Role of the Putative Osmosensor Arabidopsis *Histidine Kinase1* in Dehydration Avoidance and Low-Water-Potential Response[1][W][OA]

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The molecular basis of plant osmosensing remains unknown. Arabidopsis (*Arabidopsis thaliana*) *Histidine Kinase1* (*AHK1*) can complement the osmosensitivity of yeast (*Saccharomyces cerevisiae*) osmosensor mutants lacking Synthetic Lethal of N-end rule1 and SH3-containing Osmosensor and has been proposed to act as a plant osmosensor. We found that *ahk1* mutants in either the Arabidopsis Nossen-0 or Columbia-0 background had increased stomatal density and stomatal index consistent with greater transpirational water loss. However, the growth of *ahk1* mutants was not more sensitive to controlled moderate low water potential (ψ_w) or to salt stress. Also, *ahk1* mutants had increased, rather than reduced, solute accumulation across a range of low ψ_w severities. *ahk1* mutants had reduced low ψ_w induction of *Δ^1-Pyrroline-5-Carboxylate Synthetase1* (*P5CS1*) and *9-cis-Epoxycarotenoid Dioxygenase3*, which encode rate-limiting enzymes in proline and abscisic acid (ABA) synthesis, respectively. However, neither Pro nor ABA accumulation was reduced in *ahk1* mutants at low ψ_w. P5CS1 protein level was not reduced in *ahk1* mutants. This indicated that proline accumulation was regulated in part by posttranscriptional control of P5CS1 that was not affected by *AHK1*. Expression of *AHK1* itself was reduced by low ψ_w, in contrast to previous reports. These results define a role of *AHK1* in controlling stomatal density and the transcription of stress-responsive genes. These phenotypes may be mediated in part by reduced ABA sensitivity. More rapid transpiration and water depletion can also explain the previously reported sensitivity of *ahk1* to uncontrolled soil drying. The unimpaired growth, ABA, proline, and solute accumulation of *ahk1* mutants at low ψ_w suggest that AHK1 may not be the main plant osmosensor required for low ψ_w tolerance.

Water limitation and decreased water potential (ψ_w) caused by drought leads to numerous changes in plant growth and development. Some of these changes, such as stomatal closure to control leaf water loss, allow the plant to conserve water and avoid low ψ_w. Other changes, such as solute accumulation and osmotic adjustment, allow the plant to tolerate low ψ_w by retaining water and turgor or can ameliorate the damaging effects of tissue dehydration (Kramer and Boyer, 1995; Verslues et al., 2006). These responses depend on yet unknown mechanisms to sense water limitation and initiate downstream signaling. Such dehydration-sensing mechanisms (usually referred to as "osmosensing") have been hypothesized to involve the sensing of cell volume, shape, membrane tension, or macromolecular crowding by osmosensor proteins (Hsiao, 1973; Burg et al., 2007; Schliess et al., 2007; Wood, 2011). Most candidates for plant osmosensor proteins come from analogy to other systems, with perhaps the main candidate being two-component kinases similar to the yeast (*Saccharomyces cerevisiae*) osmosensor Synthetic Lethal of N-end rule1 (SLN1; Urao et al., 1999).

The yeast High Osmolarity Glycerol (HOG) pathway has been the subject of exceptionally detailed studies and is the best characterized osmosensing and signaling system (Saito and Tatebayashi, 2004; Muzzey et al., 2009; Pelet et al., 2011). In this pathway, the two-component sensor SLN1 (as well as a second sensor, SH3-containing Osmosensor1 [SHO1]) perceives high osmolarity via an unknown mechanism. Note that the term high osmolarity typically used in microbiology denotes the same change as low osmotic potential (ψ_s) used in plant biology. ψ_s, in turn, is a component of ψ_w used to describe plant and soil water status (Kramer and Boyer, 1995). Sensing of high osmolarity activates a sequential His phosphorelay from the transmitter to the receiver domain of SLN1 to a His phosphotransfer protein (in the HOG pathway, this is YPD1) and a response regulator (SSK1). The response regulator then activates a MAPKKK (SSK2) and a downstream

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mitogen-activated protein kinase (MAPK) signaling chain leading to activation of the HOG1 MAPK for which the pathway is named (Posas et al., 1996; Ketela et al., 1998; Li et al., 1998). This signal transduction pathway activates many stress responses, including the accumulation of glycerol as an intracellular osmoticum, and is critical for yeast growth on high-osmolarity and high-salt media (Albertyn et al., 1994; Dihazi et al., 2004). Although SLN1 is commonly referred to as an osmosensor, the exact signal that it detects is not understood. There is evidence that SLN1 may detect changes in turgor pressure (Reiser et al., 2003) or cell wall structure (Shankarnarayan et al., 2008).

The Arabidopsis (*Arabidopsis thaliana*) genome contains 11 His kinase genes. Five of these encode ethylene receptors, while the remaining six (*AHK1*, *AHK2*, *AHK3*, *CRE1/AHK4*, *CKI1*, and *CKI2/AHK5*) encode nonethylene receptor kinases. AHK2, AHK3, and CRE1 are cytokinin receptors and mediate cytokinin signaling via interaction with downstream Arabidopsis His phosphotransfer proteins and Arabidopsis response regulators (Schaller et al., 2008; To and Kieber, 2008; Perilli et al., 2010). AHK1 is not a cytokinin receptor, and the signal or ligand that activates AHK1 is not known. Interestingly, *AHK1*, *AHK2*, *AHK3*, and *CRE1* can all complement the salt-sensitive growth defect of yeast *sln1/sho1* mutants (Urao et al., 1999; Reiser et al., 2003; Tran et al., 2007) This yeast complementation has led to the idea that AHK1 has an osmosensing role in plants (Urao et al., 1999; Tran et al., 2007; Wohlbach et al., 2008) and may function in a manner analogous to yeast SLN1. However, the promiscuity of the yeast complementation assay, such that AHKs known to have differing functions in planta can all complement *sln1/sho1*, indicates that this assay by itself cannot establish an osmosensing function of AHK1 or other plant AHKs.

There is evidence for a role of *AHK1* in abscisic acid (ABA) signaling, including impaired seed desiccation tolerance and ABA-insensitive germination (Wohlbach et al., 2008). *AHK1* mutants also had an apparent increased sensitivity to soil drying (Tran et al., 2007; Wohlbach et al., 2008), although it is less clear whether the soil-drying phenotype represented an impaired ability to control leaf water loss and thus avoid the depletion of available water or a difference in tolerance of low ψ_w. The rate of water loss through leaf transpiration is mainly determined by stomatal density or control of the stomatal aperture (Casson and Hetherington, 2010). Interestingly, *ahk1* mutants have been reported to have more rapid leaf water loss (Tran et al., 2007) but no effect on ABA-induced stomatal closure (Wohlbach et al., 2008).

In contrast to water loss avoidance, differences in the tolerance of a given severity of low ψ_w involve osmoregulatory solute accumulation (also referred to as osmotic adjustment) to maintain turgor and the accumulation of specific protective solutes and proteins to maintain cellular structure and control reactive oxygen (Bartels and Sunkar, 2005). Measurement of low-ψ_w-responsive solute accumulation in the ABA-deficient mutant *aba2-1* or in the presence of exogenous ABA indicated that ABA accumulation was not required for osmoregulatory solute accumulation at low ψ_w, and ABA applied in the absence of stress was not sufficient to elicit solute accumulation (Verslues and Bray, 2006; Bhaskara et al., 2012). Thus, even though *AHK1* may affect ABA sensitivity, this does not necessarily imply a function in osmoregulation.

