Running tittle: a role for AKT1 in plant transpiration

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Disruption of the *Arabidopsis thaliana* Inward-Rectifier K^+ Channel AKT1 Improves Plant Responses to Water Stress

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Abstract

The *Arabidopsis thaliana* inward-rectifier K^+ channel AKT1 plays an important role in root K^+ uptake. Recent results show that the CIPK23-CBL1/9 complex activates AKT1 in the root to enhance K+ uptake. In addition, this CIPK-CBL complex has been demonstrated to regulate stomatal movements and plant transpiration. However, a role for AKT1 in plant transpiration has not yet been demonstrated. Here we show that disruption of AKT1 conferred an enhanced response to water stress in plants. Experiments performed in hydroponics showed that, when water potential was diminished by adding PEG, *akt1* adult plants lost less water than WT plants. Under long-term water stress in soil, adult *akt1* plants displayed lower transpiration and less water consumption than WT plants. Finally, *akt1* stomata closed more efficiently in response to ABA. Such results were also observed in *cipk23* plants. The similar responses shown by *cipk23* and *akt1* plants to water stress denote that the regulation of AKT1 by CIPK23 may also take place in stomata and has a negative impact on plant performance under water stress conditions.

Keywords: AKT1, channel, drought, potassium, stomata, transpiration.

Introduction

As autotrophic organisms, plants possess the ability to fix atmospheric CO_2, making them independent of the supply of external organic carbon sources. CO_2 can diffuse inside the plant through pores named stomata, present on the leaf surface. These pores also constitute a pathway for the release of O_2 and H_2O from the plant. By regulating stomatal aperture plants must achieve a compromise between CO_2 diffusion from the atmosphere into the inner tissues to maintain photosynthesis and the loss of water from the plant into the atmosphere (Lawson 2009).

Composed of two guard cells that form a central pore, stomata open and close by turgor changes that modify cell size and, in turn, determine the width of the pore. As guard cells are symplastically isolated, all these turgor changes are due to the movement of solutes across guard cell plasma membrane and tonoplast. During stomatal opening, guard cell volume increases by approximately 40%, due to the accumulation of solutes that lower the water potential in guard cells and drive osmotic water (Ward et al. 2009). During stomatal closure, guard cell volume decreases owing to net cellular efflux of solutes and water. Among all the solutes involved in the process of stomatal movement, K^+ is the major cation. Net entry or release of K^+ into or from guard cells contributes to stomatal opening and closing respectively (MacRobbie 1998). In addition, K^+ counteracts the negative charges of anions and organic acids that are also transported during stomatal movement, maintaining the electrical equilibrium (Marschner 1995). Expression and regulation of K^+ transport systems active at the plasma membrane of guard cells are thus of crucial importance in the control of stomatal movements (Lebaudy et al. 2007).

Among the transport systems, *Shaker*-type K^+ channels constitute important pathways for K^+ influx and efflux in guard cells (Hosy et al. 2003, Lebaudy et al. 2008a). mRNA of five inward-rectifier K^+ *Shaker*-type channel subunits, AKT1, AKT2, KAT1, KAT2, KC1 and one outward-rectifier K^+ Shaker channel GORK, are found in this cell type (Szyroki et al. 2001). Regulation of these channels is achieved by different mechanisms that include changes in cytoplasmic pH, Ca^{2+} concentrations and membrane polarization (Lebaudy et al. 2007). At the protein level, constitution of channels with different subunits (Lebaudy et al. 2008b) or phosphorylation/dephosphorylation events also become relevant (Cherel et al. 2002,

Sato et al. 2009). In this regard, CIPK-CBL regulatory complexes seem to play an important role in plant K^+ homeostasis (Weinl and Kudla 2009).

The importance of *Shaker*-type channel regulation is illustrated by the rapid changes in stomatal aperture required to prevent excessive water loss under water stress conditions. When water becomes scarce, an increase in the hormone ABA takes place. ABA signaling leads to stomatal closure via activation of anion channels (Geiger et al. 2011, Kim et al. 2010, Mori and Murata 2011, Mori et al. 2006, Vahisalu et al. 2008) which are concomitant with regulation of K+ fluxes. For instance, ABA decreases K^+ influx through inward channels and increases K^+ efflux through outward K^+ channels in guard cells (Blatt 1990, Leyman et al. 1999, Schwartz et al. 1994), The inhibition of inward channels is not essential for stomatal closure but it will speed up the process (MacRobbie 1998).

