

ATHK1 acts downstream of hydrogen peroxide to mediate ABA signaling through regulation of calcium channel activity in *Arabidopsis* guard cells

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Plants gradually develop their ability to tolerate environmental water deficit as part of the evolutionary process. Abscisic acid (ABA) plays a critical role during drought and osmotic stress. Several histidine protein kinases are regarded as osmotic sensors or regulators in the adaptive response of plants to water deficit. In this study, we report that ATHK1, which was previously shown to function as an osmotic regulator, is involved in ABA-induced stomatal signaling in *Arabidopsis*. Mutants null for *ATHK1* expression were unable to transmit normal ABA responses in guard cells, including inducing stomatal closure, producing hydrogen peroxide and activating calcium influx. Moreover, patch clamp and confocal analysis demonstrated that ATHK1 may function downstream of hydrogen peroxide in ABA-induced stomatal closure, by regulating calcium channel activity and calcium oscillation in *Arabidopsis* guard cells.

ATHK1, H₂O₂, ABA, calcium channels, guard cell

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Plant stomata are bordered by specialized cells known as guard cells, which control the exchange of carbon dioxide and oxygen, thereby regulating respiration and transpiration. When faced with environmental variation, plants maintain their growth through a series of complicated systems that involves crucial processes, one of which includes the regulation of stomatal movement. Various signal transduction pathways function in guard cells to regulate stomatal opening or closure, hence these cells are an ideal model for studying mechanisms controlling these processes [1].

The plant hormone, abscisic acid (ABA), induces stomatal closure in higher plants as an important response in resistance to drought [2]. In the process of ABA-induced stomatal closure, several signaling components are involved. These include small molecules such as hydrogen peroxide (H₂O₂), and ion channels such as calcium channels and potassium channels [3–7].

Several *Arabidopsis thaliana* histidine protein kinases (ATHKs) have been shown to act as regulators participating

in important mechanisms of stress resistance. Indeed, numerous studies have reported that *Arabidopsis thaliana* histidine kinase 1 (ATHK1) specifically responds to osmotic stress [8–11]. The *ATHK1* gene was previously cloned by Urao et al. [8,12], and encodes a heterozygous histidine protein kinase. The protein consists of two trans-membrane domains, which are sensitive to environmental osmotic changes, similar to those present in yeast histidine kinases. Researchers have shown that *ATHK1* mRNA is highly expressed in root tissue, compared with other tissues, and rapidly accumulates in response to changes in soil salinity or osmotic variation, thus making it a highly efficient sensor to environmental fluctuations [8,12,13]. Recent research revealed that ATHK1 plays a unique role in the regulation of processes controlling desiccation, which occur during seed formation. Mechanistically, this may involve regulation through ABA mediated pathways, since overexpression of *ATHK1* led to increased transcription of ABA biosynthetic genes [14]. However, while ATHK1 has been implicated in ABA signaling, the exact role and position of ATHK1 in this pathway are unclear. In previous studies, we demon-

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strated a role for secondary signaling molecules, such as hydrogen peroxide (H_2O_2) and ion channels, such as the calcium (Ca^{2+}) channel in ABA-induced stomatal movement [6,7,15]. Because *ATHK1* also interferes with stomatal movement, we investigated the potential for crosstalk between *ATHK1* and ABA- H_2O_2 signaling and the mechanisms underlying the interaction of these pathways.

1 Materials and methods

1.1 Chemicals

ABA, H_2O_2 , dichlorofluorescein diacetate (H_2DCF -DA), acetyloxymethyl 2-[5-[2-[5-[3-[4-[2-(acetyloxymethoxy)-2-oxoethyl]piperazin-1-yl]-3-oxopropyl]-2-[bis[2-(acetyloxymethoxy)-2-oxoethyl]amino]phenoxy]ethoxy]-6-[bis[2-(acetyloxymethoxy)-2-oxoethyl]amino]-1-benzofuran-2-yl]-1,3-oxazole-5-carboxylate (*FURA3-AM*), nicotinamide adenine dinucleotide phosphate (NADPH) diphenyleneiodonium Chloride (DPI), were purchased from Sigma Aldrich (USA). Analytical grade 2-*N*-morpholino ethanesulfonic acid (MES) and dimethyl sulfoxide (DMSO) (Sigma Aldrich) were used. Prior to use, all chemicals were dissolved in solvents in accordance with their qualities and manufacturer's instructions.