Transcriptional profiling of *ahk1* mutants found extensive changes in stress-related gene expression (Tran et al., 2007; Wohlbach et al., 2008). Notably, reduced induction of *Δ^1-Pyrroline-5-Carboxylate Synthetase1* (*P5CS1*), which encodes the main enzyme in stress-induced Pro synthesis (Székely et al., 2008; Sharma et al., 2011), and the ABA synthesis gene *9-cis-Epoxycarotenoid Dioxygenase3* (*NCED3*; Tan et al., 2003) was observed in both studies. Tran et al. (2007) compared *ahk1* with *ahk2*, *ahk3*, and *ahk4* mutants and proposed that *AHK1* was a positive regulator of drought resistance while the other *AHK*s were negative regulators. Wohlbach et al. (2008) proposed that reduced levels of Pro and ABA in *ahk1* may be part of the reason for its apparent stress sensitivity; however, Pro content was not reported, and only limited measurements of ABA content were reported.

These observations raise interesting possibilities and several unanswered questions about the role of *AHK1* in drought response. First, does *AHK1* affect low-ψ_w tolerance or mainly affect the control of leaf water loss and dehydration avoidance? Second, does *AHK1* control plant osmoregulatory solute accumulation? This is a critical question for knowing whether the analogy to yeast *SLN1* is useful for understanding *AHK1* function in plants; yet, there are no data on solute accumulation in *ahk1* mutants. Within this larger question, the possibility that *ahk1* may have reduced Pro is of interest because Pro is both a component of osmotic adjustment and an important drought response in its own right (Voetberg and Sharp, 1991; Zhang et al., 1999; Székely et al., 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Sharma et al., 2011). Pro accumulation is a plant response analogous to yeast osmoregulatory glycerol accumulation and thus offers another indication of whether *AHK1* function in planta is analogous to yeast *SLN1*. A third question is whether *AHK1* controls low-ψ_w-induced ABA accumulation. Given the central role of ABA in regulating many abiotic stress responses, it can be hypothesized that the primary plant osmosensors would have a role to induce and regulate ABA accumulation. Such upstream regulators of ABA accumulation are not known (Verslues and Zhu, 2007). The known signaling mutants that do affect ABA content, such as mutants of protein phosphatase 2Cs, lie downstream and affect ABA content via ABA sensitivity and feedback regulation (Verslues and Bray, 2006; Rubio et al., 2009; Bhaskara et al., 2012).

Our laboratory has a keen interest in signaling mechanisms controlling osmoregulation and low-ψ_w-induced Pro and ABA accumulation (Bhaskara et al., 2012). We undertook a series of experiments to clarify the role of *AHK1* in these processes, focusing our measurements on responses to controlled, moderate reductions in ψ_w and longer term (days rather than hours) responses, where protective mechanisms have more potential to maintain plant growth and function. Such experiments can identify different sets of genes important for adjustment to low ψ_w and growth compared with short-term stress shock or survival of severe dehydration (Skirycz et al., 2010, 2011; Des Marais et al., 2012). We found several *ahk1* phenotypes consistent with previous data, including reduced *P5CS1* and *NCED3* expression and more rapid leaf water loss. Consistent with the more rapid leaf water loss, we found that *ahk1* mutants had increased stomatal density. However, *ahk1-1* did not exhibit reduced ABA, Pro, or osmoregulatory solute accumulation or any increased sensitivity of growth at low ψ_w. Our data identify a new developmental role of *AHK1* in controlling stomatal density but question whether *AHK1* is an effector of osmoregulation and low-ψ_w tolerance.

RESULTS

ahk1 Mutants Have Decreased Ability to Avoid Dehydration during Soil Drying

We compared the soil-drying phenotype of *ahk1-1* and its wild type, Nossen-0 (Nos-0) under conditions where plants of the two genotypes were grown together and interrooted such that they would experience the same degree of soil drying even if one genotype depleted the soil water faster than the other (Verslues et al., 2006). The experimental conditions were controlled to allow a relatively slow rate of soil drying so that the response to moderate reduction in soil ψ_w could be observed. Over an 18-d drying period, soil ψ_w declined from above −0.3 MPa to approximately −1.8 MPa. The leaf relative water content

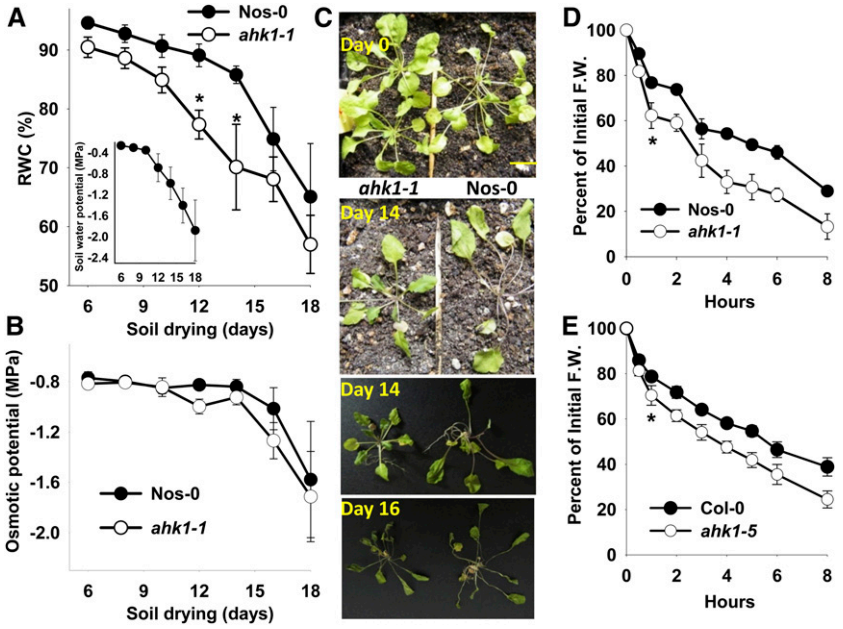

Figure 1. *ahk1* has reduced relative water content and increased leaf water loss but does not differ from the wild type in osmoregulatory solute accumulation. A, Rosette RWC over a time course of soil water withholding for *ahk1-1* and its wild type, Nos-0. Plants were 30 to 32 d old at the start of water withholding (day 0), and data were collected from day 6 of the soil-drying period. Data are means ± SE ($n = 12$) combined from three independent experiments. Asterisks indicate significant differences ($P \leq 0.05$) between the mutant and the wild type. The inset shows the soil ψ_w over the time course of soil drying. Soil ψ_w data are means ± SE combined from three independent experiments. B, Time course of osmotic potential from 6 to 18 d after the start of water withholding. Data are means ± SE ($n = 12$) combined from three independent experiments. C, Photographs of representative Nos-0 and *ahk1-1* plants before soil drying and at 14 or 16 d after soil drying. For both of the top panels, additional plants that were growing in the same pot have been removed to allow individual plants to be clearly seen. In the bottom two panels, representative plants were removed from the pots and immediately photographed. Note that *ahk1-1* was more wilty than the wild type on day 14 of soil drying, consistent with the RWC data in A. D, Water loss from detached leaves of Nos-0 and *ahk1-1*. Data are means ± SE ($n = 9$). The earliest time point where the mutant and the wild type differed ($P \leq 0.05$) is indicated by an asterisk. E, Water loss from detached leaves of Col-0 and *ahk1-5*. Data are means ± SE ($n = 6$). The earliest time point where the mutant and the wild type differed ($P \leq 0.05$) is indicated by an asterisk. F.W., Fresh weight. Water loss data of *ahk1-6* is shown in Supplemental Figure S1.