One of the inward-rectifier K^+ channel subunits, AKT1, has been demonstrated to play an important role in K^+ uptake at the root (Alemán et al. 2011, Gierth et al. 2005, Hirsch et al. 1998, Rubio et al. 2008, Spalding et al. 1999). Importantly, AKT1 is activated by phosphorylation through the CIPK23-CBL1/9 complex (Li et al. 2006, Xu et al. 2006). *cipk23* mutants showed reduced K^+ uptake (Xu et al. 2006), in agreement with the role of AKT1 in K^+ acquisition at the root. In addition, disruption of CIPK23 or CBL1/9, led to an ABA-hypersensitive and drought tolerant phenotype (Cheong et al. 2007). In the *cipk23* mutant, reduced transpirational water loss from leaves coincides with enhanced ABA sensitivity of guard cells during opening as well as closing reactions, without noticeable alterations in ABA content in the plant. Therefore, it is possible that AKT1 serves as a target for CIPK23 in the regulation of transpiration but a direct implication of AKT1 in stomatal movements and transpiration has not yet been demonstrated.

We present a study that aims at identifying the role of AKT1 in the regulation of plant transpiration and adaptation to water deficit. Here we describe the water stress tolerant phenotype of *akt1* knock-out mutants in two different backgrounds Col-0 and WS. The water stress tolerance of the mutants resulted from an ABA-hypersensitive response that produced enhanced stomatal closure in response to water stress. The results presented here point to an important role of AKT1-mediated K+ flux in stomatal movements which contributes to drought sensitivity. In addition, *cipk23* plants mirrored

the *akt1* behavior. Thus, we propose that AKT1 is regulated by CIPK23 in guard cells and it is involved water stress responses.

Results

akt1 plants showed reduced water loss from excised rosettes

It has been previously shown that *cipk23* plants displayed transpiration related phenotypes (Cheong et al. 2007) and that the CIPK23 protein kinase regulated root K+ uptake through phosphorilation of AKT1 (Li et al. 2006, Xu et al. 2006). Thus, we tested whether AKT1 may also be involved in the control of leaf transpiration. As a first approach, weight loss (mainly water) was followed from excised rosettes of WT and *akt1* plants from Col-0 (Rubio et al. 2008) and WS (Hirsch et al. 1998) backgrounds. c*ipk23* plants (Col-0) were also included as an experimental control. Regarding Col-0 background, WT plants lost approximately 50% of their fresh weight in 3h whereas *akt1* and *cipk23* plants showed significantly lower percentages of water loss during the experiment (Fig. 1A). A similar trend was observed in the plants of the WS background where WT plants nearly lost 40% of their fresh weight and *akt1* plants always presented lower values than control plants (Fig. 1B).

akt1 mutations reduced transpiration under water stress in hydroponic culture

To confirm the leaf transpiration phenotype of *akt1* plants, short term water stress induced by PEG addition to the growth solution was applied to the aforementioned plant lines grown in hydroponics. Preliminary experiments in PEG-infused agarose plates showed that a clear differential response between WT and mutant plants was observed at -0.6 MPa, and this condition was used for further experiments (Suppl fig. 1). Plants were grown for 60 d under standard conditions. Then, half of the plants was transferred to a solution in which the water potential (Ψ_π) was lowered to -0.6 MPa by the addition of PEG and the other half remained without PEG as a control. After 3h and 24h, stomatal conductance and water content were determined, respectively.

Data from Col-0 plants is depicted in Figures 2A, C and D and those from WS plants in Figures 2B, E and F. In the absence of PEG, WT, *akt1* and *cipk23* presented similar stomatal conductance values (Fig. 2A and B). PEG treatment led to an important reduction of this parameter that was significantly more marked in *akt1* and *cipk23* plants than in WT plants (Fig.2A and B).

