The Histidine Kinase AHK5 Integrates Endogenous and Environmental Signals in Arabidopsis Guard Cells

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Abstract

Background: Stomatal guard cells monitor and respond to environmental and endogenous signals such that the stomatal aperture is continually optimised for water use efficiency. A key signalling molecule produced in guard cells in response to plant hormones, light, carbon dioxide and pathogen-derived signals is hydrogen peroxide (H2O2). The mechanisms by which H2O2 integrates multiple signals via specific signalling pathways leading to stomatal closure is not known.

Principal Findings: Here, we identify a pathway by which H2O2, derived from endogenous and environmental stimuli, is sensed and transduced to effect stomatal closure. Histidine kinases (HK) are part of two-component signal transduction systems that act to integrate environmental stimuli into a cellular response via a phosphotransfer relay mechanism. There is little known about the function of the HK AHK5 in Arabidopsis thaliana. Here we report that in addition to the predicted cytoplasmic localisation of this protein, AHK5 also appears to co-localise to the plasma membrane. Although AHK5 is expressed at low levels in guard cells, we identify a unique role for AHK5 in stomatal signalling. Arabidopsis mutants lacking AHK5 show reduced stomatal closure in response to H2O2, which is reversed by complementation with the wild type gene. Over-expression of AHK5 results in constitutively less stomatal closure. Abiotic stimuli that generate endogenous H2O2, such as darkness, nitric oxide and the phytohormone ethylene, also show reduced stomatal closure in the ahk5 mutants. However, ABA caused closure, dark adaptation induced H2O2 production and H2O2 induced NO synthesis in mutants. Treatment with the bacterial pathogen associated molecular pattern (PAMP) flagellin, but not elf peptide, also exhibited reduced stomatal closure and H2O2 generation in ahk5 mutants.

Significance: Our findings identify an integral signalling function for AHK5 that acts to integrate multiple signals via H2O2 homeostasis and is independent of ABA signalling in guard cells.

Introduction

Plants are constantly exposed to a large multitude of environmental stimuli, and under adverse conditions, are mostly able to survive due to their ability to sense and transduce these signals into cellular and physiological responses. Hydrogen peroxide (H2O2) is a form of reactive oxygen species (ROS) generated by plants via several mechanisms, which include metabolic processes such as respiration and photosynthesis as well as reactions to environmental stimuli such as water deficit, high and low temperature, pollutants, UV-B light and pathogen challenge [1]. It is now well accepted that controlled or regulated production of H2O2 is beneficial to the plant. H2O2 acts as a signal and/or second messenger enabling the plant to activate physiological processes resulting in protection from and adaptation to environmental stress [2].
mediated stomatal closure, with the Cys65 residue of ETR1 in {ETR1} responding in {AHK5} in counteracting the ethylene and ABA-regulated growth in plasma membrane. Recent work has identified a function for {HK domain and receiver domain}, classifying it as a hybrid HK {HK} is predicted to be the only cytoplasmic HK, with both a canonical HK family in guard cells, the function of a least-characterised member of the signalling responses to H2O2 is not known. In recent work, we have shown that one member of the {Arabidopsis} hybrid histidine kinase (HK) family, the ethylene receptor ETR1, is a potential target for H2O2 during stomatal closure [15]. ETR1 is required for H2O2-mediated stomatal closure, with the Cys65 residue of ETR1 being essential. Intriguingly the HK domain of ETR1 was not required for H2O2-induced closure [15]. In recent developments we have also shown that ETR1 has a dual function in guard cells, that of perceiving ethylene as well as acting as a target for H2O2, thereby mediating downstream signalling processes to initiate stomatal closure [16].

The hybrid HK family of receptor proteins are part of the two-component signal perception and transduction system in plants [17]. Perception of a signal by a hybrid HK leads to autophosphorylation of a His residue in the HK domain, followed by a phosphotransfer reaction to an Asp residue on its receiver domain. Subsequently, a relay of the phosphoryl residue occurs to a His residue on a histidine phosphotransfer protein (HP) followed by phosphorylation of an Asp residue on a response regulator (RR) protein [18]. Representative HK family members in plants are the cytokinin and ethylene receptors [17]. However, the plant’s two-component signalling network appears to contribute to several other signal response pathways. For instance, recent work has demonstrated functional cross-talk between cytokinin and light (phytochrome B) signalling [19,20]. These overlaps in signalling processes induced by multiple stimuli suggest that two-component proteins are key sensors and transducers of various environmental and endogenous signals.

One mechanism by which different signalling processes and pathways may be integrated is via common secondary messenger molecules. ROS such as H2O2 are ideal candidates for such messenger molecules acting as focal points for cross-talk between a wide array of signalling cascades [3]. This is evidenced by a large overlap in the expression of genes that are regulated by ROS on the one hand and by environmental stimuli on the other. For example, H2O2-regulated genes are also regulated by drought, cold, UV-B light and pathogen attack [21,22] as well as by ABA [23]. Clearly, there exist multiple targets for H2O2 to mediate its effects in specific cells and tissues. Functional overlap is also likely to exist between these pathways, with certain targets acting as integrators of multiple stimuli.

In an attempt to identify further targets for H2O2 signalling in guard cells, the function of a least-characterised member of the HK family in Arabidopsis, namely AHK5, was investigated. AHK5 is predicted to be the only cytoplasmic HK, with both a canonical HK domain and receiver domain, classifying it as a hybrid HK [17]. Our data here indicate that AHK5 also co-localises to the plasma membrane. Recent work has identified a function for AHK5 in counteracting the ethylene and ABA-regulated growth response in Arabidopsis roots [24]. However its role in integrating signalling responses to H2O2 is not known.

Using a combination of molecular genetic, cell imaging, biochemical and physiological tools, we show that AHK5 is a key player in H2O2 homeostasis in Arabidopsis guard cells in response to environmental and endogenous signals, including NO, ethylene, darkness and bacterial flagellin. Intriguingly, AHK5 does not appear to be involved in the ABA signalling pathway in stomata. Our data suggest a regulatory function of AHK5 that is essential for guard cell response to abiotic and biotic environmental stimuli.