1.2 Epidermal strip bioanalysis and measurement of water loss

Thin strips were peeled from *Arabidopsis* leaves using medical tweezers and ~ 0.5 cm² sections were incubated in MES-KCl buffer (KCl 50 mmol L⁻¹, MES 10 mmol L⁻¹) for 1 h. Prior to treatment with reagents, stomata pores were opened by exposure to 0.3 mmol m⁻² s⁻¹ light density for 2 h and the stomatal aperture was recorded using an optical measure microscope (Nikon, magnification 400 \times). The epidermal strips were then incubated with various reagents (ABA, H_2O_2 or DPI) dissolved in MES-KCl buffer for 1 h and the stomatal aperture was measured. For measurement of water loss, rosette leaves of wild-type and *ATHK1*-deficient mutant strains were detached from 4-week-old seedlings, placed in weighing dishes, and incubated on the laboratory bench at 20°C. Weight was monitored at the indicated times (for 3 d continuously) and water loss expressed as the percentage of initial fresh weight at day one.

1.3 Patch clamp for calcium channel

Wild-type and *ATHK1*-deficient strains of *Arabidopsis thaliana* were grown in a controlled environment chamber with a 16 h/8 h light/dark cycle. Guard cell protoplasts were isolated from 4- to 6-week plants as previously described [6]. Pipettes were pulled with a vertical puller (Narishige, Japan) modified for two-stage pulls. The whole cell voltage-clamp on *Arabidopsis* guard cells was recorded using an EPC-9

amplifier (Heka Instrument, Germany) as previously described [6]. Data were analyzed using PULSEFIT 8.7 software. Standard solutions for Ca^{2+} measurements were used, including 10 mmol L⁻¹ BaCl₂, 0.1 mmol L⁻¹ DTT, 10 mmol L⁻¹ MES-Tris (pH 5.6) in the bath, and 100 mmol L⁻¹ BaCl₂ 0.1 mmol L⁻¹ DTT, 4 mmol L⁻¹ EGTA, 10 mmol L⁻¹ HEPES-Tris (pH 7.1) in the pipette. ABA, H_2O_2 and DPI were freshly added to bath solutions at the indicated concentrations. For ABA-activated Ca^{2+} current measurements, 1 mmol L⁻¹ NADPH was added to the standard pipette solution. Osmolalities of pipette and bath solutions were adjusted to 510 and 490 mmol kg⁻¹, respectively, using *D*-sorbitol (Sangon, China).

1.4 Laser scanning confocal microscopy

H_2DCFDA (50 mmol/L) and *FURA3-AM* (5 mmol/L) solutions were prepared in DMSO. These were directly added to loading buffers at a final concentration of 10 μ mol L⁻¹ for *FURA3-AM* or 50 μ mol L⁻¹ for H_2DCFDA . During the loading process, the sample temperature was maintained at 25°C. After loading (60 min for *FURA3-AM*, 30 min for H_2DCFDA), epidermal strips were transferred into fresh loading buffer to remove excess probes. The excitation and absorption wave-lengths used were 485 and 515–530 nm, respectively.

2 Results

ATHK1 mutants (*athk1-4* and *CS*) were derived from wild-type plants (WS and Col-0), by insertion of a fragment into the promoter region. We examined the T-DNA mutant, derived from SALK_000976 (insertion 88 bp fragment: 5'-CTTCTCTGTGGATGAAGCAGAGNNNTTAGTCNNTCCNANTTTTGAAGTTTTTCCTTCNTGGGNTTCTGATTGGGCTACTANAGTTTTGA-3') and CS849900 (insertion 309 bp fragment: 5'-ATTTTCCATAAGCAAAAAGATTT-CATCTTTCTATTTATTTCTTTTATAAAATTGGAACCTACTTTGCTTCTTCTTCAGTCTCCACACTTATCTTATT-TTTGCCAGTCTTCTCTGTGGATGAAGCAGAGTTTT-TAGTCAATCAAAAAGTTTGAAGCTTTCCTTCATGGG-TTTCTGATTTGTCTACTAAAGTTTTGATTTTTTTAA-TTTGGGTATTAATAAAAAAAGTGGGGAGTGACACAAGAAGCAGTATAGTCATTAGTTTTCCACTAAGCAAAGCACCCAAAAGTTCACCAACACAANACTCGA-GAAGA-3'), and observed several differences between mutant and wild-type plants.

2.1 Loss of *ATHK1* is associated with differences in water loss

To investigate the role of *ATHK1* in stomata regulation and transpiration, we used infrared cameras to examine the temperature of leaves from both wild-type and *ATHK1* mutant

plants. *ATHK1* mutants displayed a relatively lower average temperature compared with their wild-type counterparts under conditions of normal humidity (data not shown).

