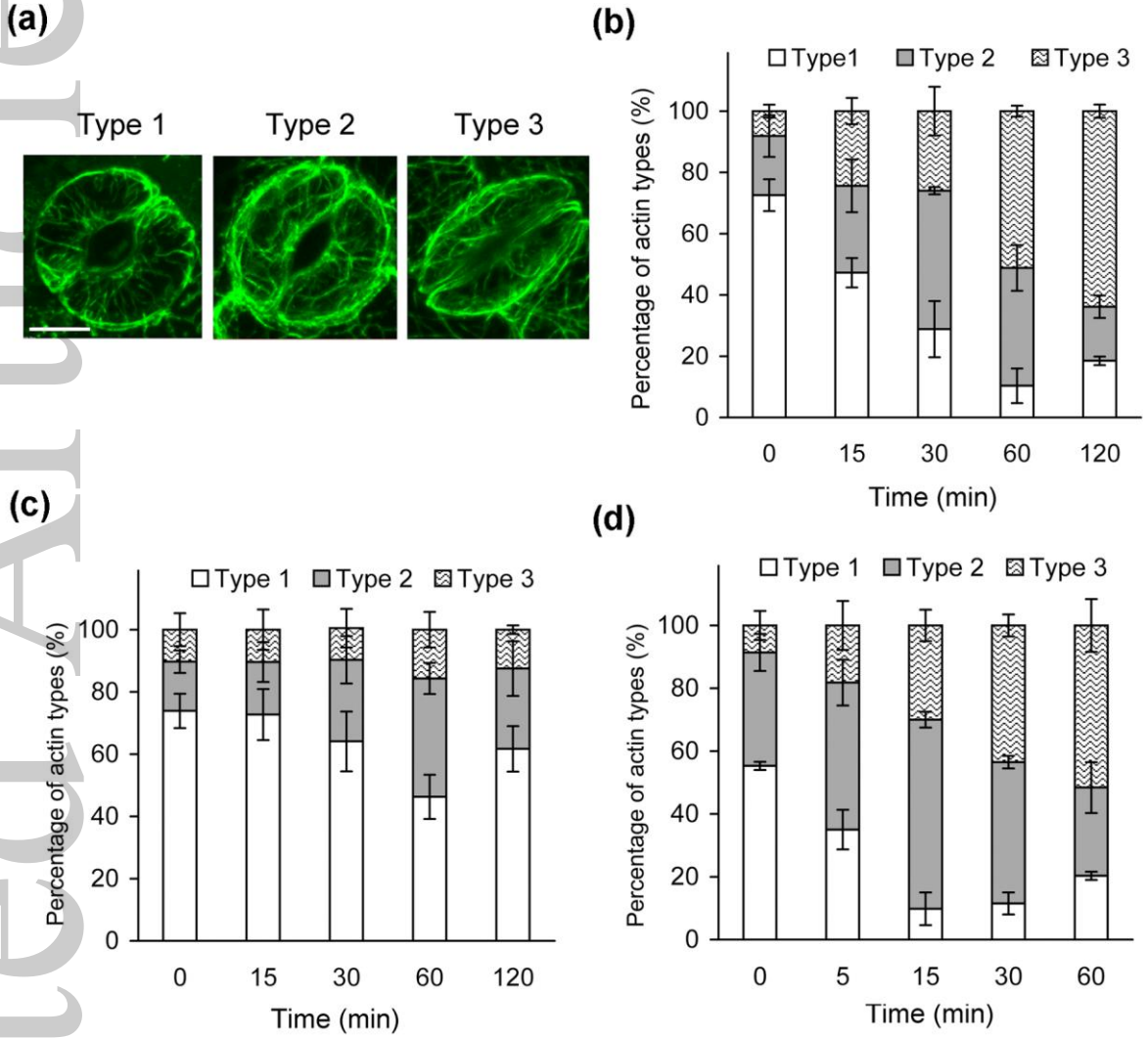


Figure 1