Results

Intracellular localisation and tissue-specific expression of AHK5

AHK5 is predicted to be the only cytoplasmic HK amongst the canonical HK class of proteins in Arabidopsis [17]. In order to confirm this, we performed in vivo localisation studies using 35S promoter-driven GFP fusion constructs of AHK5. In transiently transformed Arabidopsis protoplasts and tobacco (Nicotiana benthamiana) leaf cells the full-length fusion protein was expressed and was present in the cytoplasm, independent of whether the GFP tag was fused to the N-terminus or C-terminus of AHK5 (Fig. 1A and B, and Data S1). To substantiate our cell biological results we also performed cell fractionation experiments with extracts from transiently transformed tobacco leaf cells expressing 35S:GFP-AHK5 or several GFP-marker protein fusion genes. Whereas the ER marker ERS1-GFP [25] and the plasmalemma/endosome marker BR11-GFP [26] were detected in the microsomal fraction and the cytoplasmic/nucleoplasmic marker ARR4-GFP [19] in the soluble fraction, GFP-AHK5 was found in both fractions (Fig. 1C). This intracellular distribution could be substantiated by recording the wavelength-specific intensity distribution of a cytoplasm-plasmalemma-cell wall section of neighbouring tobacco epidermal cells co-expressing GFP-AHK5 and the red fluorescent plasmalemma marker pm-rk-CD3-1007 (Fig. 1D; [27]). Whereas pm-rk-CD3-1007 showed one distinct peak representing the two plasmalemmata of the adjacent cells, three peaks were observed for GFP-AHK5 (Fig. 1D). The medial GFP-AHK5 peak showed a perfect overlay with pm-rk-CD3-1007, whereas the other two peaks extended to the cytoplasmic sites of the adjacent cells. Our results therefore suggest that AHK5 is a HK that is localised both in the cytoplasm and at the plasmalemma of plant cells.

The expression profile of AHK5 in different Arabidopsis tissues and cell types was analysed by semi-quantitative RT-PCR. AHK5 transcript was detectable in light-grown but not in etiolated seedlings (Fig. 2A). Furthermore, AHK5 transcript was present in flowers, siliques and roots and to a lower extent in stems and leaves of 30-days-old Arabidopsis plants (Fig. 2A). Increasing the number of PCR cycles showed a detectable level of AHK5 transcript in mature leaves. As guard cells were the focus of our study, AHK5 expression was also analysed in guard-cell enriched samples [16]. Compared to whole leaves the AHK5 transcript level was significantly lower in guard cells. However, AHK5 expression was increased in guard cell RNA extracted from H2O2-treated leaves (Fig. 2A), suggesting that AHK5 might have a function in H2O2 signalling in guard cells. The guard cell expression of AHK5 was confirmed by creating an AHK5 promoter-GFP-AHK5 genomic construct (PAHK5:GFP-AHK5) and transiently expressing this in tobacco leaves. As shown in Fig. 2B and comparable to our RT-PCR results, GFP fluorescence was detected in guard cells as well as in epidermal cells indicating that the AHK5 promoter is active in stomata. Our expression data therefore correlate well with the expression profile of AHK5 observed in the AtGenExpress developmental data set [28] and adds to that reported earlier [24].
Figure 1. Subcellular localisation of AHK5 in plant cells. (A) Confocal images of Arabidopsis protoplasts and tobacco (Nicotiana benthamiana) leaf cells transiently transformed with a construct expressing P35S:GFP-AHK5 cDNA. Left panel, GFP fluorescence; right panel, bright field image. The bars represent 10 μm. (B) Western blot showing the expression of full-length GFP-AHK5 in transiently transformed tobacco leaf cells using anti-GFP antibody. Lane 1, protein standard; lane 2, extracts from cells transformed with a P35S-GFP-AHK5 construct; lane 3, extracts from cells transformed with the empty vector. (C) Cell fractionation of transiently transformed tobacco leaf cells expressing either GFP-AHK5, the microsomal marker BRI-GFP, the ER marker ERS1-GFP or the soluble marker ARR4-GFP. Two days after the infiltration of the Agrobacteria the leaf tissue was harvested and total protein...
Identification and characterisation of ahk5 T-DNA insertion mutants

The functional characterisation of AHK5 was initiated by the isolation and molecular characterisation of Arabidopsis ahk5 T-DNA insertion lines. Two independent ahk5 alleles were found, one in the INRA-Versailles T-DNA collection (ahk5-3 in Ws4 background; [29]) and one in the Syngenta SAIL T-DNA collection (ahk5-1 in Col-0 background; [30]), respectively. Plants homozygous for the insertion events were identified by PCR on genomic DNA, and the positions of the T-DNA insertions confirmed by sequencing. As shown in Fig. 2C the T-DNA of ahk5-3 is located in an intron within the predicted HK domain, whereas in ahk5-1 the T-DNA is inserted in a 3’ exon which encodes a part of the AHK5 receiver domain. The T-DNA insertions did not appear to cause any other changes within the AHK5 sequence. In addition, the presence of a single T-DNA insertion event in each line was verified by Southern blotting (Data S1). Semi-quantitative RT-PCR was used to determine the level of insertion event in each line was verified by Southern blotting (Data S9). Semi-quantitative RT-PCR was used to determine the level of expression of AHK5 in the homozygous lines. Fig. 2D shows that across the T-DNA borders there was no amplification of an AHK5 transcript. Further extensive PCR using different primer combinations (of those shown in Fig. 2C) confirmed that no full-length transcript of AHK5 was present in the mutant lines (Data S1). Therefore a fully functional AHK5 is unlikely to be expressed in ahk5-1 and ahk5-3.

For complementation and functional analyses ahk5-1 and ahk5-3 were transformed with a construct expressing the full-length GFP-AHK5 or AHK5-TAP fusion construct respectively under the control of the 35S promoter. In addition, AHK5 was ectopically expressed in the Ws4 wild type background (Fig. 2D). RT-PCR confirmed that the transformed ahk5 mutants and wild type expressed AHK5 to high levels (Fig. 2D).