Analysis of water loss also revealed that *ATHK1* mutants lost water at a faster rate than wild-type plants. Both *ATHK1* mutants lost 10% more water than wild-type plants after 60 min (Figure 1(d)). These results were not explained by differences in stomata numbers, as we observed no distinct differences in the number of stomata per square centimeter between wild-type and mutant plants (Figure 1(c)).

Taken together, these data indicate that *ATHK1* inhibits the loss of water vapor through stomata. We next asked whether *ATHK1*-regulated stomatal movement is controlled by ABA.

2.2 *ATHK1* is involved in ABA and H₂O₂-induced stomatal closure in *Arabidopsis thaliana*

To investigate the involvement of *ATHK1* in stomatal

closure induced by ABA, we assayed the response of wild-type and *ATHK1* mutant plants using the epidermal stomatal bioassay, which is typically used to test the function of ABA (Figure 1(a)). We also tested the effect of H₂O₂ on stomata in these plant lines, owing to its secondary signaling role in ABA-induced stomatal closure (Figure 1(b)).

As shown in Figure 1(a) and (b), treatment of plants with a range of concentrations of ABA (0–100 μmol L⁻¹) and H₂O₂ (0–500 μmol L⁻¹), revealed that *ATHK1* mutants were less sensitive to ABA or H₂O₂ than their wild-type counterparts. These differences were most striking for ABA at 10 μmol L⁻¹ and for H₂O₂ at 100 μmol L⁻¹. These results suggest that *ATHK1* plays a role in ABA-H₂O₂ signaling in guard cells. We next asked how *ATHK1* performs this function.