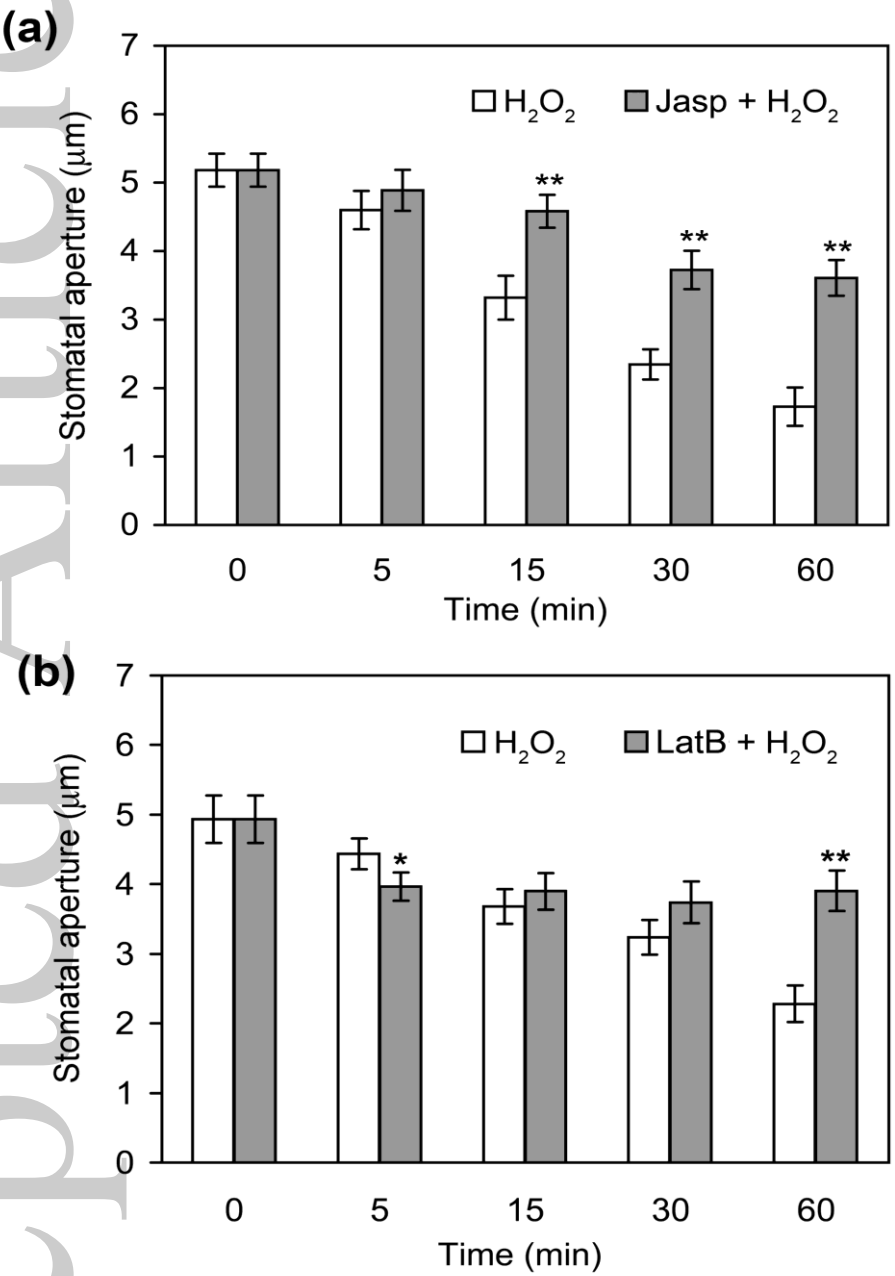
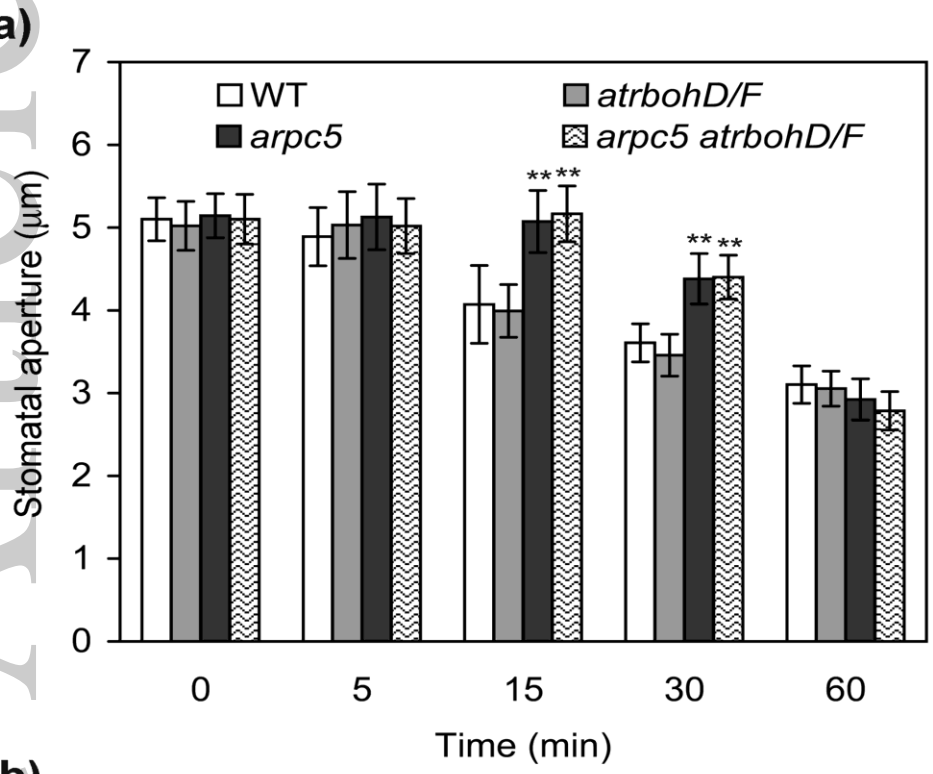