A functional AHK5 HK is required for H2O2 responses in stomatal guard cells

Initially, a pharmacological approach was used to establish whether HK activity is required for H2O2-induced stomatal closure. Arabidopsis wild type (Col-0) leaves were pre-treated with the inhibitor 3,3’,4,4’,5-tetrachlorosalicylanilide (TCSA) [31], followed by exposure to H2O2 and stomatal apertures measured. TCSA inhibited H2O2-induced closure in a dose-dependent manner (Fig. 3), thereby suggesting that HK activity is indeed required for H2O2 signalling to occur in guard cells leading to stomatal closure.

Regulation of AHK5 expression in guard cells by H2O2 led us to investigate whether AHK5 function is necessary for the response of guard cells to exogenous H2O2 by performing stomatal bioassays. Compared to wild type, guard cells of ahk5-1 and ahk5-3 were dramatically less sensitive to H2O2 at various concentrations (Fig. 4A, B). The sensitivity of both mutant lines to H2O2 was restored by the expression of the wild type AHK5 cDNA under the control of the 35S promoter (Fig. 4C) showing that the GFP-AHK5 fusion protein used for the localisation studies is functional in planta. These data also demonstrate that loss of AHK5 gene function causes the H2O2-insensitive mutant phenotype and that AHK5 is required for stomatal response to exogenous H2O2. Interestingly, ectopic expression of AHK5 in wild type background resulted in slightly smaller stomatal apertures compared to wild type Ws4 in the absence of H2O2 (Fig. 4C, right columns). This suggests that the guard cells of the AHK5 overexpressor are more sensitive to endogenous H2O2. However, we observed a response of the overexpressor to exogenous H2O2 suggesting that ectopic accumulation of AHK5 per se does not significantly alter the plant’s sensitivity to H2O2.

Stomatal responses to NO, ethylene and darkness are impaired in ahk5 mutants

The insensitivity of ahk5 mutant guard cells to H2O2 suggests that AHK5 could be an essential signalling component in the stomatal closure response of Arabidopsis to various stimuli. Recently, we have shown that H2O2 induces the generation of NO in the response of guard cells to ABA [32]. If AHK5 is acting downstream of H2O2, the response to NO might also be affected in the ahk5 mutants. As shown in Fig. 5A, ahk5-1 and ahk5-3 stomata showed a reduced sensitivity to the NO donor sodium nitroprusside (SNP). The NO insensitive phenotype of the ahk5 mutants was functionally complemented by the wild type GFP-AHK5 construct (Fig. 5A). In addition, experiments with TCSA showed that NO-induced closure required HK activity (Fig. S2). These results indicate a function for AHK5 in both the H2O2 and NO response pathway in Arabidopsis guard cells. In contrast, the sensitivity of both mutants to ABA was not changed appreciably (Fig. 5A) suggesting the possibility that stimuli other than ABA lead to H2O2 and NO generation which might act via AHK5, and that ABA signalling occurs largely independent of AHK5.

In our previous work we demonstrated that pea guard cells exposed to darkness generate H2O2 [33]. Our pharmacological data revealed that pre-treatment with TCSA inhibited dark-induced stomatal closure, suggesting a requirement for HK activity in this response (Fig. S2). To investigate the role of AHK5 in dark-induced stomatal closure, the response of ahk5 mutants to dark conditions were examined. As shown in Fig. 5A, detached leaves of both the ahk5-1 and ahk5-3 mutants showed reduced stomatal closure in response to dark conditions, with stomata of ahk5-1 responding slightly to dark conditions. The experiments were also performed with non-detached leaves. Whilst the stomata of wild type and ahk5-1 plants were open 30 min prior to transfer to darkness, the light-off conditions induced stomatal closure only in the wild type but not in the mutant (Fig. 5B). These data indicate that AHK5 function also contributes to the dark-induced stomatal closure response in Arabidopsis.

We have shown previously that ethylene perceived by the ethylene receptor ETR1 (a hybrid HK) also induces stomatal closure via H2O2 synthesis [16]. Furthermore, a functional ETR1 receptor is required to mediate H2O2-induced stomatal closure [15]. Experiments with TCSA showed that HK activity is required for ethylene-induced stomatal closure (Fig. S2). We therefore investigated the effect of ethylene on ahk5-1 and ahk5-3 guard cells. Treatment with the ethylene-generating compound ethephon induced a stomatal closure response in wild type but not in ahk5.
Figure 2. Expression pattern of AHKS and characterisation of the T-DNA insertion sites in ahk5-1 and ahk5-3. (A) Steady-state levels of AHKS transcript in different tissues and developmental stages of Arabidopsis detected by semi-quantitative RT-PCR using different cycle numbers. For the detection of AHKS, up to 40 cycles of PCR were performed using primers 3 and 10 (left panel) or primers 8 and 9 and 40 cycles of PCR (right panel). GC, cDNA from guard cell-enriched non-treated leaf sample; GC+H, cDNA from guard cell-enriched leaf samples treated with 0.5 mM H$_2$O$_2$ for 2 h; WL, cDNA from whole-leaf sample. ACTIN and EF1 were used as controls. Primer numbers indicated in panel C. (B) Expression of a PAHK5-GFP-AHK5 genomic construct in transiently transformed tobacco leaf cells. Agrobacteria carrying the PAHK5-GFP-AHK5 construct were infiltrated into the abaxial side of the leaf and the GFP fluorescence analysed by CLSM 2 days later. Top panels = GFP images, lower panels = GFP image overlaying
AHK5 Function in Guard Cells

Guard cells. The sensitivity to ethylene could be restored when the GFP- and TAP-tagged AHK5 fusion constructs were expressed in the ahk5 mutants under the control of the 33S promoter (Fig. 5C). These data show that ethylene induces stomatal closure via AHK5 function.

The data so far indicate that AHK5 function is required for H2O2, NO, darkness and ethylene-induced stomatal closure, but not for ABA-induced closure. To establish if AHK5 might be regulating redox homeostasis in response to these stimuli, the generation of both H2O2 and NO were measured in guard cells. Whilst the source of H2O2 in Arabidopsis guard cells has been established as AtbbohD and F for ABA [13], and AtbbohF for ethylene [16], it is not known how darkness induces H2O2. Figure 6A shows that guard cells of the atbbohD/F NADPH oxidase double mutant did not produce H2O2 nor close their stomata after transfer into darkness, thereby demonstrating that these homologs regulate dark-induced H2O2 synthesis in Arabidopsis guard cells. Interestingly, ahk5 guard cells did generate H2O2 following dark adaptation of leaves as in wild type (Fig. 6B), thereby suggesting that Ahk5, although involved in dark-induced stomatal closure, is not involved in regulating dark-mediated H2O2 synthesis.