Regarding water content, no significant differences were observed in shoot nor root water contents (Fig. 2C-F) under control conditions (-PEG). Water stress (+PEG) gave rise to a significant decrease in water content in both shoots and roots of all the studied lines. The reduction in water content was significantly less acute in *akt1* and *cipk23* plants than in WT plants for the shoot values (Fig. 2C and E). On the other hand, no significant differences were observed for the reduction in root water content under water stress between WT and the mutant lines (Fig. 2D and F).

The *akt1-2* mutation reduced transpiration under water stress in hydroponic culture also at 10 mM K+

The above described experiments were performed with plants grown with 1.4 mM K+. It has been described that this K+ concentration is limiting for the *akt1* line (Hirsch et al 1998, Rubio et al 2008). In fact, the K+ concentrations were lower in the shoots and roots of the *akt1-2* and *cipk23* lines in comparison with those of WT (Col-0) plants and in the shoots of *akt1-1* plants with regard to the WT (WS) plants (Table 1).

To discard that the transpiration phenotype of the *akt1* line presented above was derived from a deficient status of K+ nutrition, short term water stress was also studied in *akt1-2* and WT (Col-0) plants grown in the presence of 10 mM K+. At this external K+ concentration, *akt1* plants did not display any K+-dependent phenotype (Nieves-Cordones et al. 2010, Rubio et al. 2010, Rubio et al. 2008) and no differences in tissue K+ concentrations were observed between *akt1-2* and WT (Col-0) plants (Table 1). A similar experiment to the one described in the previous section was performed: after growing the plants for 60 d in standard conditions in the presence of 10 mM K+, half of the plants were transferred to containers with PEG (Ψ_π = -0.6 MPa) during 24h, while the rest of the plants were kept in a solution without PEG. Stomatal conductance and water content were determined at the same time points as in the previous experiment.

Measurements of stomatal conductance revealed differences between mutant and WT plants. Like in the previous experiment, in the absence of stress (-PEG), *akt1-2* and WT plants exhibited comparable values of transpiration (Fig. 3A). Exposure to a low water potential (+PEG), reduced stomatal conductance in both plant lines but in *akt1-2* plants this parameter was significantly reduced to lower values than in WT plants.

At the end of the experiment, shoot and root water contents were similar in *akt1-2* and WT plants in -PEG conditions (Fig. 3C and D). When plants were subjected to water stress (+PEG), shoot and root water contents significantly decreased in the two plant lines. Shoot water contents of *akt1-2* plants were much less affected by water stress than those of WT plants, and significantly higher values in mutant plants were observed (Fig. 3C). Changes in water content may also be reflected in plant macroscopic appearance. Representative pictures (Fig. 3B) show that both plant lines grew similarly in control conditions (Fig. 3B, upper panel) but when PEG was added, WT plants were more affected than *akt1-2* plants, with wilting symptoms observed in the former line (Fig. 3B, lower panel).

The *akt1-2* mutation reduces transpiration under drought stress in plants grown in soil

WT (Col-0) and *akt1-2* plants grown in soil were used to further study long-term effects of water stress on adult plants. Seeds were sown in individual pots and irrigated for 60 d with 1/5 Hoagland solution, which contained 10 mM K^+ to avoid K^+ deficiency in the *akt1-2* line. Then, irrigation was withheld for 29 d for a group of plants while another group remained regularly watered as a control. Several harvests and physiological analyses were performed during this period. Plant growth, shoot K^+ concentrations and shoot water contents did not show differences between WT and *akt1-2* plants (Suppl. Table1). In well-watered control plants, stomatal conductance did not show significant differences between the two plant lines (Fig. 4A). However, under water stress, *akt1-2* plants exhibited lower stomatal conductance than WT plants (Fig. 4A). Water consumption was calculated in water stressed plants and it was observed that WT plants showed higher water consumption for the periods studied (days 0-14 and 14-29) than *akt1-2* plants (Fig. 4B).