2.3 *ATHK1* does not inhibit ABA-induced H₂O₂ production in guard cells

ABA induces H₂O₂ production as a classic response to

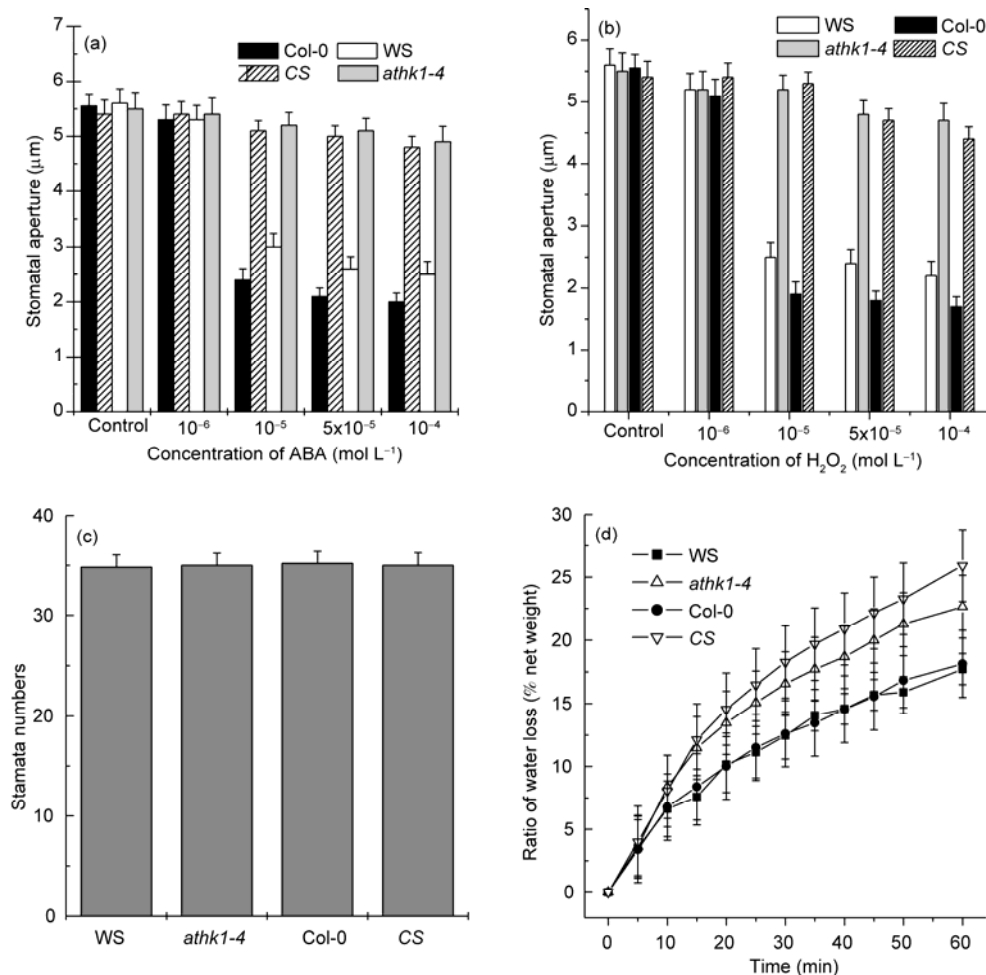


Figure 1 *ATHK1* plays a role in ABA and H₂O₂-induced stomatal closure. (a) Stomatal aperture of wild-type plants (WS and Col-0) or *ATHK1* mutants (*athk1-4* and CS) at given ABA concentrations ($n \geq 250$). (b) Stomatal aperture of wild-type or *ATHK1* mutant plants at given H₂O₂ concentrations ($n \geq 250$). (c) Determination of stomatal cell patterning between wild-type and *ATHK1* mutant plants. (d) Water loss ratio of detached plants of *ATHK1* mutants ($n=5$) was higher than that of wild-type plants ($n=5$). Data for (c) and (d) represent the average of 5 independent experiments \pm SEM ($P \leq 0.05$).

drought in guard cells [2]. To assess the role of ATHK1 in H₂O₂ production, we performed H₂DCF fluorescence assays to measure H₂O₂ levels in both wild-type and mutant plants. Laser scanning confocal microscopy (LSCM) analysis of guard cells revealed that fluorescence levels were 10-fold higher in ABA treated cells compared with controls, in both wild-type and *ATHK1* mutant cells. The NADPHase inhibitor, DPI, was capable of inhibiting the production of H₂O₂ in both systems (Figure 2). These results suggest that ATHK1 does not affect H₂O₂ production in response to ABA treatment.

2.4 ATHK1 regulates ABA and H₂O₂-induced Ca²⁺ channel activity in guard cells

Previous studies by Pei et al. [4] revealed that H₂O₂ controls ABA function via the regulation of Ca²⁺ channels in guard cells. Based on this, we hypothesized that there may be differences in ABA or H₂O₂-regulated Ca²⁺ channel signaling between wild-type and *ATHK1* mutant plants.

To test this, we performed a whole-cell ramp patch clamp. In wild-type plants, treatment with ABA significantly induced Ca²⁺ channel activity for inward Ca²⁺ currents compared with control, which rose from < 70 pA at time zero, to 350 pA at 10 min. Treatment with DPI led to a decrease in activity to 170 pA (Figure 3(a) and (c)). In contrast, treatment of mutants with ABA had minimal effect on Ca²⁺ channel activity compared with controls, with changes no greater than 100 pA observed (Figure 3(b) and (c)).

Similarly, treatment of wild-type guard cells with H₂O₂

led to an increase in the Ca²⁺ current to approximately 350 pA (Figure 3(a) and (d)), whereas in *ATHK1* mutant cells, this current was not significantly changed compared with controls, varying less than 100 pA (Figure 3(b) and (d)).

2.5 ATHK1 affects ABA and H₂O₂-induced Ca²⁺ concentration variation in guard cells

Previous research has shown that ABA and H₂O₂ are capable of inducing changes in the Ca²⁺ concentration of guard cells, preceding stomatal closure [4,7]. Ca²⁺ channel signaling on the guard cell membrane is regarded as the main source for elevation of inner-cell Ca²⁺ concentrations [1]. ABA and H₂O₂ induce stomatal closure by affecting the cytoplasmic concentration and dynamic variation of Ca²⁺. *ATHK1* deficiency may inhibit Ca²⁺ channel signaling and the cytoplasmic concentration of Ca²⁺, thus rendering guard cells insensitive to ABA and H₂O₂. To test this hypothesis, we assessed *FURA3-AM* fluorescence, which is an indicator of intracellular Ca²⁺ levels, in wild-type and *ATHK1* mutant plants using confocal microscopy.

As shown in Figure 4, treatment with ABA led to an increase in *FURA3-AM* fluorescence in wild-type WS guard cells, but did not significantly affect fluorescence in *ATHK1* mutants. Indeed, ABA-induced fluorescence was 3-fold higher in wild-type compared with mutant plants. Treatment with both ABA and DPI inhibited *FURA3-AM* fluorescence induced by ABA in wild-type plants, without affecting fluorescence in mutant plants (Figure 4).