Figure 2



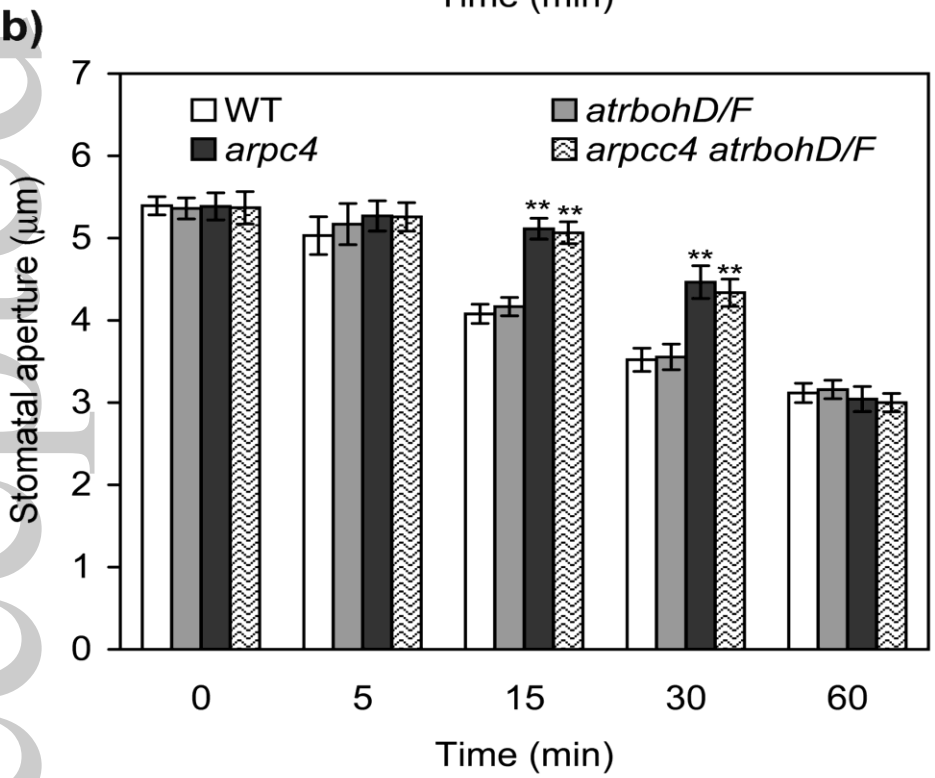
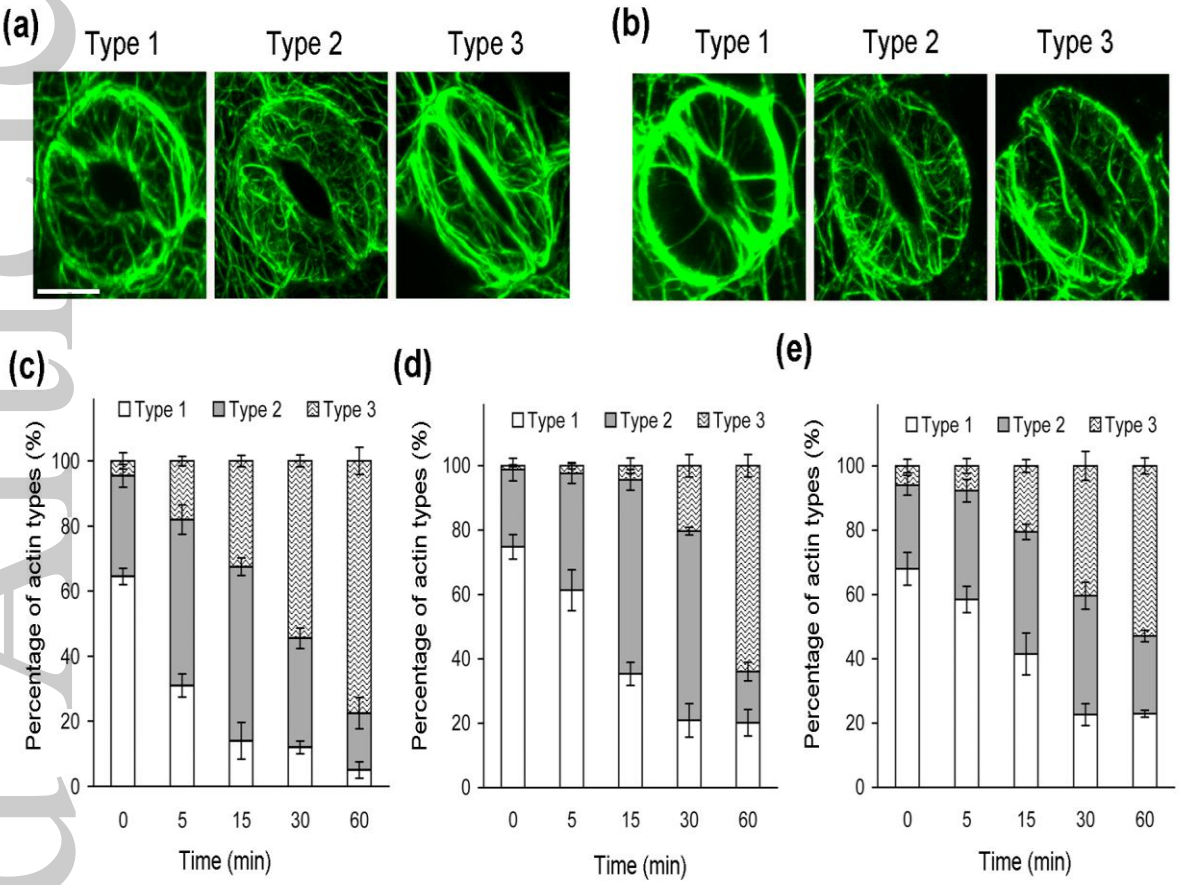


