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Physiological and transcriptional memory in guard cells during repetitive dehydration stress

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Summary

• Arabidopsis plants subjected to a daily dehydration stress and watered recovery cycle display physiological and transcriptional stress memory. Previously stressed plants have stomatal apertures that remain partially closed during a watered recovery period, facilitating reduced transpiration during a subsequent dehydration stress.
• Guard cells (GCs) display transcriptional memory that is similar to that in leaf tissues for some genes, but display GC-specific transcriptional memory for other genes. The rate-limiting abscisic acid (ABA) biosynthetic genes NINE-CIS-EPOXycAROTENOID DIOXYGENASE 3 (NCED3) and ALDEHYDE OXIDASE 3 (AAO3) are expressed at much higher levels in GCs, particularly during the watered recovery interval, relative to their low levels in leaves.
• A genetic analysis using mutants in the ABA signaling pathway indicated that GC stomatal memory is ABA-dependent, and that ABA-dependent SNF1-RELATED PROTEIN KINASE 2.2 (SnRK2.2), SnRK2.3 and SnRK2.6 have distinguishable roles in the process. SnRK2.6 is more important for overall stomatal control, while SnRK2.2 and SnRK2.3 are more important for implementing GC stress memory in the subsequent dehydration response.
• Collectively, our results support a model of altered ABA production in GCs that maintains a partially closed stomatal aperture during an overnight watered recovery period.

Introduction

Plants alter their responses during recurring stresses via an adaptive process termed ‘stress memory’ (Bruce et al., 2007; Byun et al., 2014; To & Kim, 2014). A pre-exposure (priming) to different types of stress may alter subsequent responses by displaying a faster and/or stronger activation of the various response pathways (Liu et al., 2014). This can provide the benefits of enhanced protection against disease or insects (Ton et al., 2005, 2007; Conrath et al., 2006; van Hulten et al., 2006; Jaskiewicz et al., 2011). After a dehydration stress, the extent and speed of the physiological recovery are affected by the severity of the dehydration stress endured (Miyashita et al., 2005; Grzesiak et al., 2006). Despite a fairly rapid recovery in the plant’s water status, a slower rate of photosynthesis and a slow reopening of the stomatal aperture are observed for a period extending up to several days (Galle & Feller, 2007; Galle et al., 2007; Galmes et al., 2007; Flexas et al., 2009; Xu et al., 2009). Arabidopsis plants subjected to a repetitive dehydration stress also display a slower rate of transpiration during a subsequent dehydration stress (Ding et al., 2012). These plants also display genome-wide transcriptional stress memory in leaves as 1963 genes have altered transcript levels during a subsequent dehydration stress relative to their levels in a first dehydration stress (Ding et al., 2013).

Guard cell (GC) regulation of stomatal opening and closing is environmentally responsive to drought, CO₂, light, humidity, biotic stresses and different plant hormones (Melotto et al., 2008; Wilkinson & Davies, 2010). Most of the signaling pathways responding to these stimuli include signal transduction via the abscisic acid (ABA) signaling pathway (Joshi-Saha et al., 2011; Lee & Luan, 2012; Nakashima & Yamaguchi-Shinozaki, 2013; Roychoudhury et al., 2013). ABA biosynthesis increases during dehydration stress and both nine-cis-epoxycarotenoid dioxygenase 3 (NCED3) and aldehyde oxidase 3 (AAO3) appear to catalyze the key rate-limiting steps (Tan et al., 2003; Wan & Li, 2006; Melhorn et al., 2008). ABA biosynthesis appears to be primarily active in vascular tissues and GCs in the aerial parts of the plant (Tan et al., 2003; Koiwai et al., 2004; Ikegami et al., 2009; Bauer et al., 2013).

Once present in a cell, ABA binds to the Pyrabactin Resistance/ PYR1-like protein/Regulator component of the ABA receptor (PYR/PYL/RCAR) family of ABA receptor proteins (Ma et al., 2009; Park et al., 2009; Klingler et al., 2010). The ABA-bound receptors bind members of the clade A protein phosphatase 2C (PP2C), which are involved in negatively regulating ABA responses (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). The ABA receptor-bound PP2C phosphatases, including ABA Insensitive 1 (ABI1), ABA Insensitive 2 (ABI2) and Hypersensitive to ABA 1 (HAB1), are no longer able to dephosphorylate Snf1 (Sucrose non-fermenting 1)-related protein kinases 2 (SnRK2s), allowing the SnRK2s to achieve and maintain an active, phosphorylated state to propagate ABA
signaling (Klingler et al., 2010; Yunta et al., 2011; Xie et al., 2012).