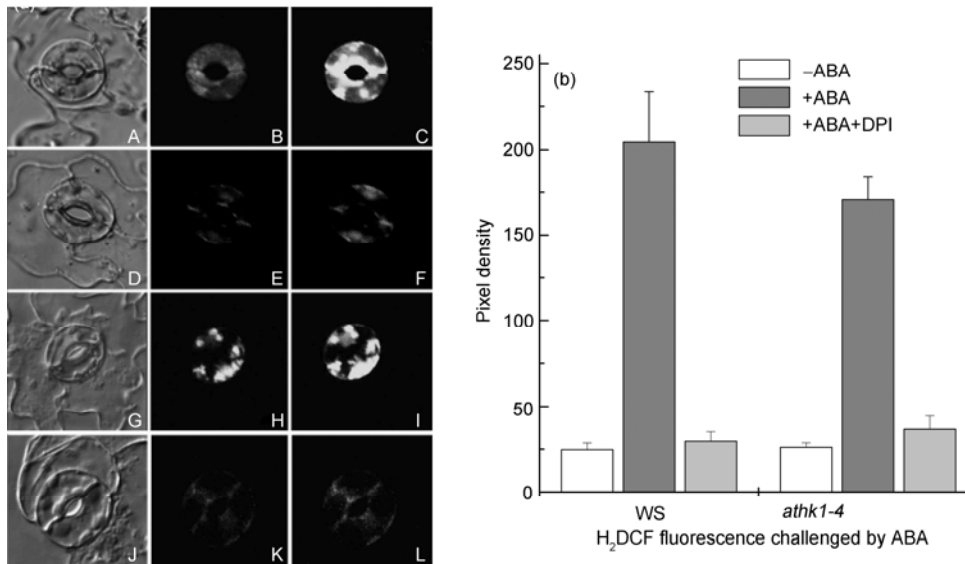


Figure 2 H₂DCF fluorescence showing the production of H₂O₂ before (B, E, H, K) and after abscisic acid (ABA) treatment (C, F, I, L) in wild-type WS and *ATHK1* mutant guard cells. (a) Laser scanning confocal microscopy (LSCM) revealed variations in H₂DCF fluorescence. A–C, WS+ABA; D–F, WS+ABA+DPI; G–I, *ATHK1*+ABA; J–L, *ATHK1*+ABA+DPI. (b) Changes in H₂DCF fluorescence pixel density in both wild-type and *ATHK1* mutant plants after treatment with ABA and ABA + DPI ($n=10$). Data represent the mean fluorescence intensity for 10 independent guard cells \pm SEM ($P \leq 0.05$). The concentration of reagents are as follows: ABA, 10 $\mu\text{mol L}^{-1}$; DPI, 50 $\mu\text{mol L}^{-1}$.