H2O2 can also induce NO synthesis in the ABA signal transduction pathway in guard cells [32]. Although ahk5 mutants do not respond to either H2O2 or NO (Fig. 4 and 5A), H2O2 induced NO synthesis in both mutant alleles (Fig. 6C), thereby positioning AHK5 downstream of H2O2 and NO in the signal response pathway. In addition, ethylene-induced H2O2 production was investigated in ahk5. Upon treatment with ethephon we observed a strong increase in H2O2-fluorescence in wild type guard cells (Fig. 7D). In contrast, ethylene caused a decrease of H2O2 levels in ahk5-1 in comparison to that of the mock-treated control (Fig. 7D). These data demonstrate that AHK5 contributes to both, the ethylene-induced H2O2 production and stomatal closure response in Arabidopsis. However, ABA induced H2O2 synthesis in ahk5 mutant, as in wild type guard cells (25% increase over controls; Data S1).

In summary so far, the data presented show that darkness-induced H2O2 synthesis, which is generated by ATRBOHD/F, signals through AHK5 to mediate stomatal closure, and that AHK5 is positioned downstream of H2O2 and NO in the signal response pathway. Moreover, AHK5 regulates ethylene-induced H2O2 synthesis leading to stomatal closure, but is not involved in the ABA signal transduction pathway.

Stomatal responses to PAMPs are impaired in the ahk5 mutants

Bacteria-derived pathogen-associated molecular patterns (PAMPs) such as flagellin and EF-Tu have been shown to induce the synthesis of ethylene [34] and an oxidative burst in Arabidopsis leaves, which is mediated by ATRBOHD [35]. Furthermore, recent work by Melotto and colleagues (2006) demonstrated that stomata act as sites of entry for bacteria, and that bacteria and bacteria-derived PAMPs caused a primary stomatal closure response followed by a re-opening, when the plant was attacked by a virulent bacterium [36].

Experiments with the Pseudomonas syringae strains pv DC3000 and the hrpD mutant, which lacks a functional type III secretion apparatus, revealed that ahk5-1 mutant guard cells showed reduced stomatal closure in response to both bacterial strains (Fig. 7A). The reduced stomatal closure response to the hrpD mutant strain suggests that AHK5 is involved in the basal defence of Arabidopsis, which is mediated by PAMPs such as the flagellin peptide 22 (flg22) and the EF-Tu peptide 26 (elf26; [37]).

Flagellin-induced stomatal closure in wild type plants was inhibited by pre-treatment with TCSA (Fig. S2), indicating a requirement for HK activity to mediate this response. To investigate this in more detail, leaves of the ahk5-1 mutant (Col-0 background) were exposed to flg22 and the stomatal apertures measured. The response to flg22 was not investigated in the ahk5-3 mutant as the Ws background lacks a functional FLS2 receptor [38]. As reported [36], flg22 induced stomatal closure in Col-0 wild type guard cells (Fig. 7B). This response was not observed in the ahk5-1 mutant. The loss-of-function phenotype of ahk5-1 was complemented by the expression of the GFP-AHK5 fusion construct, which is under the control of the 35S promoter (Fig. 7B). Interestingly, elf26 caused an identical stomatal closure response in wild type (Col-0, Ws4) as well as in the ahk5-1 and ahk5-3 mutants (Fig. 7C). These data suggest that AHK5 plays a specific role in the flagellin signal response pathway in guard cells.

PAMP-induced H2O2 production was also investigated to study the role of AHK5 in redox homeostasis during basal defence. Interestingly, whereas elf26 caused an identical increase in H2O2 fluorescence in guard cells of wild type and ahk5-1, treatment with flg22 actually caused a decrease in H2O2 fluorescence in ahk5-1, when compared with that of the mock-treated control (Fig. 7D). Therefore AHK5 appears to play a role in both the flg22-induced regulation of H2O2 production and stomatal closure.

Discussion

An AHK5-dependent signalling pathway acts in stomatal guard cells

We have characterised the canonical HK AHK5 as being a cytoplasmic/membrane protein differentially expressed in various tissues of Arabidopsis. Despite the predicted cytoplasmic location
Figure 4. *ahk5* mutant guard cells show reduced sensitivity to H$_2$O$_2$. (A) Stomatal closure in wild type Col-0 (white bars) and *ahk5*-1 (black bars) leaves 2.5 h after exposure to increasing concentrations of H$_2$O$_2$. (B) Stomatal closure in wild type Ws4 (white bars) and *ahk5*-3 (black bars) leaves 2.5 h after exposure to increasing concentrations of H$_2$O$_2$. (C) Stomatal closure in leaves of wild type (Ws4, Col-0), *ahk5*-3 and *ahk5*-1 as well as in the *ahk5* mutants and wild type (Ws4) transformed with a construct expressing the *AHK5* cDNA under the control of the 35S promoter (P$_{35S}$-AHK5). The leaves were either mock-treated (white bars) or exposed to or 100 μM H$_2$O$_2$ (black bars). Data are expressed as mean ± S.E. derived from measuring the apertures of at least 60 guard cells from 3 independent experiments.

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Figure 5. Guard cells of *ahk5* mutants show an altered response to NO, ethylene and darkness. (A) Stomatal closure in wild type (Col-0, Ws4), *ahk5-3* and *ahk5-1* in response to buffer (white bars), 50 μM sodium nitroprusside, (SNP, black bars), 10 μM abscisic acid (ABA, grey bars) and 2.5 h exposure to darkness (striped bars). Data from *ahk5-1* dark treatment are statistically significant (Student’s t-test; p<0.05) versus dark treatment.
of wt, and data from ahk5 ABA treatment are statistically significant (p < 0.05) versus controls. (B) Guard cell response in plant-attached leaves in light and after transfer to darkness. Stomatal apertures were measured from wild type (Col-0) or ahk5-1 leaves either 30 min prior to light off (white bars) or 1 h after transfer to darkness (black bars). Data are expressed as mean +/- S.E. from 3 independent experiments (n = 60 guard cells). (C) Stomatal closure response to ethylene in guard cells of wild type (Col-0, Ws4), ahk5-1, ahk5-3 and ahk5 mutants transformed with the construct PINS/AHK5. White bars, mock-treated; black bars, 3 h treatment with 100 μM ethephon. Data are expressed as mean +/- S.E. from 3 independent experiments (n = 60 guard cells).