The SnRK2s are a gene family of serine/threonine protein kinases that mediate ABA-dependent and-independent responses and are organized into three subfamilies: ABA independent group I (SnRK2.1/2.4/2.5/2.9/2.10); weakly ABA-activated group II (SnRK2.7/2.8), and strongly ABA-activated group III (SnRK2.2/2.3/2.6) (Boudsocq et al., 2004; Kulik et al., 2011). The Arabidopsis SnRK2 ABA-activated group III kinases are expressed in GCs (Fujii et al., 2007) and their critical role in stomatal control was demonstrated via a triple mutation in the group III snrk2.2/2.3/2.6 kinases that resulted in a loss of stomatal control and severely wilted plants (Fujii & Zhu, 2009; Fujita et al., 2009). SnRK2.6 (Open stomata 1, OST1) can directly phosphorylate the outward slow anion channel SLAC1 in vitro, leading to the activation of the channel to induce stomatal closure (Geiger et al., 2009; Brandt et al., 2012; Lee et al., 2013). SnRK2.6 also negatively regulates the activity of the K+ channel (KAT1) K+ inward ion channel to inhibit its ability to open stomata (Sato et al., 2009). Group III SnRK2s also regulate the activity of various transcription factors that bind the ABA-response elements (ABREs) present in many ABA-inducible promoters, such as the ABRE-binding factors (ABFs) (Furukawa et al., 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Fujii & Zhu, 2009; Fujita et al., 2011).

The occurrence of GC stomatal stress memory, together with the ability to isolate RNA specifically from GCs, provides a system for comparing the cellular response of GC stress response with GC-specific transcriptional memory responses in a single type of cell (Sirichandra et al., 2009a,b). Here we examine the GC-specific regulation of selected genes to determine their transcriptional memory patterns relative to their behavior in leaf tissues, which are predominantly mesophyll cells. We demonstrate that GCs can display transcriptional memory patterns distinct from those measured in leaves. We also analyzed GC stomatal regulation during repetitive dehydration stress in wild-type plants and in plants with mutations in ABA signaling pathways to gain insight into the genetic control of GC stomatal memory. The results from these experiments emphasize that genes have functionally distinct roles in a first or subsequent dehydration stress, and that ABA-dependent SnRK2.2 and SnRK2.3 play a critical role in GC stomatal memory.

Materials and Methods

Plant growth and treatments

Wild-type (WT; Columbia-0), abf2/3/4 (Yoshida et al., 2010), snrk2.6, snrk2.2/2.3, snrk2.2/2.3/2.6, snrk2.1/2.4/2.5/2.7/2.8/2.9/2.10 (Fujii et al., 2011) and aba2 (CS6147 allele) mutant Arabidopsis thaliana plants were grown in potting soil in growth rooms at 22°C with a 12-h light photoperiod and light intensity of 180 μmol m⁻² s⁻¹. The day before any treatment, the plants were removed from the soil, any remaining soil from their roots was washed away and their roots were placed in water overnight. The dehydration stress was applied as described previously (Ding et al., 2012). Briefly, 3-wk-old watered plants (W) were gently blotted onto filter paper to remove water and subjected to an initial air-drying stress for 90 or 120 min (S1). The plants were placed in water for 22 h, corresponding to the recovery period (R1). For a subsequent stress treatment, R1 plants were blotted onto filter paper to remove water and air-dried for 90 or 120 min (S2). One leaf from each of eight to 10 plants was harvested at the different stages, frozen in liquid nitrogen and stored at −80°C for subsequent RNA isolation.

Stomatal aperture and water loss measurements

Stomatal aperture size measurements were performed on 3-wk-old plants by a modification of the glutaraldehyde leaf-fixation methods (Yamaguchi et al., 2007; You et al., 2013). Leaves were fixed in a solution of 4% glutaraldehyde, 10 mM PIPES, pH 7, 5 mM MgCl₂, and 5 mM EGTA for 6 h at 4°C. The samples were then washed five times in a phosphate-buffered saline (PBS) solution. Forty to sixty stomata from three fixed leaves were examined in each condition using a confocal microscope and cellular autofluorescence (Russo et al., 2010). The autofluorescence of the walls of the leaf epidermal cells and of guard cell extracellular components is highly visible (Supporting Information Fig. S1C; Methods S1). The width of the stomatal pores was measured using IMAGEJ software. The means of the stomatal apertures for three leaves were then used for ANOVA using the statistical software R (R Core Team, 2014) to compare the different conditions for a specific genetic background (Table S1). A comparison of stomatal aperture widths measured by this glutaraldehyde leaf-fixation method to those measured by a method using a rapid (≤ 1 min) confocal microscopic analysis of fresh, unfixed leaves indicated that the two methods provided equivalent results (Fig. S1).