Figure 3



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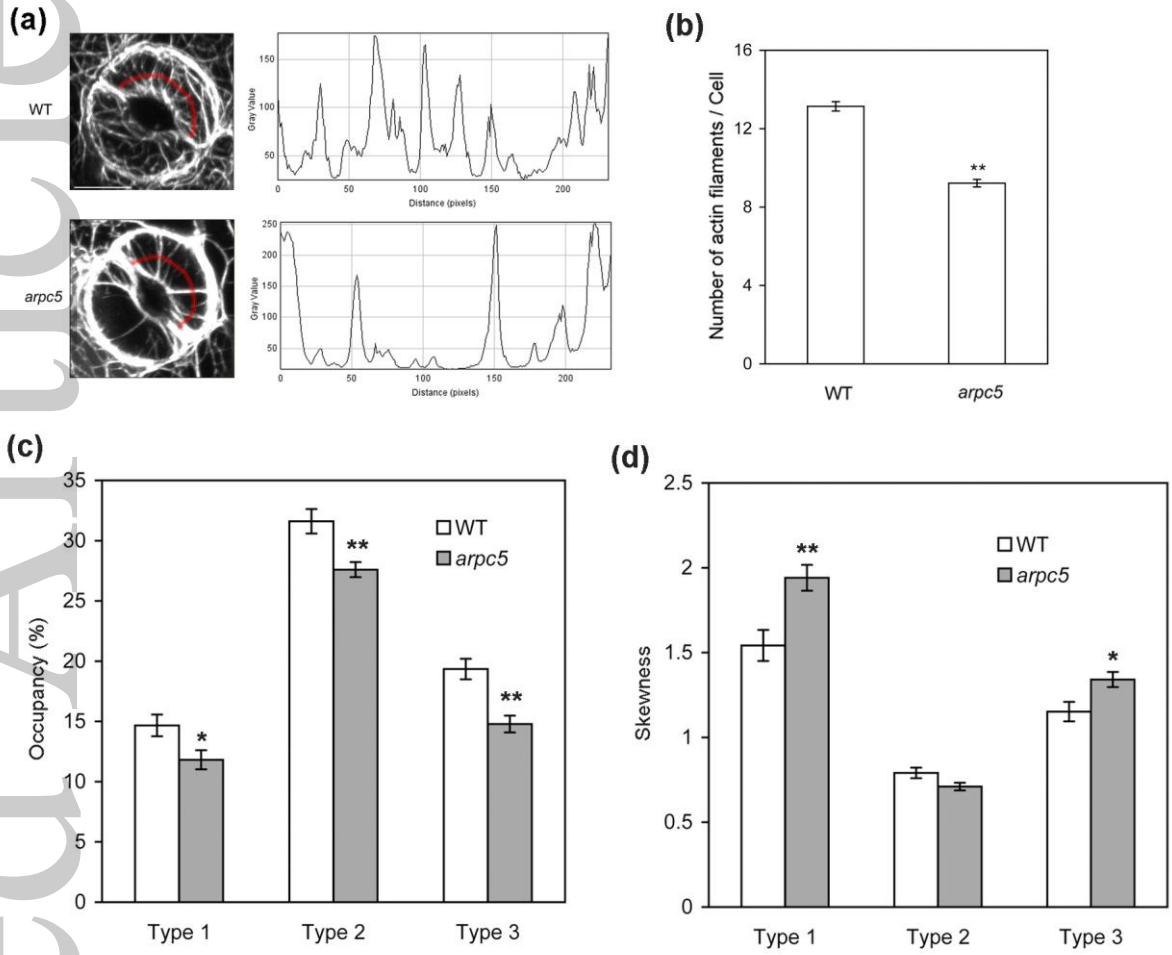