(RWC) of *ahk1-1* was lower than that of the wild type, especially during the middle of the drying cycle (days 12 and 14; Fig. 1A), when soil ψ_w was declining (Fig. 1A, inset). We did not detect any significant difference in ψ_s between *ahk1-1* and Nos-0 that would indicate a difference in solute accumulation and osmotic adjustment (Fig. 1B). We do not rule out the possibility that the ψ_s of *ahk1-1* may have been slightly lower than that of the wild type, as may be expected from its lower leaf RWC; however, the difference was small enough to be obscured by variability in soil drying between the experimental replicates. Also, *ahk1-1* appeared to wilt sooner than the wild type during the soil drying (days 12–14; Fig. 1); however, overall appearance and apparent sensitivity to soil drying were similar between *ahk1-1* and Nos-0 by the end of the drying cycle, when both genotypes were exposed to an equally severe stress (Fig. 1C). This pattern suggested that the main difference of *ahk1-1* compared with the wild type was a decreased ability to avoid tissue dehydration during soil drying. Consistent with this, *ahk1-1* had more rapid water loss from detached leaves than the wild type (Fig. 1D). This more rapid leaf water loss may explain differences between *ahk1-1* and the wild type when plants were grown in separate pots such that *ahk1* could more rapidly deplete soil water (Tran et al., 2007).

ahk1 Mutants Have Increased Density of Stomates and Leaf Epidermal Cells

Two additional *ahk1* transfer DNA insertion alleles in the Columbia-0 (Col-0) background (Supplemental Fig. S1A) were isolated for further study. Both of these alleles lacked *AHK1* transcript (Supplemental Fig. S1B) and were designated as *ahk1-5* (CS857854) and *ahk1-6* (CS849900) to distinguish them from *ahk* mutants used in other studies (Wohlbach et al., 2008). We performed additional physiological assays with all three alleles to better establish *AHK1* function.

Both *ahk1-5* (Fig. 1E) and *ahk1-6* (Supplemental Fig. S1C) had higher rates of leaf water loss similar to *ahk1-1*. The basis for this more rapid leaf water loss was unclear, as *ahk1* mutants did not differ from the wild type in ABA-induced stomatal closure (Wohlbach et al., 2008). Examination of leaf morphology using cryo fixation and scanning electron microscopy (Cryo-SEM) found that *ahk1* mutants had increased densities of both stomates and epidermal cells and higher stomatal index compared with their respective wild types (Fig. 2; additional leaf tracings and scanning electron microscopy [SEM] images can be seen in Supplemental Fig. S2). This difference could be seen on both the adaxial and abaxial leaf surfaces but was most pronounced on the abaxial surface. There were also differences in cell density between the two accessions, Nos-0 and Col-0. The stomatal density and stomatal index of Col-0 were similar to previous reports (Baxter et al., 2009; Yoo et al., 2010). By way of comparison, mutation of *GT-2 LIKE1* (*GTL1*), which reduced stomatal index to an extent similar to the increase seen in *ahk1-1* (25%), caused reduced transpiration rate and enhanced survivability of a period of water withholding because of reduced soil water depletion by *gtl1* (Yoo et al., 2010). Thus, the increased stomatal

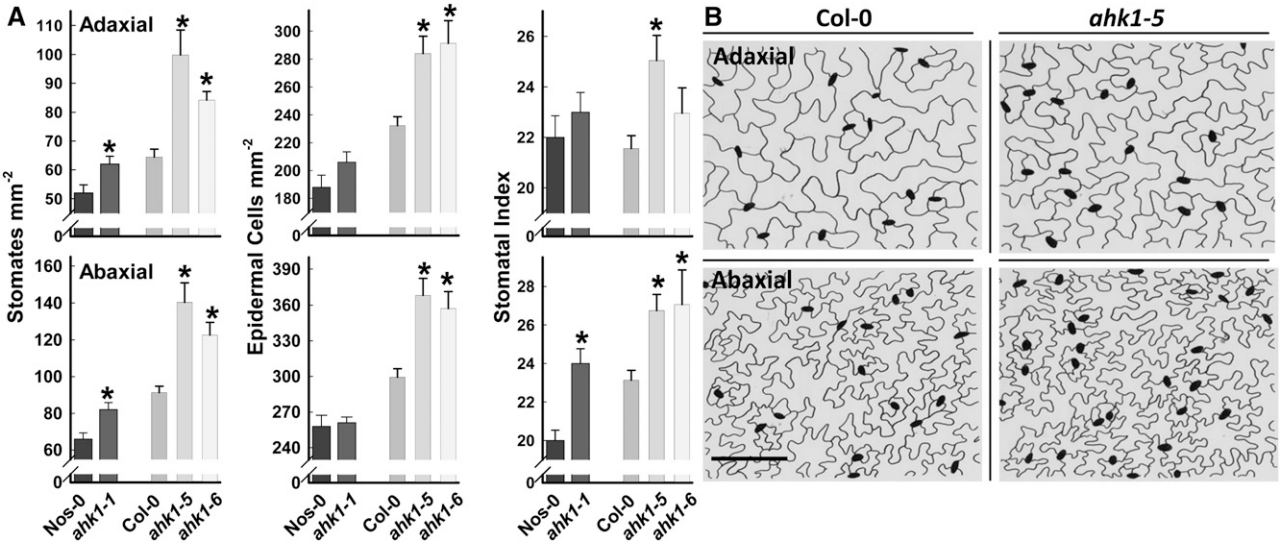

Figure 2. *ahk1* mutants have increased stomatal density and stomatal index. A, Stomatal density, epidermal cell density, and stomatal index compiled from SEM images. Data are means ± SE (n = 7–14) from two independently grown sets of plants. Asterisks indicate significant differences ($P \leq 0.05$) between *ahk1-1* and its wild type, Nos-0, and between *ahk1-5* or *ahk1-6* and their wild type, Col-0. B, Representative tracings of adaxial and abaxial leaf surfaces prepared from Cryo-SEM images of Col-0 and *ahk1-5*. Stomata are colored in black. Bar = 200 μm. Additional representative tracings and Cryo-SEM images of all genotypes shown in A can be found in Supplemental Figure S2.

density of *ahk1* mutants was consistent with their greater leaf water loss.

ahk1 Mutants Are Not Impaired in Growth under Low-ψ_w or Salt Stress

To more directly determine if *AHK1* is required for low-ψ_w tolerance, we quantified root growth and fresh weight after transfer of seedlings to polyethylene glycol (PEG)-agar plates. This experimental system allows ψ_w to be held constant over the course of the experiment and mimics soil drying, because high-M_r PEG causes cytorrhysis similar to soil drying rather than plasmolysis (Carpita et al., 1979; Oertli, 1985; Verslues et al., 2006). Also in contrast to other osmotica, PEG itself does not alter metabolism other than through its effect on ψ_w (Wormit et al., 2012). Such experiments quantifying growth in response to moderate levels of low-ψ_w stress are a more effective way to test the role of *AHK1* in low ψ_w or salt resistance than assays of survival after severe stress (Skirycz et al., 2011). Root elongation and fresh weight in control, moderate low ψ_w (−0.7 MPa), or more severe but not lethal low ψ_w (−1.2 MPa) were used to assay low-ψ_w growth sensitivity. Under these conditions, fresh weight gives a reliable measure of total growth and water uptake/retention because transpiration is low (also see below for measurements of RWC in this experimental system).