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[18] and lack of transmembrane domains within the AHK5 sequence, our data suggest that AHK5 co-localises at the plasmalemma as well. Interestingly, putative N-myristoylation sites are predicted for AHK5, suggesting that an association of AHK5 to the plasmalemma is possible. In addition, we cannot exclude the possibility that the interaction with intrinsic membrane proteins including other HKs locates AHK5 to the plasmalemma. Expression of AHK5 appears to be regulated by H2O2 in guard cells. In addition, we have shown using functional approaches that AHK5 plays a crucial role in mediating H2O2-dependent processes in stomatal guard cells which are induced by environmental and hormonal signals such as NO, ethylene, adaptation to darkness and the PAMP flagellin (flg22). Despite its low level of expression, mutations in the AHK5 gene appear to have profound effects on the guard cell phenotype. The pattern of low expression of a gene in guard cells still resulting in distinct stomatal phenotypes has been observed before – e.g. the nitric oxide (NO)-generating enzyme nitrate reductase (NR). There are two NR genes, NR1 and NR2, in the Arabidopsis genome. Although NR1 is expressed at much lower levels than NR2 in guard cells, mutations in NR1 but not NR2 appear to affect ABA-induced NO responses in stomata [39]. Although cis elements found in the promoters of guard cell specific genes [40] are present in the promoter region of AHK5, it is possible that other cis elements which repress promoter activity in guard cells cause the weak expression pattern, as seen with other guard cell genes [41]. It is also likely that phenotypic effects may be explained by specific protein-protein interactions between a low-expressed protein in guard cells and other proteins of higher abundance. Preliminary data show that expression of the phosphotransfer protein AHP2 and the response regulator ARR4 that AHK5 interact with are of higher abundance than AHK5 in guard cells (Data S1). Further studies will analyse the functions of AHK5 promoter, as well as studying the protein interacting two-component partners of AHK5.

AHK5 functions as integrator of H2O2-dependent signalling in stomatal guard cells

A lack of functional AHK5 results in altered stomatal responses not only to exogenous H2O2 but also to multiple stimuli which are known to generate H2O2 in plant tissues. These signals include ethylene [16], the light-off signal (darkness; data shown here and [33]) and the PAMP flg22 [35].

Previously we provided evidence for a role for the plant hormone ethylene in mediating stomatal closure via H2O2 signalling [16]. Although the HK function of ETR1 is not required for H2O2 signalling, the null-term of ETR1 appears to be essential for this signalling to occur in guard cells [15]. The pharmacological data presented here with TCSA indicate that HK activity is required for H2O2 (and NO)-induced stomatal closure in Arabidopsis. As ethylene is able to produce H2O2 in wild type guard cells [16], and as shown here, AHK5 is also involved in ethylene-dependent signalling leading to H2O2 synthesis and stomatal closure, it is possible that the ethylene-sensing N-terminus of ETR1 functionally and/or physically interacts with H2O2-activated AHK5 during ethylene signal transduction in guard cells. This is in agreement with recent work by Iwama et al. [24], who demonstrated a functional interaction of AHK5 with the ethylene and ABA response in the control of root growth in Arabidopsis. The authors propose an "unidentified" stimulus as being sensed by AHK5, which could integrate the ABA and ethylene signalling pathways in roots. On the basis of our data it is likely that this unknown stimulus for AHK5 is H2O2, although the ahk5 phenotype in roots in response to H2O2 remains to be determined.

Synthesis of H2O2 upon transfer of plants to darkness was found to depend on NADPH oxidase orthologues in pea [33]. By using an atrbohD/F double mutant we demonstrate here that dark-induced H2O2 formation occurs by a similar mechanism in Arabidopsis. We also show that ahk5 mutants do not close their stomata in response to darkness. This is substantiated by the pharmacological data showing inhibition by TCSA, of dark-induced closure in wild type Arabidopsis. Stomata of the H2O2-insensitive etr1-1 mutant still respond to darkness (Data S1), suggesting the possibility that as far as HKs are concerned, AHK5 might have a unique role in the dark-H2O2 signalling pathway in guard cells. Although of fundamental physiological and ecological relevance, little is known of the dark-induced signalling processes leading to stomatal closure. The type 2C protein phosphatases ABI1 and ABI2 [42], the outward potassium channel GORK [43] and the MYB transcription factor AtMYB61 [44] have functions in guard cell responses in the dark. However, the mechanism by which AHK5-dependent phosphorylation is linked to proteins such as ABI1, 2, GORK and AtMYB61 in the guard cell signalling network is not yet known.