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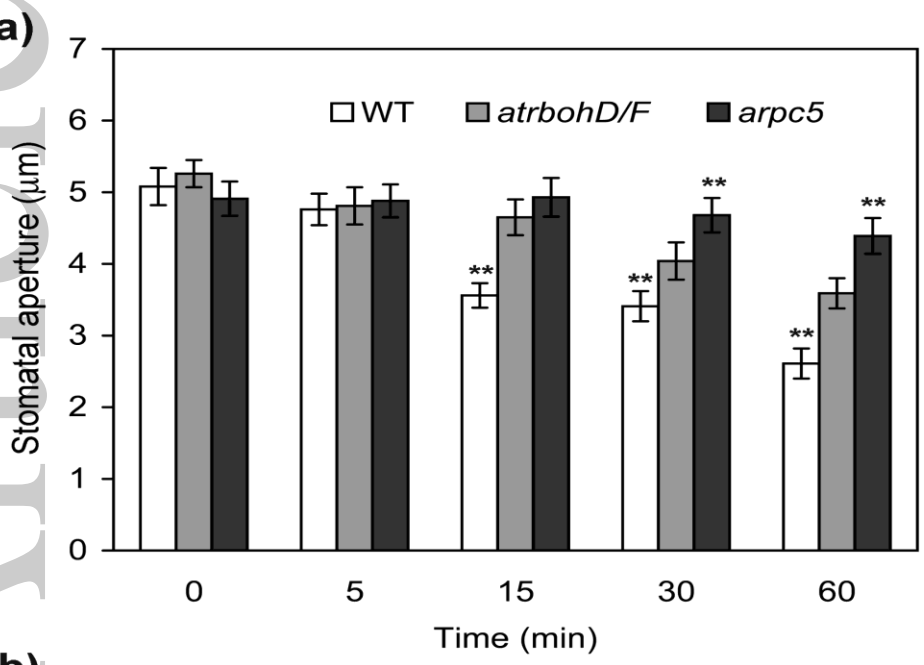