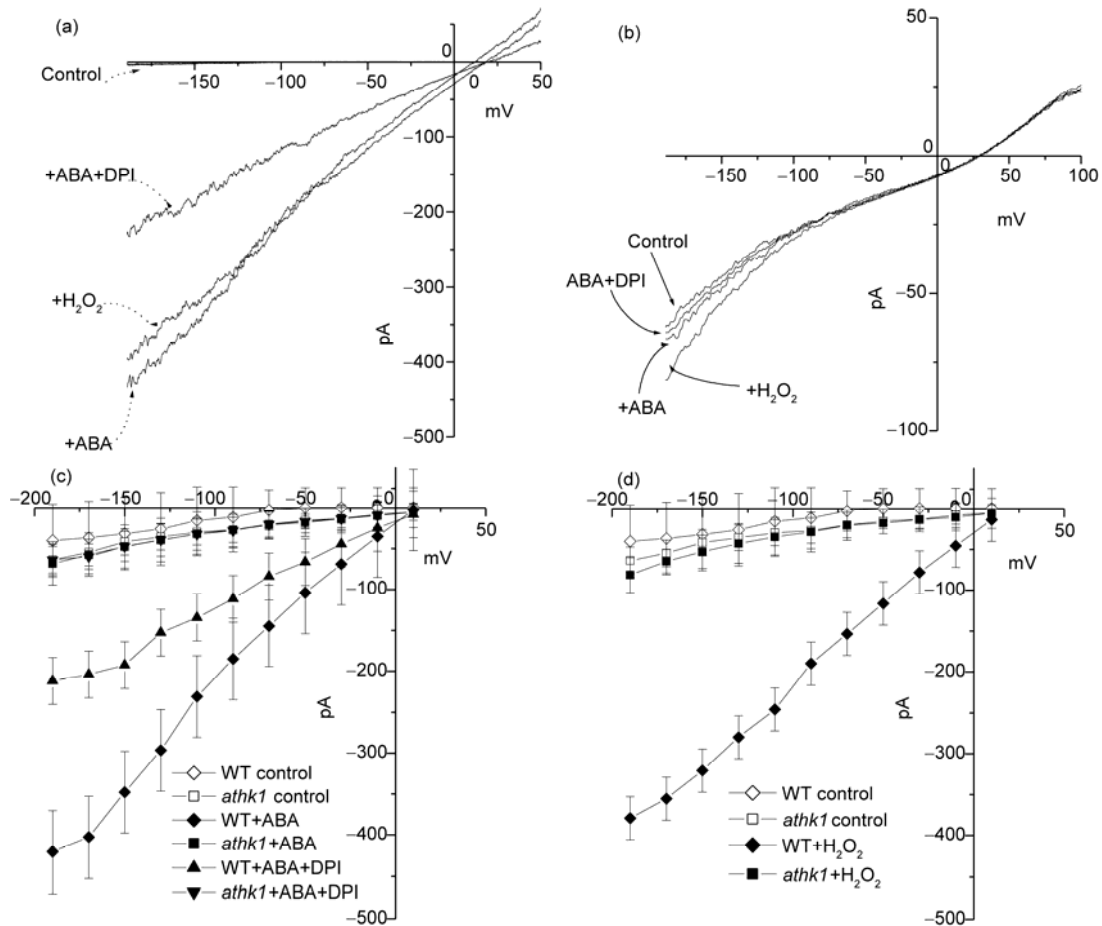


Figure 3 Ca^{2+} channel activity on guard cell membranes is affected by ABA and H_2O_2 in wild-type WS and *ATHK1* mutant plants. (a) Ca^{2+} channel activity in WS membrane before and after ABA (and DPI) or H_2O_2 treatment. (b) Ca^{2+} channel activity in *ATHK1* mutants before and after ABA (and DPI) or H_2O_2 treatment. (c) Statistical analyses of Ca^{2+} channel currents following ABA exposure in wild-type and *ATHK1* mutant plants ($n=10$). (d) Statistical analyses of Ca^{2+} channel current following H_2O_2 treatment in wild-type and *ATHK1* mutant plants ($n=10$). The final concentration of reagents was: ABA, $10 \mu\text{mol L}^{-1}$; H_2O_2 , $100 \mu\text{mol L}^{-1}$; DPI, $50 \mu\text{mol L}^{-1}$.

2.6 *ATHK1* regulates the rhythm of Ca^{2+} concentration fluctuation

Oscillation of Ca^{2+} levels is characteristic of many biological reactions. To test the idea that *ATHK1* may affect Ca^{2+} oscillation in guard cells, we performed a time course experiment to assess calcium levels in wild-type and mutant cells. Our results revealed a fluctuating pattern of Ca^{2+} concentration in wild-type guard cells upon exposure to ABA (Figure 5(a)). In contrast, no such fluctuations were observed in *ATHK1* mutant guard cells (Figure 5(b)). We also observed that the oscillation in Ca^{2+} concentration only occurred at higher concentrations of Ca^{2+} (Figure 5(c)).

3 Discussion

In this study, we provide evidence that *ATHK1* plays a role in ABA signal transduction and control of H_2O_2 -induced calcium channel activity in guard cells, mediating stomatal

closure. *ATHK1* mutants displayed less sensitivity to ABA under normal conditions, in contrast with wild-type plants. Compared with wild-type plants, *ATHK1* mutants displayed impaired ABA-induced stomatal closure and more rapid water loss in response to water deficit stress (Figure 1). Previous studies have demonstrated the effect of H_2O_2 on stomatal closure [4,6]. In this study, we found that H_2O_2 production induced by ABA was not decreased in *ATHK1* mutant guard cells, suggesting that the loss-of-function mutation in *ATHK1* does not impair H_2O_2 homeostasis in guard cells (Figure 2). It is possible that *ATHK1* acts downstream of H_2O_2 , modulating stomatal closure in guard cells. *ATHK1* is not only critical for mediating the effects of H_2O_2 , but is an essential element of the ABA signaling pathway that mediates stomatal closure. The finding that *ATHK1* is involved in ABA signaling in guard cells raises the questions of how ABA and H_2O_2 signals are transduced, and the identities of the components of the cascade involved in this process.