These measurements gave no indication that *ahk1* mutants were more sensitive to low ψ_w than the wild type. In fact, *ahk1-5* and *ahk1-6* had greater root elongation and fresh weight than the Col-0 wild type at −0.7 and −1.2 MPa (Fig. 3). All of the mutants had increased root elongation in the high-ψ_w control (−0.25 MPa; Fig. 3), and *ahk1-5* and *ahk1-6* also had increased fresh weight in the control. These results were consistent for all three *ahk1* alleles, with the caveat that the *ahk1-5* and *ahk1-6* mutants had stronger increases in root elongation and fresh weight at low ψ_w than *ahk1-1*. The likely cause of this difference is the differing genetic backgrounds (Col-0 versus Nos-0) of the *ahk1* alleles.

We also quantified root elongation and fresh weight after transfer of seedlings to plates containing 75 or 150 mM NaCl as an additional measure of stress sensitivity. Here again, we did not observe any evidence for greater stress sensitivity of *ahk1* mutants, and root elongation and fresh weight were again higher for the *ahk1* mutants than for the wild type (Fig. 3, bottom panels). This was particularly true for *ahk1-5* and *ahk1-6* at 150 mM NaCl. Both the salt-stress and low-ψ_w results differed from previous studies that reported stress sensitivity of *ahk1* mutants (Tran et al., 2007; Wohlbach et al., 2008).

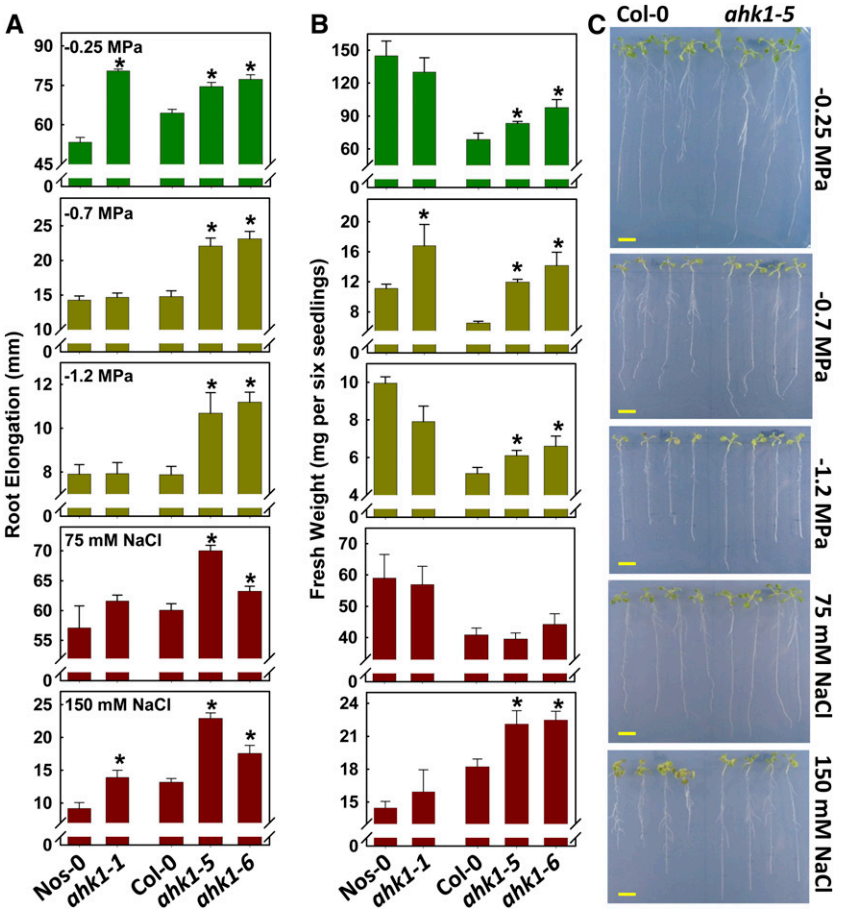

Figure 3. *ahk1* mutants are not more sensitive to growth inhibition by low ψ_w or salt stress and have greater root elongation and fresh weight than the wild type in some conditions. A, Root elongation of *ahk1* mutants and their respective wild types under control conditions (−0.25 MPa), at low ψ_w (−0.7 and −1.2 MPa), or at high salt (75 and 150 mM NaCl). Four-day-old seedlings were transferred to the indicated treatments, and root elongation over the subsequent 8 d was quantified. Data are means ± SE (n = 24–36) combined from three independent experiments. Significant differences ($P \leq 0.05$) between mutants and their corresponding wild types are indicated by asterisks. Note the differences in y axis scaling between the different panels. B, Seedling fresh weight quantified at the end of the root elongation experiments. Data are means ± SE (n = 4–6) of measurements combined from three independent experiments. Significant differences ($P \leq 0.05$) between mutants and their corresponding wild types are indicated by asterisks. Note the difference in y axis scaling between the different panels. C, Representative examples of seedlings used for the measurements in A and B. Bars = 1 cm.

Probable reasons for this difference were our approach of measuring the growth response of seedlings transferred to moderate, sublethal severities of low ψ_w or NaCl rather than counting of survival after severe stress shock or assays of germination on osmoticum-containing medium. Germination on high osmoticum is controlled by ABA sensitivity and not necessarily reflective of postgermination stress tolerance (for discussion, see Verslues et al., 2006).

The hypothesis that *AHK1* may be a positive regulator of stress signaling was based in part on RNA gel-blot analysis, which found a transient induction of *AHK1* over the initial 40 min to 2 h of dehydration (Tran et al., 2007). It has also been proposed that *AHK1* expression is positively regulated by ABA (Wohlbach et al., 2008). To better relate *AHK1* expression to the growth phenotypes that were measured over much longer time periods, we quantified *AHK1* expression over a longer time course of low ψ_w. We found that Nos-0 *AHK1* expression was repressed by as much as 5-fold over 2 to 96 h of low-ψ_w treatment (Fig. 4). *AHK1* expression was also repressed in Col-0 and the ABA-deficient mutant *aba2-1*, indicating that *AHK1* repression at low ψ_w did not require ABA accumulation (Fig. 4; note that *aba2-1* is in the Col-0 genetic background). Such decreased *AHK1* expression is perhaps consistent with the growth phenotypes of *ahk1* mutants at low ψ_w but also unexpected given previous hypotheses of the role of *AHK1* in osmosensing and drought resistance.

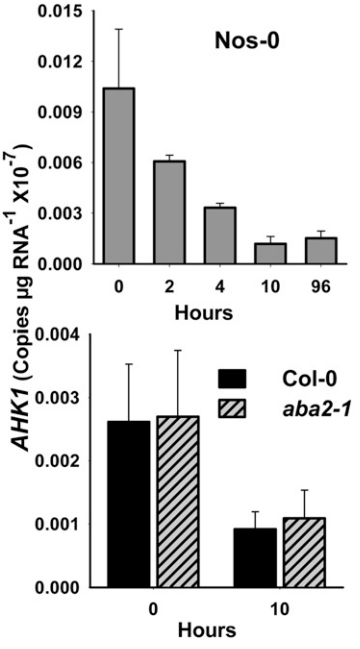

Figure 4. *AHK1* gene expression is reduced after transfer of seedlings to low ψ_w. Quantitative reverse transcription-PCR using standard curves of *AHK1* DNA to calculate absolute copy numbers of *AHK1* transcript was performed to quantify *AHK1* expression at the indicated times after transfer of seedlings to low ψ_w (−1.2 MPa). The top panel shows *AHK1* expression in Nos-0, and the bottom panel shows expression in Col-0 and the ABA-deficient mutant *aba2-1*. Data are means ± SE (n = 3–4) combined from two experiments.