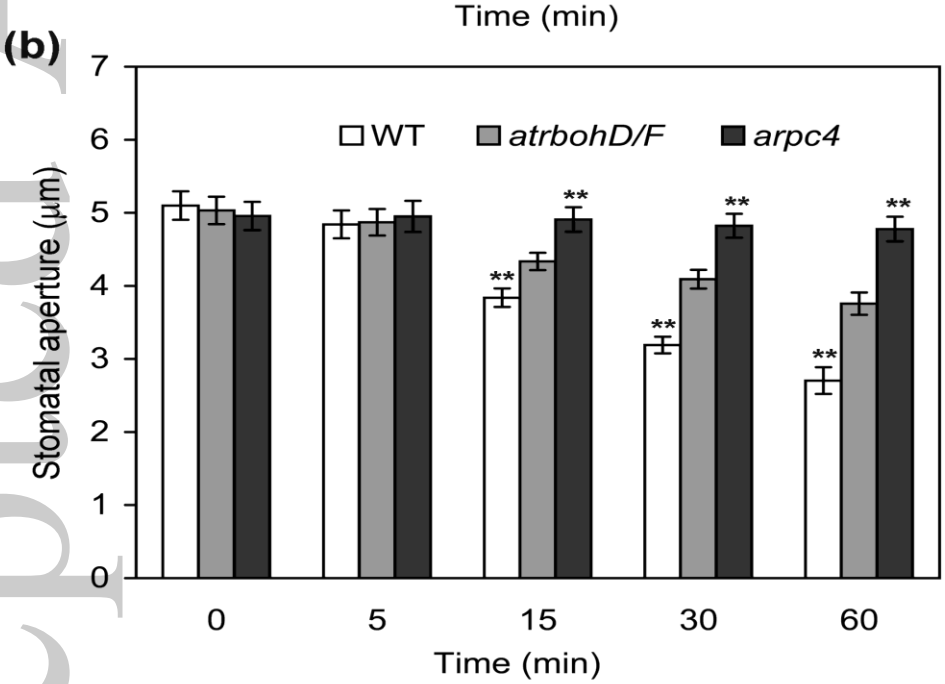

Figure 6