Disruption of *AKT1* enhances stomatal closure in response to ABA

To determine whether AKT1 could be involved in the regulation of stomatal movements, abaxial epidermal peels from leaves of WT (Col-0), *akt1-2 and cipk23* plants and of WT (WS) and *akt1-1* plants were exposed to ABA to induce stomatal closure. Plants were grown in hydroponics at the non-limiting K+ concentrations of 10 mM. Firstly, stomatal opening was induced by incubating epidermal peels in a solution with 50 mM KCl in the presence of light. After 3 h, epidermal peels were transferred to fresh medium in the absence or in the presence of ABA at two different concentrations (10 and 20 µM), and incubated for another 3 h. During this period, stomata were observed under an inverted microscope at different time points to obtain a time trend of stomatal closure.

Regarding Col-0 plants, in control conditions (no addition of ABA) similar stomatal apertures were registered in WT, *akt1-2* and *cipk23* plants (Fig. 5A and B, closed symbols). Exposure to ABA, induced stomatal closure in these three plant lines but such response was more pronounced in *akt1-2* and *cipk23* plants than in WT plants. At 10 µM ABA the mutant lines showed significantly narrower stomatal pores than WT (Fig. 5A, open symbols). At 20 µM ABA, similar results were obtained although no significant differences were observed (Fig. 5B, open symbols). It should be noted that at 10 µM ABA, WT stomata closed at a slower rate (Fig. 5A, minutes 30 and 60) than at 20 µM ABA (Fig 5B). By contrast, mutant plants exhibited a similar behaviour, irrespective of the ABA concentration.

With respect to WS plants, stomatal aperture remained constant with time in WT and *akt1-1* plants in control conditions (Fig. 5C and D, closed symbols). Exposure to micromolar concentrations of ABA, led to a sharp decrease of stomatal aperture in both WT and mutant plants (Fig. 5C and D, open symbols). However, a significantly higher degree of closure was observed in the mutant stomata with respect to the WT at both ABA concentrations (10 µM and 20 µM).

Discussion

AKT1 has been previously demonstrated to play a key role in K^+ nutrition by constituting a pathway for K^+ uptake at the root (Alemán et al. 2011, Gierth et al. 2005, Hirsch et al. 1998, Rubio et al. 2010, Rubio et al. 2008, Spalding et al. 1999). This pathway was regulated by CIPK/CBL complexes (Xu et al. 2006), being CIPK23 and CBL1 and CBL9 the proteins involved. Moreover, the activity of these complexes was critical for K+ uptake through AKT1 since KO mutants, for instance *cipk23* or *cbl1/cbl9*, were phenocopies of *akt1* plants (Xu et al. 2006). In addition to the phenotype for root K+ acquisition, it was shown that *cipk23* and *cbl1/cbl9* plants displayed water stress resistant phenotypes (Cheong et al. 2007). This was due to the fact that their stomata were hypersensitive to ABA, which resulted in drought-tolerant plants because of a reduced transpiration. The involvement of these CIPK-CBL complexes in the regulation of transpiration (Cheong et al. 2007) and the demonstration that they interact with AKT1 for regulation of root K+ uptake (Xu et al. 2006), left open the possibility that AKT1 might be also a target of such complexes in stomatal function. This would indicate a role for AKT1 in plant transpiration. However, albeit AKT1 is well expressed in stomata (Szyroki et al. 2001), a direct involvement of AKT1 in stomatal movements has not yet been demonstrated. In the present manuscript we provide substantial evidence that AKT1 plays an important role in stomatal movements and water stress responses. We show that this function of AKT1 in transpiration is probably regulated by interaction with CIPK23 because of the phenocopying phenomenon observed between *cipk23* and *akt1* plants.

As a first simple approach to study water stress in *akt1* adult plants, water loss was followed in excised rosettes. A significant decrease in water loss was obtained in *akt1-2* and *cipk23* plants with respect to WT plants (Col-0 ecotype) (Fig.1A) pinpointing that the absence of AKT1 or CIPK23 reduced water loss from excised rosettes. Experiments performed in a different background showed similar results and confirmed that an AKT1 mutant displayed reduced water loss with respect to WT plants in the WS ecotype (Fig. 1B). The *cipk23* behaviour observed here (Fig. 1) was comparable to that previously described (Cheong et al. 2007).