ahk1 Mutants Are Not Impaired in ABA Accumulation at Low ψ_w

ahk1 mutants have also been reported to have reduced ABA content after short-term treatment with mannitol (Wohlbach et al., 2008), and two studies have noted that *NCED3* gene expression was reduced in *ahk1* mutants (Tran et al., 2007; Wohlbach et al., 2008). We found that both *ahk1-1* and *ahk1-5* had reduced induction of *NCED3* expression at low ψ_w compared with their respective wild types (Fig. 5A). However, none of the *ahk1* mutants had reduced ABA accumulation at either 10 or 96 h after transfer to low ψ_w, and *ahk1-1* had slightly higher ABA content than its wild type, Nos-0 (Fig. 5B). The high ABA accumulation at 96 h is likely induced by a loss of turgor that occurs upon transfer of seedlings to the low-ψ_w medium (see below for further discussion). At the 96-h time point, the seedlings have recovered from the initial transfer and ABA content stabilizes at a lower level that is correlated with ψ_w of the medium (Verslues and Bray, 2004). It is interesting that *ahk1* mutants were not impaired in either the initial short-term increase in ABA or the longer term ABA level.

Previous data showed that *ahk1* mutants were ABA insensitive in seed germination (Tran et al., 2007; Wohlbach et al., 2008). We found that *ahk1-1* maintained greater root elongation in the presence of ABA (Supplemental Fig. S3). This is consistent with ABA insensitivity; however, caution must be used in interpreting these data because of the greater root elongation of *ahk1-1* in the absence of ABA. We also assayed the expression of three marker genes (*RD29A*, *COR15*, and *RAB18*), which we have previously shown to be induced by low ψ_w in an ABA-dependent manner (Sharma and Verslues, 2010). For *RAB18* and *COR15*, we saw decreased expression in *ahk1-1* at 10 h after transfer of seedlings to low ψ_w (Supplemental Fig. S4). Because ABA content is unaffected, the reduced stress response of these genes is also consistent with ABA insensitivity. Thus, several phenotypic assays indicate that *ahk1* mutants have reduced ABA sensitivity but do not have reduced ABA content.

We also tested whether AHK1 may be involved in activation of the ABA- and stress-responsive kinases MPK3 and MPK6 (Pitzschke et al., 2009). Such activation could be analogous to signaling through the HOG1 MAPK in yeast, and MPK3 and MPK6 are involved in environmental control of stomatal density (Pillitteri and Torii, 2012). However, we did not observe any difference in MPK3 or MPK6 activation (Supplemental Fig. S5).

ahk1 Mutants Do Not Have Reduced Pro Accumulation or P5CS1 Protein Level Despite Reduced *P5CS1* Gene Expression at Low ψ_w

Reduced *P5CS1* expression has been observed in *ahk1* mutants in short-term dehydration experiments

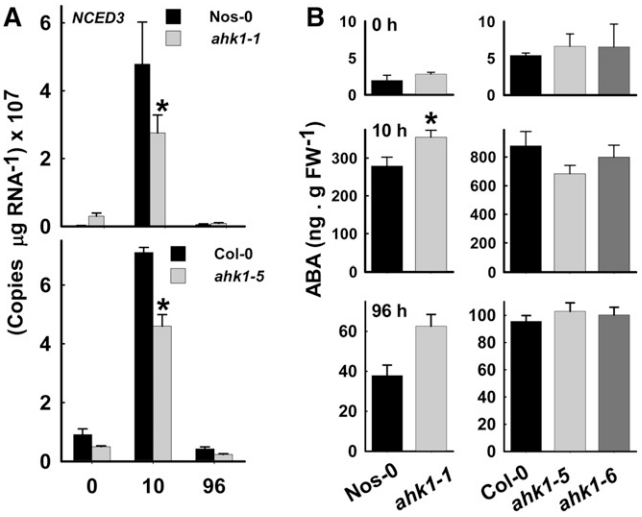

Figure 5. *ahk1* mutants have reduced *NCED3* expression but are unaffected in low-ψ_w-induced ABA accumulation. A, Expression of *NCED3* at 0, 10, and 96 h after transfer of 7-d-old seedlings of *ahk1-1* (Nos-0 background) from control (−0.25 MPa) to low ψ_w (−1.2 MPa). Data are means ± SE (n = 4–6) of measurements combined from two or three independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. B, ABA content of *ahk1* mutants and their corresponding wild types at 0, 10, and 96 h after transfer of 7-d-old seedlings from control (−0.25 MPa) to −1.2 MPa. Data are means ± SE (n = 3–9). Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. FW, Fresh weight.

(Tran et al., 2007; Wohlbach et al., 2008), and it has been hypothesized that *ahk1* mutants have less Pro accumulation than the wild type (Wohlbach et al., 2008). We found that low ψ_w induction of *P5CS1* gene expression was reduced in both *ahk1-1* and *ahk1-5* compared with their respective wild types (Fig. 5A). However, neither *ahk1-1* nor *ahk1-5* had reduced Pro content at any point in a 96-h time course after transfer to −1.2 MPa. In fact, *ahk1-1* had transiently higher Pro than Nos-0 at 48 and 72 h after transfer to −1.2 MPa (Fig. 5B; Pro accumulation of *ahk1-6* is shown in Supplemental Fig. S7A). In addition, *ahk1* mutants had similar levels of salt-induced Pro accumulation as their respective wild types (Supplemental Fig. S7B).

Western blotting with P5CS1-specific antisera (Kesari et al., 2012) found no difference in P5CS1 protein level between Col-0 and *ahk1-5* or *ahk1-6* (Fig. 6C) or between Nos-0 and *ahk1-1* (Supplemental Fig. S6A). These data explained the apparent discrepancy between the reduced *P5CS1* gene expression but high Pro accumulation in *ahk1* mutants. It also suggested that P5CS1 protein level is controlled, in part, by posttranslational regulation unaffected by *AHK1*. We also examined the expression of other Pro metabolism genes in *ahk1-1* but found only small and transient differences (Supplemental Fig. S6B).

ahk1 Mutants Are Not Impaired in Osmoregulatory Solute Accumulation

Pro accumulation is part of plant osmoregulatory solute accumulation (Verslues and Sharma, 2010; Bhaskara et al., 2012). If *AHK1* functions in plant osmoregulation, similar to the osmoregulatory glycerol accumulation controlled by yeast SLN1, then it would be hypothesized that *ahk1* mutants have reduced solute accumulation at low ψ_w. To test this hypothesis, ψ_s of *ahk1* mutants and the wild types was measured on PEG-agar plates where ψ_w was held constant and transpiration was held low such that the more rapid transpirational water loss of *ahk1* was not a confounding factor. Contrary to the hypothesized result, these assays found that seedlings of both *ahk1-1* and *ahk1-5* had decreased ψ_s (increased solute content) compared with the wild type at several severities of low ψ_w (Fig. 6D). The difference in ψ_s between Nos-0 and *ahk1-1* was between −0.15 and −0.38 MPa. Using the van't Hoff equation to convert ψ_s to solute concentration ($\psi_s = -RTC$, where R is the gas constant, T is the absolute temperature, and C is the molar solute concentration), this corresponds to a 60 to 150 mM higher solute content in *ahk1-1*. To rule out any confounding effects of differing seedling water contents on solute concentration, we assayed RWC in seedlings of Nos-0 and *ahk1-1* but did not find any difference between the mutant and the wild type (Supplemental Fig. S8). Thus, *AHK1* may be a negative regulator of solute accumulation. Alternatively, the differences in epidermal cell density (Fig. 2) as well as root growth (Fig. 3) of *ahk1* mutants suggest differences in cell expansion or division that may indirectly influence solute content. In any case, the results are not consistent with a role of AHK1 as an inducer of osmoregulatory solute accumulation analogous to the induction of glycerol accumulation by SLN1 (Dihazi et al., 2004).