AHK5 also appears to be essential for mediating flagellin- (flg22) induced stomatal closure in the Col-0 ecotype, again correlating with the TCSA data demonstrating inhibition of flg22-induced stomatal closure. Surprisingly, the AHK5-mediated response seems to be specific for flg22 because the mutants showed a wild type stomatal closure response to the PAMP elf26. This is unexpected because the signalling cascades of flg22 and elf26 overlap considerably in non-guard cell tissue in respect to ethylene and H2O2 production, alkalinisation, activation of MAPKs and changes in gene expression [34,37]. However, as noted recently, there are likely to be differences between guard cells and mesophyll cells mediating pre- and post-invasive immunity [45]. Recent work by Melotto et al. [36] indicates that bacterial PAMPs such as flagellin induce stomatal closure in Arabidopsis. Our data, therefore, position AHK5 in a signal transduction cascade specific to flagellin in guard cells. The exact mechanism by which AHK5 interacts with this pathway remains to be determined, but it is likely that the interaction occurs with the flg22 receptor FLS2. It is interesting to note that the expression of FLS2 is abundant in guard cells [46]. Given that FLS2 is a plasmalemmbound and AHK5 is likely to be located at the plasma membrane as well as the cytoplasm, one may speculate that both receptors could physically interact at the plasma membrane allowing AHK5 to perceive high local H2O2 concentrations induced by the flg22-activated FLS2/BAK1 receptor complex [47,48]. Importantly, flg22 but not elf26 was unable to induce H2O2 accumulation in ahk5 mutant guard cells. Thus, AHK5 also appears to contribute
Figure 6. Regulation of H$_2$O$_2$ homeostasis by AHKS. (A) Stomatal responses to darkness in wild type (Col-0) and the atrbohD/F mutant. White bars, stomatal apertures of guard cells from leaves exposed to light (light) or transferred to darkness for 2 h (dark). Black bars, H$_2$O$_2$ fluorescence determined by confocal microscopy from epidermal peels exposed to light (light) or transferred to darkness for 30 min (dark). Data are expressed as mean +/- S.E. (n = 60 guard cells for aperture measurements; n = 40–80 guard cells for average H$_2$O$_2$ fluorescence from confocal experiments). (B) Darkness-induced H$_2$O$_2$ synthesis in wild type (Ws4) and ahk5-3 mutant guard cells. H$_2$O$_2$ fluorescence from epidermal peels exposed to ambient light (white bars) or darkness (black bars) for 30 min was determined by confocal microscopy using the H$_2$O$_2$-sensitive fluorescent dye H$_2$DCFDA. Data are expressed as mean +/- S.E. (n = 40–80 guard cells for each treatment). (C) H$_2$O$_2$-induced NO fluorescence from guard cells of wild type and ahk5 mutants using confocal microscopy and the NO-sensitive fluorescent dye DAF2-DA. White bars, mock-treated; black bars, 15 min treatment with 100 μM H$_2$O$_2$. Data are expressed as mean +/- S.E. (n = 75–130 guard cells for each treatment).

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Figure 7. Guard cells of ahk5 mutant show a differential response to PAMPs. (A) Stomatal aperture of wild type (Col-0) and ahk5-1 3 h after exposure to P. syringae pv. DC3000 (black bars) or hrpA^- mutant (grey bars). White bars, mock-treatment. (MgCl2). (B) Stomatal closure of wild type (Col-0), ahk5-1 and ahk5-1 transformed with a P35S-AHK5 construct after a 3 h treatment with either buffer (white bars) or 10 nM flg22 peptide (flg22,
to the flagellin-induced regulation of H$_2$O$_2$ levels in guard cells (see below for detailed discussion). This also implies that an additional H$_2$O$_2$ sensor is required for the perception of the H$_2$O$_2$ signal derived from the EF-Tu/EFR receptor complex.

As demonstrated by the wild type behaviour of both ahk5 mutants to ABA, the AHK5-dependent signalling pathway does not contribute to the ABA response pathway in guard cells. This is not entirely surprising, as we have previously observed that mutants of ETR1 also respond normally to ABA [16]. Although ABA signalling requires the synthesis and action of H$_2$O$_2$ [13], our data indicate that this function is independent of AHK5.

The complexity of redox signalling in stomatal guard cells

Redox signalling in guard cells is likely to be regulated via a number of signalling pathways. This may be because of the nature of H$_2$O$_2$ as such, being able to diffuse freely between cellular compartments, and also due to the fact that H$_2$O$_2$ is likely to be generated in localized “hot spots” within the cell, thereby leading to localized effects of H$_2$O$_2$ on its target proteins [2]. Generation of H$_2$O$_2$ in guard cells in response to ABA and darkness occurs via the NADPH oxidase orthologues ATRBOHD and ATRBOHF ([13] and this study). ATRBOHF is essential for H$_2$O$_2$ generation in response to ethylene and ETR1 functions as a central mediator of H$_2$O$_2$ responses [16]. In leaves, flg22-induced H$_2$O$_2$ production occurs via ATRBOHD [35]. RBOH proteins are localised at the plasma membrane [49], and the proteins involved in H$_2$O$_2$ signalling are located at the ER (ETR1; [25,50]), plasma membrane (AHK5, FLS2, [51]) or in the cytosol (AHK5). We have observed that AHK5 function is crucial for ethylene and flg22-induced but not for darkness or elf26-induced H$_2$O$_2$ accumulation. Further work using ahk5 plants crossed with atrboh mutants is required to confirm how AHK5 and RBOH signalling interact. However, AHK5 transcript appears to be regulated by H$_2$O$_2$ in guard cells, and AHK5 function is essential for H$_2$O$_2$ and NO-dependent signal transduction. Together, the data suggest that AHK5 acts to maintain H$_2$O$_2$/redox homeostasis in guard cells in response to multiple stimuli. A positive feedback loop is possible, whereby H$_2$O$_2$ generated via different stimuli (from RBOH), regulates AHK5 expression (or activity), which in turn regulates H$_2$O$_2$ synthesis (for ethylene and flagellin pathways) and action leading to stomatal closure. Regulation of the expression of HKs by the stimulus that induces their activity is not uncommon – e.g. perception of the cytokinin receptor CRE1 and ethylene receptors is regulated by cytokinin and ethylene treatments, respectively [22]. Detailed investigations are necessary to elucidate the molecular mechanisms of redox regulation of AHK5, via mass spectrometry of the purified protein.

The dual function of AHK5, in regulating H$_2$O$_2$ synthesis and action is reminiscent of the role of ETR1, which we have shown previously to have a dual function in guard cells, that of perceiving ethylene as well as H$_2$O$_2$ [16]. AHK5 could therefore have multiple functions as well: firstly, AHK5 may contribute to the flagellin- and ethylene-induced H$_2$O$_2$ accumulation and, secondly, may sense H$_2$O$_2$ produced in the course of the ethylene-, NO-, flg22- and darkness-regulated stomatal closure response. Our evidence that AHK5 plays a role in the inducible accumulation of H$_2$O$_2$ comes from our observation that the H$_2$O$_2$ level is decreased in ahk5-1 guard cells upon treatment with ethylene or flg22. It is not yet known whether AHK5 interacts with receptors such as ETR1 or FLS2, H$_2$O$_2$-generating enzymes such as ATRBOHD/F and redox-active proteins such as ATGXP3, ABI1 and ABI2 which are located in distinct sub-cellular compartments [9,10,52] or whether AHK5 co-ordinates its functions to integrate H$_2$O$_2$ signalling. However, our study provides evidence that AHK5 acts to integrate multiple H$_2$O$_2$-dependent processes at different molecular levels.