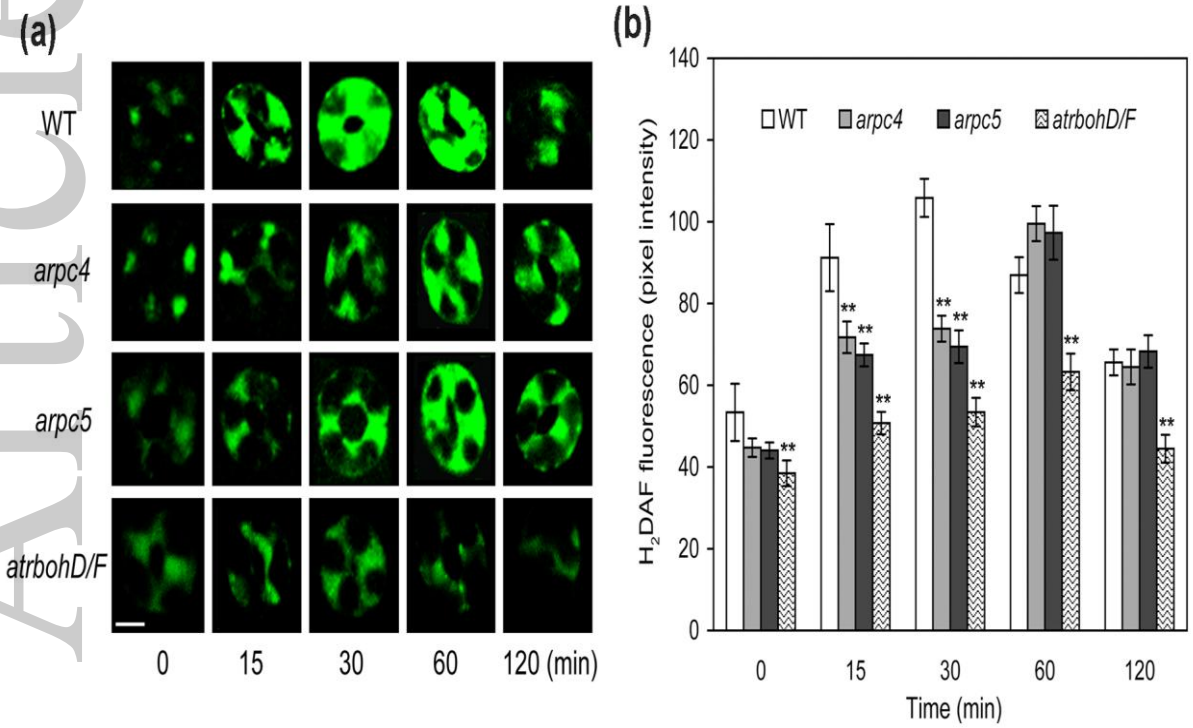

Figure 7

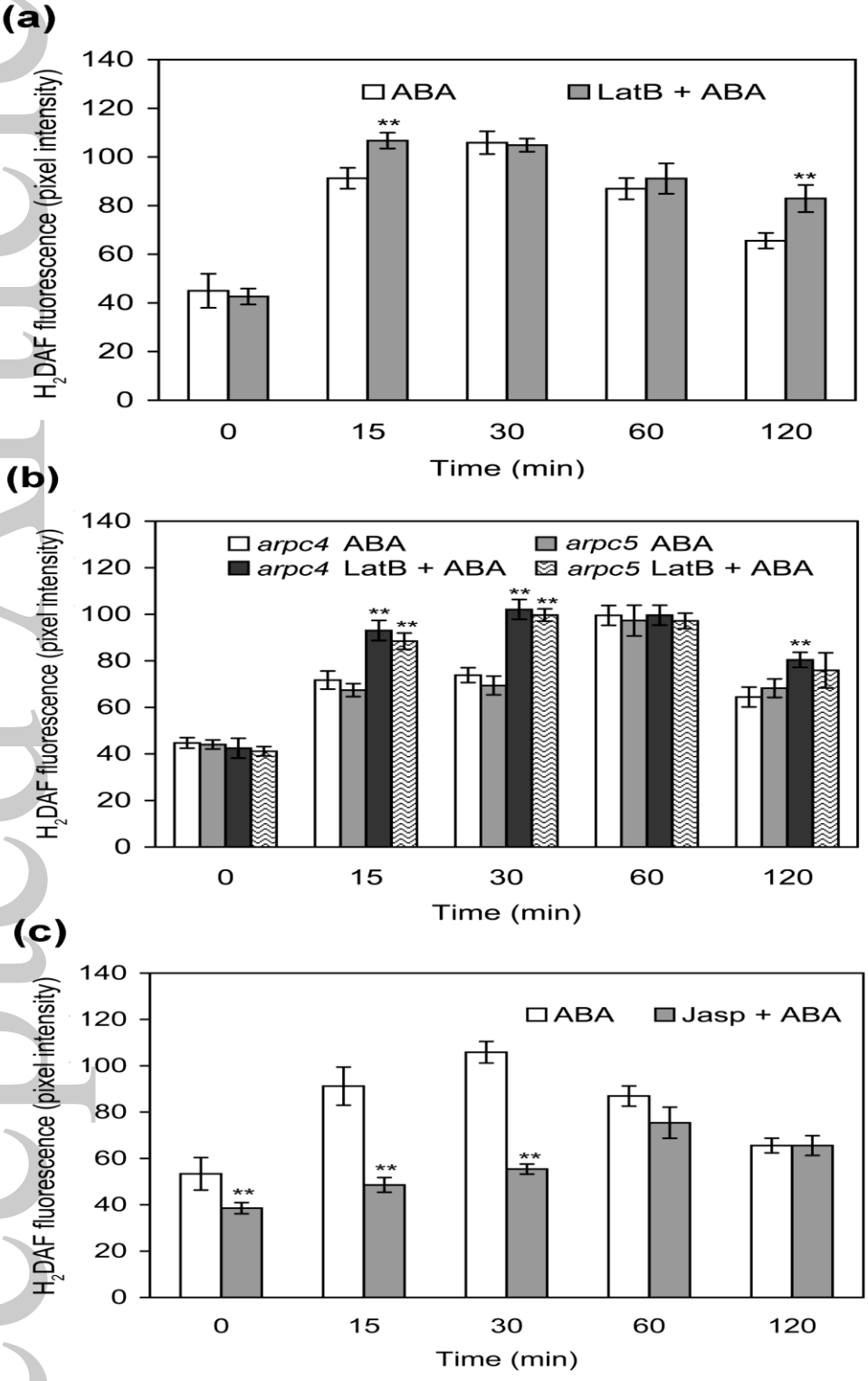