DISCUSSION

The identity of osmosensors responsible for the initial detection of water limitation and the initiation of upstream stress signaling is one of the most critical questions in plant stress biology. AHK1 is one of the leading candidates for such an osmosensor and has been proposed to play a role analogous to yeast SLN1. Our analysis of *ahk1* mutants found a new role of *AHK1* in determining stomatal density and phenotypes that were both consistent and contrasting with the existing hypothesis of *AHK1* function. These data allow a fresh assessment of *AHK1* function.

AHK1 Is a Regulator of Stomatal Density and Dehydration Avoidance

We found increased stomatal density and stomatal index in *ahk1* mutants that could explain their more rapid leaf water loss. Stomatal density is regulated by

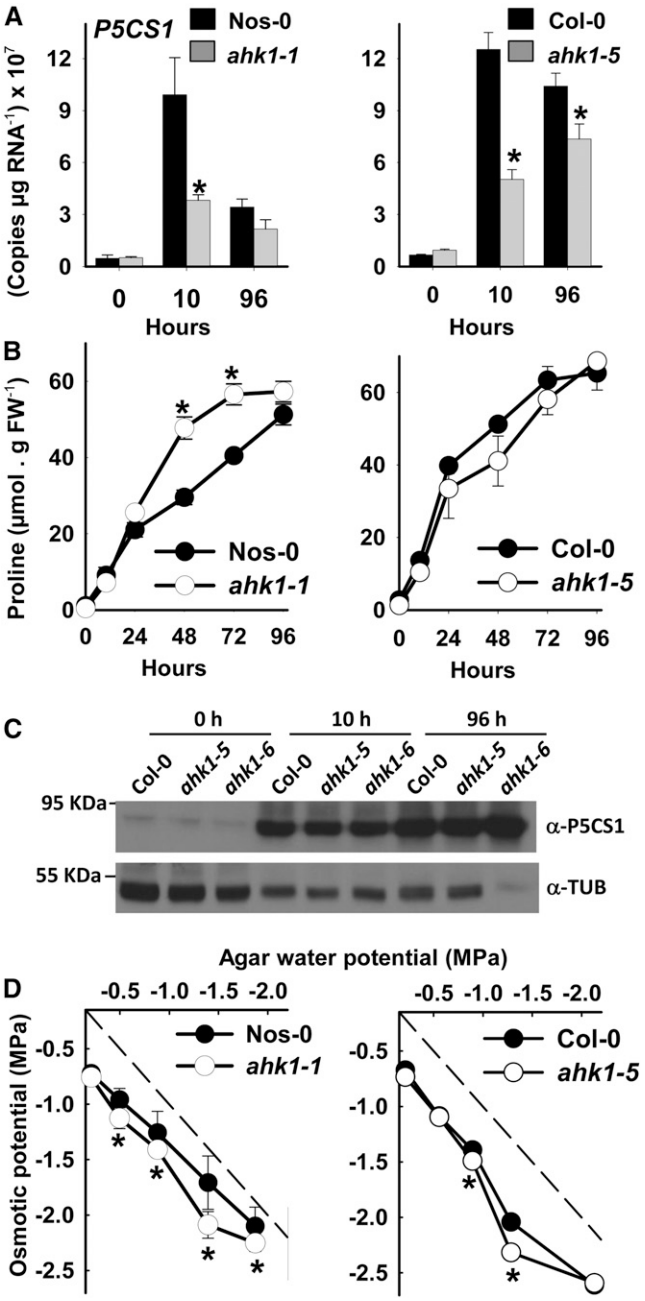

Figure 6. *ahk1* mutants accumulate wild-type levels of Pro and P5CS1 at low ψ_w despite reduced *P5CS1* expression. A, Expression of *P5CS1* in *ahk1* mutants and their corresponding wild types at 0, 10, and 96 h after transfer from control (−0.25 MPa) to low ψ_w (−1.2 MPa). Data are means ± SE (n = 4–6) of measurements combined from two to three independent experiments with significant differences ($P \leq 0.05$) indicated by asterisks. FW, Fresh weight. B, Time course of Pro content over 0 to 96 h after transfer of 7-d-old seedlings of *ahk1* mutants and their wild types to −1.2 MPa. Data are means ± SE (n = 6–9) of measurements combined from two to three independent experiments. Significant differences between each mutant and its corresponding wild type ($P \leq 0.05$) are indicated by asterisks. Additional Pro measurements of *ahk1-5* and *ahk1-6* are shown in Supplemental Figure S7A. C, Western blotting of P5CS1 in Col-0, *ahk1-5*, and *ahk1-6*. A total of 50 µg of protein was loaded. Blots were stripped and reprobed with anti-tubulin antibody as a loading control. Note that tubulin protein levels were altered by low ψ_w, so tubulin levels should only be compared across genotypes within a given treatment. Western-blot detection of P5CS1 in Nos-0 and *ahk1-1* can be seen in Supplemental Figure S6A. In both cases, the experiment was repeated with consistent results. D, Seedling ψ_s of *ahk1* mutants and their wild types measured 96 h after transfer of seedlings from control medium to the indicated ψ_w on PEG-infused agar plates. Dashed lines indicate the point where ψ_s equals the agar ψ_w. Seedling ψ_s below this line is consistent with a positive turgor pressure. Data are means ± SE (n = 6–9) of measurements combined from two to three independent experiments. Significant differences between each mutant and its corresponding wild type ($P \leq 0.05$) are indicated by asterisks.

light and CO_2 as well as other environmental factors through mechanisms that are not well understood (Casson and Hetherington, 2010; Pillitteri and Torii, 2012). For example, overexpression of a *Medicago truncatula* dehydrin, a class of stress-associated genes not known to be involved in stomatal patterning, decreased stomatal density and allowed plants to survive a longer period of water withholding than the wild type (Xie et al., 2012). This is similar to the effect of *AHK1* overexpression (Tran et al., 2007). Membrane receptors such as *Too Many Mouths* and *Erecta* family proteins as well as secreted peptide signals are key players in stomatal development (Casson and Hetherington, 2010; Pillitteri and Torii, 2012). Additional membrane receptors such as AHK1 may be a way for other environmental or developmental inputs to influence stomatal density. Thus, control of stomatal density and stomatal index is an important physiological role of *AHK1*. A role of His kinases in stomatal density was also found by Wang et al. (2012), who observed that ectopic expression of a maize (*Zea mays*) His kinase, *ZmHK9*, decreased stomatal density and also allowed plants to survive longer periods of water withholding. Interestingly, we also observed substantial differences in stomatal density between the two accessions used in this study, Nos-0 and Col-0. This is consistent with a previous report of natural variation in stomatal density (Vile et al., 2012) and with numerous observations of extensive variation between accessions in traits important for environmental adaptation.