Summary and concluding remarks

We have shown that AHK5 functions in guard cells to mediate stomatal responses to various stimuli that generate H$_2$O$_2$. Evidence is slowly emerging that implicate overlapping signalling pathways during abiotic and biotic stress responses in plants, which include hormone signalling, ROS signalling and protein phosphorylation [22,53]. Our data position AHK5 both upstream and downstream of ROS in integrating bacterial, darkness and hormonal-induced responses which could be achieved by differential protein-protein interactions. This is the first demonstration of a role for a HK two-component signalling pathway in integrating abiotic and biotic signals. Moreover, it is the first identification of a HK mediating H$_2$O$_2$ homeostasis to integrate multiple stress responses in guard cells. The data presented here highlight the mechanistic role of the AHK5 two-component signal transduction pathway in stomata, which are ideal model systems to study integration of multiple stimuli.

Materials and Methods

Growth and maintenance of plants

Wild type and mutant seeds of Arabidopsis thaliana ecotype Columbia (Col-0) and Wassilewskija (Ws4) were sown on Levington’s F2 compost and grown under a 16 h photoperiod (100–150 µE m$^{-2}$ s$^{-1}$), 22°C and 65% relative humidity in controlled environment growth chambers (Sanyo Gallenkamp, UK). Arabidopsis thaliana (Col-0) and Wassilewskija (Ws4) seeds were crossed to A. thaliana (Col-0). The T-DNA insertion lines in AHK5 are as follows: ahk5-1 mutant seeds (SAIL 50_H11) were originally obtained from Syngenta (SAIL collection, now available at ABRC/NASC), and the ahk5-3 seeds (FLAG_271G11) were obtained from the INRA/FLAG-FST collection at Versailles [29,30].

Stomatal bioassays

Stomatal assays were performed on leaves essentially as described in [15]. Leaves were floated for 2.5 h under continuous illumination (100–150 µE m$^{-2}$ s$^{-1}$) in Mes/KCl buffer (5 mM KCl/10 mM Mes/50 µM CaCl$_2$, pH 6.15). Once the stomata were fully open, leaves were treated with various compounds for a further 2.5 h. The leaves were subsequently homogenised individually in a Waring blender for 30 s and the epidermal fragments collected on a 100 µm nylon mesh (SpectraMesh, BDH-Merck, UK). Stomatal apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (Leica QWin software, Leica, UK). Flg22 and
elf-26 peptides were a kind gift from J Mansfield (Imperial College London). Elicitors were added to the incubation buffer at 2.5 h and stomatal apertures measured after a further 3 h.

For bacterial experiments, *Pseudomonas syringae* pv DC3000 or *P. syringae* hrpA− mutant were grown overnight in LB media and overnight cultures centrifuged, resuspended in 10 mM MgCl₂ at an OD₆₀₀ = 0.2 (equivalent to 2 x 10⁸ cfu/ml). Silwet (0.002% v/v) was added to cultures or MgCl₂ to act as a wetting agent. Bacteria were gently coated onto the abaxial side of leaves on intact plants (controls were MgCl₂ with Silwet alone). Plants were left in the growth chambers with a covered lid (to increase humidity) for 3 h, inoculated leaves subsequently detached and stomatal apertures measured.

**Measurement of H₂O₂ and NO using confocal/fluorescent microscopy**

Epidermal peels from mature leaves, prepared as described above, were incubated in Mes/KCl buffer for 2–3 h. Following this, the fragments were loaded by incubation in 50 μM of the H₂O₂-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes, Leiden, The Netherlands) for 10 min. After washing in fresh buffer for a further 20 min, the fragments were challenged with various compounds as indicated in the figure legends. For dark treatments the peels were incubated in darkness for 30 min and microscopy performed.

Confocal laser scanning microscopy was used to visualise fluorescence, using an excitation wavelength of 488 nm and an emission wavelength of 515–560 nm (Nikon PCM2000, Nikon Europe B.V. Badhoevedorp, The Netherlands). Images were acquired and analysed using Scion Image software (Scion Corp., USA) to measure the relative fluorescence intensities in the cells following various treatments. For the data in Figure 7D, fluorescent microscopy (Zeiss Axioskop2, Zeiss, UK) was used with filter set 10 (excitation filter BP 450–490 nm, beam splitter FT 510 nm and emission filter BP 515–565 nm). Images were acquired and analysed using Image J software (NIH, USA). Data represent fluorescence intensities expressed as average fluorescence or as a percent of the control values, from several guard cells analysed in different experiments. For NO fluorescence, epidermal peels were loaded with 10 μM of the NO-sensitive dye diaminofluorescein diacetate (DAF2-DA, Calbiochem, UK) using exactly the same dye loading procedure, and images acquired using confocal microscopy as described above.

**Cloning, expression analysis of AHKS and characterisation of T-DNA mutants**

The *AHK5* Entry clone was constructed using Gateway™ technology (Invitrogen, UK). It was obtained through TOPO-reaction using the pENTR/D-TOPO vector (Invitrogen). PCR was performed using Phusion™ polymerase (Finzymes, UK) and cDNA from Arabidopsis roots as template. Primers were as follow: 5′-CACC-TCTGAGACCTCCACGGCGGATA-GATTATCG-3′ and *AHK.REV.*GGTGAACAGCTCCCTGGCTGCAG-CATTTCACAGACCATTGATCAAGGTTTCTC-3′ (underlined). The construct for the expression of the GFP fusion proteins under the control of 35S promoter (35S::GFP::AHK5) was cloned via LR-reaction into the destination vector pMDC99 [56] using the LR reaction. This binary vector was then transformed into the *Agrobacterium tumefaciens* strain GV3101, and used in tobacco transient expression studies as described above.

For identification and characterisation of homozygous insertion mutants, genomic DNA isolated from appropriate wild type and mutant plants was used for PCR analysis, using various PCR combinations and primers. T-DNA primers used were those already described [29,30]. Individuals were chosen from homozygote lines by selection on BASTA, Southern analysis confirmed the presence of single T-DNA insertions in these lines and at least 3 generations were followed through to get a homozygote population.