Previous studies have shown that ABA-induced H_2O_2

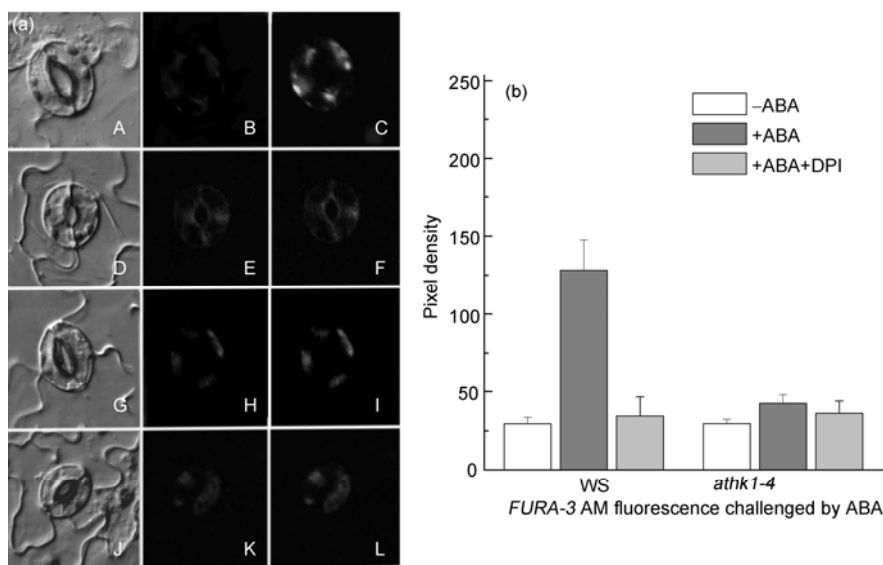


Figure 4 *FURA3-AM* fluorescence showing changes in intracellular guard cell Ca^{2+} concentration before (B, E, H, and K) and after ABA treatment (C, F, I, L) in wild-type WS and *ATHK1* mutant cells. (a) LSCM images display the variation of *FURA3-AM* fluorescence following treatment with ABA and ABA + DPI. A–C, WS+ABA; D–F, WS+ABA+DPI; G–I, *ATHK1*+ABA; J–L, *ATHK1*+ABA+DPI. (b) Changes in *FURA3-AM* fluorescence pixel density in both wild-type and *ATHK1* mutant plants after treatment with ABA and ABA + DPI. Data points are expressed as the mean \pm SEM ($n=10$, $P\leq 0.05$). The final concentration of reagents was: ABA, $10\ \mu\text{mol L}^{-1}$; DPI, $50\ \mu\text{mol L}^{-1}$.

generation in the guard cells of *Vicia* and *Arabidopsis* leads to the activation of plasma membrane Ca^{2+} channels, thus permitting Ca^{2+} influx in guard cells [4,5,15]. The link between ABA-induced H_2O_2 production and *ATHK1* is further strengthened by our observations. In this study, we found that *ATHK1* functions downstream of ABA and H_2O_2 , but upstream of Ca^{2+} channel signaling. Mutations in *ATHK1* impaired ABA-induced stomatal closure and the activation of Ca^{2+} -permeable channels in the plasma membrane. Indeed, calcium currents were not affected by ABA and H_2O_2 in the *ATHK1* mutant plants compared with their wild-type counterparts (Figure 3). One possibility for this observation is that loss of function of *ATHK1* affects downstream events mediated by H_2O_2 and ABA signaling, thereby blocking the activation of Ca^{2+} channels within guard cells and disrupting stomatal closure. The intimate connection between *ATHK1* and Ca^{2+} channel activity implies that *ATHK1* may play an important role in sensing and monitoring intracellular Ca^{2+} levels in guard cells.

Previous research in our laboratory showed that ABA induces H_2O_2 production, thereby regulating calcium influx into guard cells, which in turn regulates changes in stomata by altering osmotic conditions [5,6,15]. In this study, we demonstrate that *ATHK1* also regulates the activity of Ca^{2+} channels, and the NADPHase inhibitor, DPI was capable of reversing this function (Figure 3). These data suggest that H_2O_2 -regulated channels may be controlled by *ATHK1* in wild-type plants, whereas this function is lost in mutant plants.

We also performed confocal experiments to clarify the

relationship between ABA, H_2O_2 and Ca^{2+} . We employed two biochemical probes, H_2DCFDA and *FURA3-AM*, to measure intracellular reactive oxygen species and Ca^{2+} levels respectively. We found that H_2DCFDA fluorescence continuously increased in both wild-type plant and mutant guard cells in the presence of ABA, and treatment of plant tissue with DPI decreased the trend of H_2O_2 production (Figure 2). In contrast, *FURA3-AM* fluorescence increased in wild-type plant guard cells but changed minimally in *ATHK1* mutants, implying that *ATHK1* may be an upstream regulator for Ca^{2+} signal transduction (Figure 4).

In addition, we also observed that *ATHK1* regulated not only intracellular guard cell Ca^{2+} concentration, but also the rhythm of concentration variation. Interestingly, Ca^{2+} oscillation was only observed at high Ca^{2+} concentrations, such as that present in wild-type guard cells, but not at low concentrations present in *ATHK1* mutant guard cells (Figure 5). This indicates that there must be a threshold of Ca^{2+} concentration for Ca^{2+} oscillation to occur, and *ATHK1* may play a key role in controlling this threshold.