Similar to other mutants and transgenics that have altered stomatal density (Yoo et al., 2010), the increased stomatal density of *ahk1* mutants makes them less able to control water loss and avoid tissue dehydration as water supply is depleted. More rapid transpiration and faster soil water depletion suggest that in experiments where the wild type and *ahk1* were grown in separate containers and soil water content was not controlled, *ahk1* mutants were actually exposed to more severe low-ψ_w stress than the wild type over time. Experiments where *ahk1* mutants were exposed to controlled low ψ_w on PEG-infused agar plates did not show any indication of hypersensitivity to low ψ_w

and in fact showed greater growth of *ahk1* mutants under conditions of moderate low-ψ_w stress. Thus, the more prominent physiological role of *AHK1* is in dehydration avoidance rather than low-ψ_w tolerance. Dehydration avoidance can explain many previously observed *ahk1* phenotypes (Tran et al., 2007; Wohlbach et al., 2008).

In addition to its effect on stomatal density, *AHK1* may have a broader role in cell expansion, as the density of epidermal cells was also increased in *ahk1*. Such an increased cell density while retaining wild-type leaf morphology implies a coordinated change in cell division and expansion patterns. It will be of interest to see if such differences also may be involved in the increased root elongation seen in *ahk1* mutants. Now that key traits regulated by *AHK1* have been identified, the identity of the signal that AHK1 responds to and downstream signaling targets will be key questions for further analysis.

AHK1 Affects Gene Expression and ABA Sensitivity But Is Not a Positive Regulator of ABA, Pro, or Osmoregulatory Solute Accumulation

Both our results and previous studies (Tran et al., 2007; Wohlbach et al., 2008) indicate a role of *AHK1* in ABA sensitivity. This altered ABA sensitivity may be related to the stomatal density phenotypes of *ahk1* mutants. However, altered ABA sensitivity does not itself suggest a difference in osmoregulation, and we measured ψ_s and water contents to directly analyze the effect of *AHK1* on osmoregulation. Here, it is informative to compare the phenotypes of *AHK1* with those of the clade A protein phosphatase 2C *Highly ABA-Induced1* (*HAI1*), which we found to be a negative regulator of osmoregulatory solute accumulation using similar experiments as those presented here (Bhaskara et al., 2012). In soil-drying experiments, *hai1-2* had decreased osmotic potential but did not differ from the wild type in RWC or in the rate of leaf water loss. This was the opposite of the effect seen in *ahk1-1*, which had reduced RWC but little or no change in ψ_s compared with the wild type. On PEG-infused plates, *hai1-2* had decreased ψ_s (up to −0.6 MPa lower than that of the wild type) and increased seedling fresh weight across a range of ψ_w. This indicated that *HAI1* was a negative regulator of osmoregulatory solute accumulation, and greater solute deposition in *hai1-2* drove greater water uptake at low ψ_w. *ahk1* mutants had lower ψ_s than the wild type (although the difference was not as large as in *hai1* mutants) and seedling fresh weights that were similar to or increased compared with the wild type. This is the opposite of the reduced water content and higher ψ_s (lower solute content) that we expected to see if AHK1 functioned to induce osmoregulatory solute accumulation. Thus, our data indicate a negative effect of *AHK1* on osmoregulation.

Previous studies have found extensive gene expression changes in *ahk1* mutants after exposure to short-term dehydration or osmotic stress (Tran et al., 2007; Wolhbach et al., 2008). We found similar reduced induction of *P5CS1*, *NCED3*, and ABA-dependent marker genes (*COR15* and *RAB18*) across a longer time course of low ψ_w. The reduced *P5CS1* and *NCED3* may be expected to result in reduced Pro and ABA levels. However, we saw no decrease in levels of ABA and Pro in *ahk1* mutants. For Pro accumulation, the apparent discrepancy between gene expression and Pro content could be explained by the fact that P5CS1 protein level was not affected in *ahk1* mutants. The similar ABA levels in *ahk1* and the wild type may be similarly explained by discordance between gene expression and protein levels or by differences in ABA catabolism or conjugation that allow high levels of ABA accumulation despite reduced NCED3. For example, Tsugama et al. (2012) have proposed that the ABA catabolism genes *CYP707A1* and *CYP707A3* are regulated by signaling mechanisms responsive to changes in turgor.

AHK1 May Not Be the Main Plant Osmosensor

Such data raise anew the question of whether AHK1 acts as a direct osmosensor and positive regulator of drought resistance in plants, as has been suggested by previous studies and by analogy to yeast SLN1. If one uses a broad definition of an osmosensor as a protein that can directly sense osmotic change (and/or low ψ_w), either directly or through change in turgor pressure, cell volume, or cell shape (or other signals), and initiate a downstream cellular response, then there is nothing in our data to preclude such a role. It is possible that AHK1 directly senses plant water status and controls ABA sensitivity and stomatal density. However, one key function of yeast SLN1 is to induce osmoregulatory glycerol accumulation, a process analogous to Pro and osmoregulatory solute accumulation in our experiments. The observation that *ahk1* mutants did not have reduced Pro or solute accumulation argues against a role of AHK1 as a sensor controlling these key low-ψ_w responses.

The actual signal detected by a plant osmosensing system may be a change in turgor pressure (Hsiao, 1973). Two types of experiments have shown that loss of turgor pressure is the likely trigger for ABA accumulation. Pierce and Raschke (1980) found that leaf ABA accumulation correlated with loss of turgor in several species. Species that accumulated more solutes and could maintain turgor at lower ψ_w only initiated ABA accumulation at very low ψ_w when turgor was lost (when leaf ψ_w equaled ψ_s). In contrast, species that had less solute accumulation and lost turgor at higher ψ_w started to accumulate ABA at these higher ψ_w. Similarly, Creelman and Zeevaart (1985) found that treatment with the membrane-permeable solute ethylene glycol, which reduced ψ_w but did not affect turgor, did not cause ABA accumulation. In contrast, non-membrane-permeable solutes, which reduced both ψ_w and turgor, elicited high levels of ABA accumulation.

Performing the ethylene glycol experiment with Arabidopsis gives a similar result: treatment with ethylene glycol alone did not increase ABA content despite reduced ψ_w, while treatment with a combination of ethylene glycol and PEG elicited only the level of ABA expected from treatment with PEG alone (Verslues, 2004). Thus, a main plant osmosensing system that detects changes in turgor (or other stimulus related to turgor loss) would be expected to control ABA accumulation. Our observation that *ahk1* mutants were not impaired in ABA accumulation also argues against the involvement of AHK1 in the main plant osmosensing, or turgor-sensing, mechanism(s).

Overall, our observations indicate that interpreting *AHK1* function by analogy to yeast *SLN1* should be treated with caution, as AHK1's in planta role differs substantially from the role of *SLN1*. The unimpaired ABA and Pro accumulation and growth at low ψ_w in *ahk1* mutants indicate that even if AHK1 does act as a direct sensor of water status in plants, it is probably not the only or the main sensor. Interestingly, a recent analysis of two-component kinases and cytokinin signaling proteins in rice (*Oryza sativa*) did not find a rice ortholog of *AHK1* (Tsai et al., 2012), suggesting that AHK1 function is not essential or is taken over by other proteins in rice. Also, it was observed that *CRE1*, but not *AHK1*, was involved in osmoresponsive changes in metabolism and gene expression caused by the inhibition of cellulose biosynthesis (Wormit et al., 2012). It should also be considered that the physiological response to low ψ_w and dehydration is likely to involve the integration of signals from multiple osmosensing systems, as is known to be the case for other organisms (Kültz, 2012). The identification of water status-sensing mechanisms that control ABA accumulation, Pro, osmoregulation, and overall tolerance of low ψ_w remains one of the key unsolved questions of plant stress biology.