Figure 8

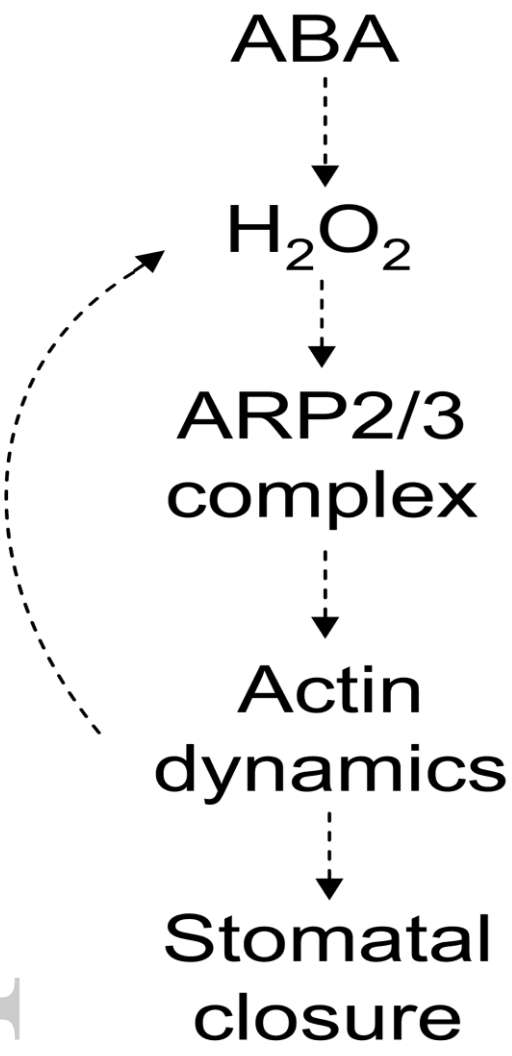

Figure 9