4 Conclusion

On the basis of the results presented here as well as those reported previously, we present a model describing the inter-relationships between *ATHK1*, ABA, H_2O_2 and Ca^{2+} channel signal transduction pathways. We demonstrate that the function of *ATHK1* in ABA signal transduction is closely related to the activity of NADPHase, which controls

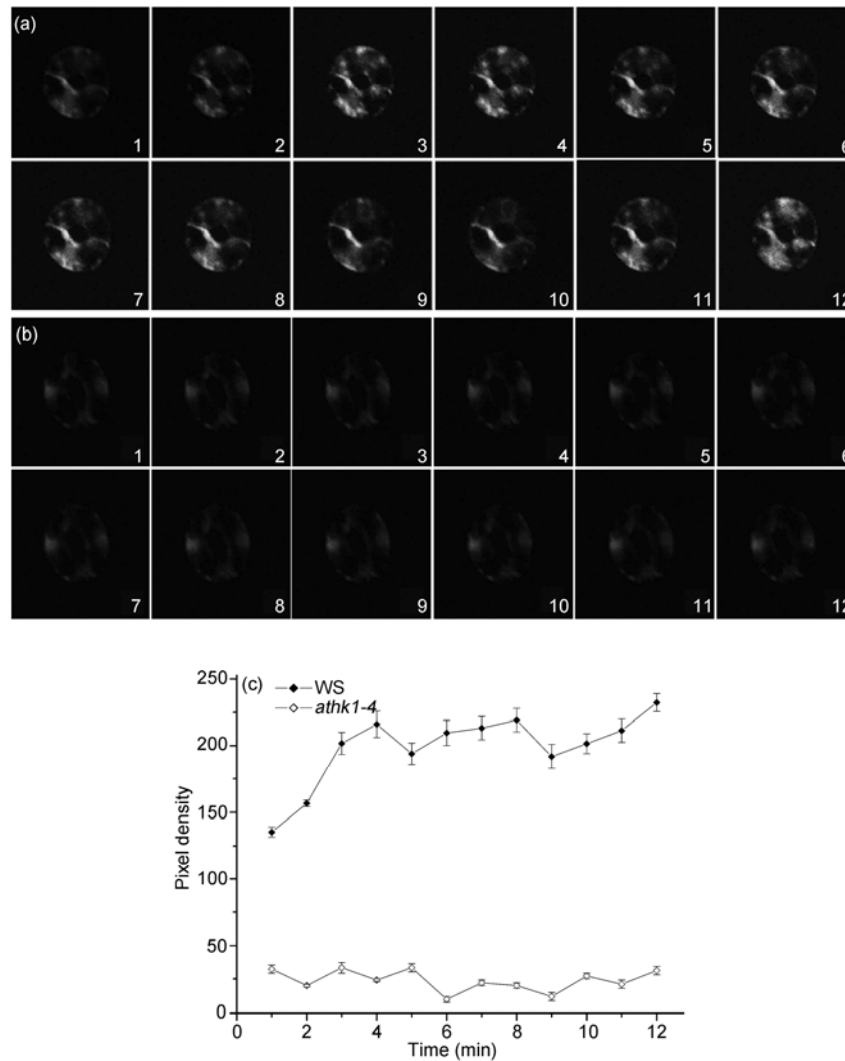


Figure 5 *FURA3-AM* fluorescence revealing the fluctuation of Ca^{2+} concentration after ABA treatment in wild-type WS and mutant *ATHK1* guard cells. (a) LSCM images displaying the fluorescence levels in a WS guard cell. (b) Fluorescence levels in an *ATHK1* mutant guard cell. (c) Oscillations of Ca^{2+} concentration as assessed by changes in *FURA3-AM* fluorescence pixel density in both wild-type and *ATHK1* mutant plants. Data points are expressed as the mean \pm SEM ($n=5$, $P \leq 0.05$). ABA was used at a final concentration of $10 \mu\text{mol L}^{-1}$. Images were captured 1–12 min after ABA treatment using a FV1000S (Olympus, Japan).

the production of H_2O_2 and directly influences Ca^{2+} channel activity. Thus, *ATHK1* may act as a sensor for H_2O_2 , transducing this signal to downstream components. *ATHK1* also controlled Ca^{2+} channel signaling, by regulating the concentration of Ca^{2+} in guard cells, thus regulating stomatal closure.

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