For RT-PCR, total RNA from corresponding tissues and developmental stages of *A. thaliana* was isolated using RNAwiz™ (Ambion, UK) or TRIZOL reagent (Invitrogen, UK) and genomic DNA was removed using TURBO DNA-free™ (Ambion, UK). RNA was isolated from guard cell-enriched epidermal fragments and whole leaves as described previously [16]. Subsequently, 1.5 μg of total RNA was reverse transcribed using oligo-dT primer with SuperScript™ III Reverse Transcriptase (Invitrogen, UK) and the resulting cDNA was used as template for the PCR with

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HooStart Taq polymerase (Genaxxon, Germany). PCR products were separated on agarose gel electrophoresis after different number of PCR cycles for comparison with ACTIN2 or EF1 as described in [39]. The sequences of the primers shown in Figure 2 are in Supplementary data.

Transient transformation of tobacco leaf cells and Arabidopsis protoplasts, GFP and RFP analyses

The p19 protein from tomato bushy stunt virus cloned in pBIN61 [60] was used to suppress gene silencing in tobacco (Nicotiana benthamiana). All plasmids were transformed in Agrobacterium tumefaciens strain GV3101 pMP90, which was grown in YEB medium to OD600 1.0 and prior to infiltration resuspended in AS medium (10 mM MgCl2, 150 mM acetosyringone and 10 mM MES pH 5.7) to OD600 = 0.8. The Agrobacterium strains containing the GFP or p19 construct were mixed in a 1:1 relationship and co-infiltrated into leaves of 4-week-old tobacco plants as described in [60]. The abaxial epidermis of infiltrated tobacco leaves was assayed for fluorescence by CLSM (confocal laser-scanning microscopy) 2 to 3 day post infiltration according to [61]. Arabidopsis protoplasts were transformed using PEG mediated transformation procedure and assayed for fluorescence by CLSM after 20 h [61]. CLSM was performed using a Leica TCS SP2 confocal microscope (Leica Microsystems, Germany). These CLSM images were obtained using the Leica Confocal Software and the HCX PL APO 63×/1.2 W CORR water-immersion objective.

For recording the RFP and GFP intensity profiles a homemade confocal laser-scanning microscope, based on a Zeiss Axiovert was used [62,63]. The microscope was equipped with an avalanche photodiode (APD, SPCM-AQR-14, Perkin Elmer, USA) as a spectrally integrating detector. A pulsed 473 nm diode laser (Picoquant LDH-P-C470) operating at a repetition rate of 10 MHz served as excitation source. Fluorescence intensity images were obtained by raster scanning the sample and detecting emission intensity for every spot on the sampled area. The setup was equipped with a 480 nm long pass filter (Semrock Razor Edge LP02-473RU-25) to block back-scattered excitation light, a 500 nm bandpass filter (Semrock BrightLine BL500/24) to detect GFP-fluorescence and a 590 nm bandpass filter (Semrock FF01-590/20-25) to detect RFP-fluorescence in front of the APD. The processing of the obtained fluorescence intensity images was accomplished with the WSxM software [64].

Protein extraction, cell fractionation, SDS-PAGE and western blotting

For cell fractionation 100 mg tissue of transiently transformed tobacco leafs were homogenised in liquid nitrogen and the homogenate was extracted in 2 ml homogenization buffer (25 mM MOPS, 0.1 mM MgCl2, 8 mM L-cysteine, 2.5 mM EDTA, 2× protease inhibitor mix (Roche), 250 mM sucrose; pH 7.8). The crude extract was cleared from debris by centrifugation (4000×g 40 min, 4°C). The microsomal fraction was separated from the soluble fraction by ultracentrifugation (100,000×g 30 min, 4°C).

The pellet was washed three times in homogenization buffer supplemented with 0.05% Triton X-100 and resuspended in 50 μl SDS-PAGE sample buffer. The soluble fraction was mixed with SDS-PAGE sample (ratio: 2:1 v/v). For SDS-PAGE 18 μl of the soluble fraction and 10 μl of microsomal fraction were loaded. Western blot analysis and immunodetection were performed according to [61] using anti-GFP antibody (Roche, Switzerland) to detect GFP-AHK5, BRI1-GFP, ERS1-GFP and ARR4-GFP. An anti-mouse-AP conjugate (BioRad, UK) was used as secondary antibody.

Statistical analysis

All data from stomatal bioassays and fluorescence measurements were statistically analysed by using Student’s t-test analysis. Data are statistically significant (p<0.01) for all treated versus control responses for wild type and complemented lines, and not significant (p>0.01) for mutant treated (H2O2, ethephon, NO, darkness and flg22) versus mutant controls, unless otherwise indicated.

Supporting Information

Figure S1 The NO insensitive stomatal closure response phenotype of the ahk5-1 mutant is complemented by the 35S promoter-driven expression of the AHK5 cDNA. Stomatal closure in wild type Col-0, ahk5-1 mutant or ahk5-1 transformed with a construct expressing GFP-AHK5 under the control of the 35S promoter (P35S-AHK5/ahk5-1) in response to mock treatment (white bars) or SNP (50 μM, black bars) for 2.5 h.

Found at: doi:10.1371/journal.pone.0002491.s001 (1.42 MB TIF)

Figure S2 Histidine kinase (HK) activity is required for NO-, dark- and flg22-induced stomatal closure. Effect of the HK inhibitor 3,3′,4,4′,5-tetrachlorosalicylanilide (TCSA) on stomatal closure in wild type Arabidopsis (Col-0). Arabidopsis leaves were incubated in stomatal opening buffer for 2.5 h followed by treatment for 15 min with 10 μM of TCSA prior to exposure to darkness, ethephon (eth, 100 μM), flg22 (100nM) or SNP (50 μM) for 2.5 h. Control, buffer alone. Data are expressed as mean +/- S.E. from 3 independent experiments (n = 60 guard cells).

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Data S1

Found at: doi:10.1371/journal.pone.0002491.s003 (0.02 MB DOC)

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Author Contributions

Conceived and designed the experiments: SN KH RD RH JH. Performed the experiments: RD JH CC VM CG JW KE MC. Analyzed the data: KH AM. Wrote the paper: SN KH RD RH JH.

References