MATERIALS AND METHODS

Plant Materials, Growth, and Stress Treatments

The Arabidopsis (*Arabidopsis thaliana*) *ahk1-1* mutant, in the Nos-0 background, was obtained from Prof. Tatsuo Kakimoto (Osaka University). The *ahk1-5* (*CS857854*) and *ahk1-6* (*CS849900*) alleles in the Col-0 background were obtained from the Arabidopsis Biological Resource Center. Homozygous plants were identified by PCR, and lack of *AHK1* transcript was verified by reverse transcription-PCR. For plate experiments, seeds were vapor sterilized for 1.5 to 2 h and plated on sterile nylon mesh overlaid on half-strength Murashige and Skoog medium with 2 mM MES buffer (pH 5.7), stratified for 3 d, placed vertically, and grown at 25°C under continuous light of 70 to 90 μmol photons m^{-2} s^{-1}. PEG-infused agar plates were prepared using PEG-8000 as described previously (Verslues et al., 2006). ABA treatment was performed by adding *S*(+)-ABA to medium after sterilization. No sugar was included in any of the growth media. For assay of gene expression, Pro, ABA, or ψ_s, 7-d-old seedlings were transferred from control medium to PEG-infused plates. For root growth and fresh weight measurements, 4-d-old seedlings were transferred to PEG or NaCl plates, root elongation was measured over the subsequent 8 d, and fresh weight was quantified at the end of the experiment.

For soil-drying experiments, standard Arabidopsis potting mix was supplemented with 30% fine sand to allow more even soil drying. Seeds of the wild type and mutants were sown in the same pot to allow them to interroot and be exposed to the same ψ_w regardless of differing rates of water loss between the genotypes. Water withholding was started when plants were 5 weeks old, and samples for RWC and ψ_s were collected every other day starting on day 6 of soil drying. Plants were grown in 8-h-light/16-h-dark conditions (22°C–26°C, 95–100 μmol photons m^{-2} s^{-1}).

Stomatal Density

Stomatal density was determined using abaxial and adaxial sides of leaves from wild-type and mutant plants grown in the same pot. Leaves were collected from 5- or 6-week-old plants, and two to three plants were sampled in each experiment. For Cryo-SEM imaging, fresh leaves were dissected, frozen by liquid nitrogen slush, and transferred to a sample preparation chamber at −160°C. After 5 min, the temperature was raised to −85°C, and sublimation was allowed to proceed for 15 min. Samples were then coated with platinum at −130°C, transferred to a cryo-stage SEM chamber, and imaged at −160°C using a Cryo-SEM apparatus (Quanta 200 SEM/Quorum Cryo System PP2000TR; FEI).

Quantification of RWC, ψ_s, Pro, and ABA

To measure RWC, rosettes were collected and immediately weighed to obtain fresh weight (FW), floated on cold water at 4°C for 8 to 9 h and reweighed to obtain the hydrated weight (HW), and then dried at 60°C overnight and dry weight (DW) was quantified. RWC was calculated as $((FW - DW)/(HW - DW)) \times 100$. A similar procedure was used to estimate RWC of seedlings grown on agar plates (Verslues, 2010). For ψ_s quantification, leaf or seedling samples were macerated, frozen and thawed, and centrifuged to obtain cell sap, and ψ_s was quantified using a Psypro system with c52 sample chambers (Wescor). Agar plate or soil ψ_w was also quantified using the Psypro instrument.

Pro was quantified by ninhydrin assay modified for 96-well plates (Bates et al., 1973; Verslues, 2010). ABA analysis was performed by extracting freeze-dried seedlings (50–200 mg) in 80% methanol with 25 pmol of [D_6]ABA (Plant Biotechnology Institute) as an internal standard. Extracts were passed through a C_{18} solid-phase extraction cartridge (Supelco), evaporated to dryness, resuspended in diethyl ether:methanol (9:1), and derivatized by the addition of trimethylsilydiazomethane (Sigma). After derivatization, remaining trimethylsilydiazomethane was destroyed by the addition of 0.5 M acetic acid in hexane (Schmelz et al., 2003). The samples were then evaporated, resuspended in a small volume of ethyl acetate, injected onto a VF-14MS (Varian/Agilent) column, and analyzed by tandem mass spectrometry. Methanol chemical ionization was used to generate precursor ions (261 mass-to-charge ratio [*m/z*] for ABA and 267 *m/z* for [D_6]ABA). Daughter ions of 229 *m/z* (ABA) and 233 + 234 *m/z* ([D_6]ABA) were used for quantification (Müller et al., 2002). The ABA content of samples was quantified by a standard curve over 2 to 60 pmol of ABA calculated using the ratio of the 229 and 233 + 234 peak areas.

Gene Expression

Plant tissue samples were ground in liquid nitrogen, and RNA was extracted using the Plant RNeasy kit with DNase treatment (Qiagen). RNA was quantified by a Nanodrop spectrophotometer, and complementary DNA was synthesized using 1 μg of total RNA and SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR of stress marker genes and Pro metabolism genes was performed using Taqman primer and probe sets and standard curves to calculate absolute copy numbers as described by Sharma and Verslues (2010). *AHK1* expression was quantified in the same manner using a newly designed Taqman primer and probe set (forward primer, 5′-CCAGATTCTGGAGGCTGTAATAAAA-3′; reverse primer, 5′-ATCACTT-CCGTTTCCTCTGTTTCTT-3′; Taqman probe 5′-CCGGAAGAGAGGCTTG-TGTAATGAC-3′ labeled with FAM/BHQ [Biosearch Technologies]). Complementary DNA samples were appropriately diluted to fall within the range of the standard curves for each gene assayed.

Western Blotting and MAPK Activation

P5CS1 protein content was determined by western blotting using P5CS1-specific antisera (Kesari et al., 2012). Protein was extracted by grinding 50 to 100 mg of seedlings using liquid nitrogen with 100 μL of extraction buffer (125

mm Tris buffer, pH 8.8, 1% SDS, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration in the supernatant was quantified using the Pierce bicinchoninic acid protein assay kit (Thermo Scientific), 50 μg of protein was loaded onto 10% SDS-PAGE gels, and western blotting was performed as described previously (Kesari et al., 2012). For determination of MAPK activation, protein was extracted from seedlings at different time points of low-ψ_w treatment (Romeis et al., 1999), and 5 μg of protein was separated on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Western-blot analysis was performed using phospho-p44/p42 MAPK antibody (Cell Signaling Technology), which recognizes phosphorylated MPK6 and MPK3 (Brock et al., 2010). Protein loading was confirmed by Coomassie blue staining.

Statistical Analysis

Data typically represent the combined results of two to three independent experiments. Significant differences were determined by either Student's *t* test or two-factor ANOVA (for experiments involving multiple treatments or genotypes) performed using SigmaPlot 11.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Characterization of additional *ahk1* transfer DNA mutants in the Col-0 background.

Supplemental Figure S2. Representative tracings and SEM images of leaves from *ahk* mutants and their respective wild types.

Supplemental Figure S3. *ahk1-1* is insensitive to ABA inhibition of root elongation.

Supplemental Figure S4. Expression of ABA-regulated genes in *ahk1-1*.

Supplemental Figure S5. MPK6 and MPK3 activation by low water potential in Nos-0 and *ahk1-1*.

Supplemental Figure S6. P5CS1 protein level and expression of Pro metabolism genes in *ahk1-1*.

Supplemental Figure S7. Low water potential and salt responsive Pro accumulation of *ahk1* mutants.

Supplemental Figure S8. *ahk1-1* does not differ from the wild type in relative water content on PEG-agar plates at low water potential.

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