In another experiment, water stress induced by PEG in hydroponics allowed us to study the response of mutant plants, maintaining whole-plant features. Water potential of the solution was adjusted by PEG addition to -0.6 MPa. Absence of stress (-PEG) in hydroponics, reported no differences among WT, *akt1* and *cipk23* plants in plant water content or stomatal conductance (Fig. 2A-F). By contrast, water stress (+PEG) resulted in significant differences between mutant and WT plants. Shoots of *akt1* and *cipk23* plants showed higher water contents and lower stomatal conductances than WT plants in both Col-0 and WS backgrounds (Figure 2A, B, C and E). Importantly, lower K+ concentrations were obtained in plants of the mutant lines (Table 1). The reason for this is that, at an external concentration of 1.4 mM, K+ uptake in the mutant lines is reduced because the remaining K+ uptake systems in the root are not able to compensate for AKT1 absence (Rubio et al. 2010, Rubio et al. 2008). It has been shown that plant transpiration may be affected by the K+ status of the plants (Benlloch-González et al. 2008, Harvey and van den Driessche 1999, Kanai et al. 2011). Therefore, to discard any effect of an impaired K+ nutrition of akt1 plants on water stress responses, further experiments were performed. Such experiments were conducted in a similar fashion but growing the plants with 10 mM K+, since with this K+ supply, *akt1* plants exhibited comparable K+ concentrations to WT plants (Nieves-Cordones et al. 2010, Rubio et al. 2010). Similarly to the experiment at 1.4 mM K+, water stress (+PEG) applied to plants grown at 10 mM K+ led to higher shoot water content and lower stomatal conductance in the mutant plants with respect to WT plants (Fig. 3A, C). Moreover, the magnitude of the differences could be easily spotted macroscopically (Fig. 3B). In addition, similar K+ concentrations between mutant and WT plants were confirmed by mineral analyses (Table 1), thereby discarding the effect of different K+ tissue concentrations on the observed water stress responses.

Water stress was then studied in plants grown in soil in long-term experiments to check whether the phenotypes observed resulted only from an altered short term water response. Furthermore, this approach exerted a water stress without PEG addition, discarding any specific effect of this chemical. Less water consumption and less stomatal conductance were observed in non-irrigated pots of *akt1* plants with respect to WT plants (Fig. 4). These results confirmed the low transpiration phenotype of *akt1* plants even in the long-term stress conditions. Interestingly, no significant differences were observed in plant weight, water content or K+ concentrations between the mutant

and the WT plants (Supplementary Table S1). This indicated that, despite *akt1* exhibited lower water consumption and stomatal conductance, these differences were not important enough under these conditions to provide changes in, for instance, water content.

Finally, an enhanced response to ABA was observed in *akt1* and *cipk23* stomata. Application of this hormone produced a larger stomatal closure in *akt1* and *cipk23* plants than in WT plants, and this was observed in both Col-0 and WS backgrounds (Fig. 5). The *cipk23* responses were in agreement with a previous published study (Cheong et al. 2007). In our conditions, differences in stomatal pore widths in *akt1-2* and *cipk23* plants in comparison to WT plants (Col-0) were better observed at 10 µM ABA than at 20 µM ABA (Fig. 5A, B). WT stomata closed at a slower rate at 10 µM ABA than at 20 µM ABA, which clearly evidenced different stomatal apertures between mutant and WT plants at the lower ABA concentration (Fig. 5A). By contrast, in WS plants, narrower stomatal apertures upon addition of ABA were always observed in *akt1-1* plants with respect to WT plants, regardless the ABA concentration employed (Fig. 5C, D). Importantly, the narrower stomatal apertures in the presence of ABA of mutant plants could account for the lower transpiration values registered under water stress (Fig. 2A and B, Fig. 3A, Fig. 4A). Moreover, the accumulative effect of this lower transpiration was finally observed in higher water contents (Fig 2C and E, Fig. 3C) and less water consumption (Fig. 4B) in mutant plants. It is worth to note that in all the measurements presented here for stomatal aperture, the absence of AKT1 did not affect stomatal opening, a process governed by inward-rectifying K^+ channels (Lebaudy et al. 2008a), nor transpiration under control conditions, and the *akt1* phenotype was only observed in the presence of the stress. This absence of phenotype under control conditions was also reported in *cipk23* plants (Cheong et al. 2007).

The parallel behaviour of *akt1* and *cipk23* plants observed throughout the present study strongly suggested that the CIPK23-CBL1/9 network described in guard cells by Cheong et al (2007) acted upon AKT1 in guard cells to regulate leaf transpiration. The fact that *cipk23* plants phenocopied here *akt1* plants recalled well the results obtained when studying such a network in root K+ uptake (Xu et al. 2006) and points to a critical role of CIPK23 in the regulation of AKT1 activity in guard cells in addition to in root cells. Interestingly, KO mutants of the cited network displayed a better performance than WT plants in water stress adaptation while they are impaired in K+ acquisition at

low K+ supply. This is indicative that the AKT1 activation by the CIPK23-CBL1/9 complex diminishes water stress adaptation capacities of the plant.

How the AKT1-CIPK23-CBL1/9 network is integrated in guard cells to regulate water stress adaptation through ABA-induced stomatal closure is unclear. It should be taken into account that stomatal closure is achieved, in addition to other events, by the coordinated inactivation and activation of inward- and outward K+ currents respectively, that lead to a net outward flux of K+ (Pandey et al. 2007). It has been described that K+-inward rectifying currents, like those mediated by AKT1, are downregulated after ABA exposure in guard cells, promoting stomatal closure (Blatt 1990, Leyman et al. 1999). This inhibition is not thought to be essential for stomatal closure but it would accelerate such a response (MacRobbie 1998). In fact, the results presented here suggest that the absence of an active AKT1 because of a mutation in the gene encoding the channel itself (*akt1* line) or in a kinase that enhances its activity (*cipk23* line), led to an improved stomatal closure in response to ABA. On the other hand, the AKT1-CIPK23-CBL1/9 network is activated by Ca2+ (Li et al. 2006) which is a well-known signalling element in guard cells (Israelsson et al. 2006). However, increases in Ca_{cyt}^{2+} inhibited guard cell K+ inward rectifying channels (Pandey et al. 2007). Therefore, Ca2+ signalling and ABA inhibition of K+ inward currents in guard cells represent two promising targets to focus further research in order to ascertain the role of AKT1 and its regulatory complex in stomatal closure. Nevertheless, involvement of other signalling molecules like sucrose (Rolland et al. 2006) add even more complexity to the role of this pathway in controlling leaf transpiration. Importantly, *akt1* seedlings grown in sealed agarose plates coped better with transplantation in a under a sudden desiccation in a laminar flow hood in the absence of sucrose than WT seedlings (Suppl Fig. 2). By contrast, presence of sucrose in the agarose plates prevented problems derived from transplantation in WT seedlings.

In conclusion, by employing KO mutants, we have been able to study the overall role of AKT1 in water stress adaptation and to envisage that its activity, regulated by CIPK23-CBL1/9, was opposed to the other responses that took place during water stress, since *akt1* plants always exhibited better performances, for instance, less stomatal conductance, than WT plants. The data presented here provide new insights into plant adaptation to drought. Regulation of AKT1-mediated K^+ inward rectifying activity has turned out to be crucial in this process. Further studies in this research line

will contribute to deepen our knowledge in this issue and will focus research in order to obtain plants better adapted to drought conditions.

Materials and Methods

Plant growth and water stress induced by PEG

Seeds of wild-type Arabidopsis plants ecotypes Columbia (Col-0) and Wassilewskija (WS) and the mutants *akt1-2* (Rubio et al. 2008) and *cipk23* in Col-0 background and *akt1-1* in WS background (Hirsch et al. 1998) were employed. The *cipk23* line was obtained from the Arabidopsis Biological Resource Center (Salk_138057), checked for homozygosis and propagated. Seeds of the different lines were surface sterilised briefly in a solution of 70% ethanol, followed by 20% (v/v) commercial bleach for 15 min. They were then washed with sterilised water four times and suspended in sterile water at 4 ºC for 72 h. Plants were grown for 60 d in 2 l containers with a modified one fifth strength Hoagland solution with the following macronutrients (mM): 1.4 or 10 KCl (as indicated), 1.4 $Ca(NO_3)_2$, 0.35 $MgSO_4$ and 0.1 $Ca(H_2PO_4)_2$ and the following micronutrients (µM): 50 $CaCl_2$, 12.5 H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.5 $CuSO_4$, 0.1 H_2MoO_4, 0.1 $NiSO_4$ and 10 Fe-EDDHA. Plants always remained in a controlled conditions chamber (8 h/16 h day/night cycle at 150 mmol m^{-2} s^{-1} light, 22 ºC, relative humidity of 60%).Then, some plants were transferred for 24 h to containers to which PEG (MW= 8000, Sigma-Aldrich Chemie Gmbh Munich, Germany), was dissolved until they reached a $\Psi\pi$ =-0.6 MPa and measurements were then performed as described in the text.

Water loss from excised rosettes

Plants of the different lines were grown in hydroponics in the presence of 1.4 mM K+ for 60 d. Then, plant rosettes were excised from the roots and exposed to desiccation by placing them on trays on the bench. A piece of parafilm sealed the excised stem to avoid water loss through it. Rosettes were weighted at different time points, their fresh weight

determined and the water loss calculated from the difference between the weight at each time point and the initial one.

Growth on pots with soil

90 g of the mixture 1:1 vermiculite:peat were placed in pots and seeds of WT and *akt1-2* plants were sown. Pots were placed in groups of 12 on trays that were irrigated periodically (500 ml week-1 tray-1) with the Hoagland solution described above (10 mM KCl) for 60 d and kept in the same controlled conditions chamber previously described. Before irrigation was stopped, pots were irrigated to saturation (50 ml of growth solution in every pot) and the drained solution was removed twice with a 1 h interval between removals. A group of plants remained irrigated (control plants). During the period of irrigation withdrawal, there were pots without plants that were used to calculate evaporation from the substrate. The water consumption by the plants was deduced from the difference between the water loss of the pots with plants minus the average value of the water loss of the pots without plants for the periods indicated.

Plant physiological determinations

Plants grown under the treatments indicated were separated into root and shoot. Then, the plant material was dried at 65ºC for 4 d, weighed and digested with HNO_3:$HClO_4$ (2:1, v:v) and K^+ concentrations were then determined by atomic absorption spectrometry in an AAnalyst 400 Perkin-Elmer spectrometer (Perkin-Elmer, Waltham, MA, USA). The data reported are the averages of 5-7 values per treatment and error bars denote standard errors. Water content was calculated from the difference between fresh and dry weights. Stomatal conductance was measured on intact leaves of plants subjected to the treatments indicated above by using a portable porometer (AP4 model, Delta-T Devices, Cambridge, UK).

Stomatal aperture assays

Leaves from 8-week-old Arabidopsis plants grown at 10 mM K+ were excised and epidermal strips were obtained by pulling the epidermis with forceps. After peeling, epidermal strips were placed in Petri dishes containing 10 ml of incubation solution (50 mM KCl, 10 mM MES-KOH, pH 6.5, 10 µM $CaCl_2$). Epidermal strips were kept in the incubation solution for 3 h in the light (150 µmol m^{-2} s^{-1} light, 22ºC). Then, they were transferred to fresh medium with or without abscisic acid (ABA, Sigma Aldrich), at 10 µM or 20 µM as indicated, for another 3 h. ABA was dissolved at a concentration of 100 mM in methanol so the final concentration of the solvent was not higher than 0.01%. Then, stomatal apertures were measured (pore width) with an optical microscope (Olympus CKX41) fitted with a camera (ALTRA20) linked to a personal computer. Each experiment was performed in triplicate and consisted of at least 4 epidermal strips from different leaves and 100 measurements per treatment and per genotype.

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