Water loss curves were obtained by weighing the whole plants at different times during the dehydration stress interval. The percent water loss was calculated using the formula: water loss (%) = FWi / FW₀ min × 100, where FWᵢ is the fresh weight for the indicated time, and FW₀ min corresponds to the initial plant weight for the watered or recovery conditions. For all the experiments, two or three replicates, each containing five to eight plants, were used and each experiment was repeated at least three times. The statistical analyses were performed by using an ANOVA, at the P=0.05 level, to compare the significance of the differences between the S1 and S2 curves for each genotype (Table S1).

Isolation of the guard cells

Approximately 2 g of 3-wk-old plants leaves was excised and blended for 3 × 1 min with a Waring blender in cold water in the presence of actinomycin D (10 mg l⁻¹) and cordycepin (50 μM). Epidermal fragments were collected, washed, and filtered through a 100-μm mesh in three 1.5-ml Eppendorf tubes with cold water in the presence of transcription inhibitors. Blended peels were sonicated to remove contaminating mesophyll and epidermal cells, for 30 s at a power setting of 31%
(Digital Sonifier 450; Branson Ultrasonic Corp., Danbury, CT, USA) for five to six times or until no green chlorophyll contamination was observed. Sonicated tissues were washed after three sonications. A subsequent treatment with RNAse A, to remove the RNA which was present from the disrupted epidermal and mesophyll cells, was performed by incubation with 50 μg ml⁻¹ RNAse A and transcription inhibitors for 10 min at room temperature. The guard cells were washed with cold water in the presence of transcription inhibitors, frozen in liquid nitrogen and stored at −80°C until further use.

Reverse transcription and real-time PCR
RNA isolation with TRIzol (Invitrogen, Grand Island, NY, USA), DNase treatment, and reverse transcription were carried out as described previously (Virlouv et al., 2011). Briefly, the isolated GCs or total leaf tissues were pulverized by bead beating in the presence of TRIzol reagent before the addition of chloroform. Real-time PCR analysis was performed with the cyclerIQ real-time PCR instrument (Bio-Rad, Hercules, CA, USA) and SYBR Green mixture (Bio-Rad). The relative expression of specific genes was quantified with the 2⁻DDCt calculation according to the manufacturer’s instructions (Bio-Rad (Livak & Schmittgen, 2001)), where DDc is the difference in the threshold cycles of the specific gene and the reference housekeeping gene, which was actin2 for expression analyses, and then of the specific condition and the well-watered condition. The mean threshold cycle values for the genes of interest were calculated from three technical replicates. The values obtained for the technical replicates (measurements of the same RNA sample) were used for ANOVA analysis with R software to compare the different conditions for a specific tissue or cell type (Table S1). The primers used are given in Table S2.

Results
The GC stomatal aperture memory response
Arabidopsis plants subjected to a prior dehydration/recovery cycle show a decreased rate of water loss in a subsequent dehydration stress (Ding et al., 2012). This physiological memory suggests that regulation of the stomatal aperture is occurring. To gain insight into this process, stomatal apertures were measured at the end of the watered (W) period, as well as the end of the first (S1) and second (S2) dehydration stresses, and the end of the 22-h watered recovery period (R1) between S1 and S2 stresses (Fig. 1). We observed that the stomata partially closed during S1, and the extent of the closure was not significantly different from this level at the end of the 22-h R1 watered recovery period or during the subsequent S2 stress (Fig. 1). These results suggest that partially closed stomatal apertures during the watered recovery period are a dominant feature of GC stress memory after dehydration stress, that is, a R1/W GC stomatal stress memory (we will denote the stages being compared in a stress memory analysis). This partially closed state during R1 presumably helps reduce water loss when plants enter the subsequent S2 dehydration stress.

Isolation of GC-specific RNAs
We next sought to examine selected gene transcript levels in GCs. We developed a nonprotoplast method that rapidly isolates intact GCs while removing and/or disrupting the other leaf cells. This GC RNA isolation method combines two steps from previously published protocols. First, a Waring blender is used to chop leaves into small epidermal fragments (Pandey et al., 2002). After filtering out larger leaf fragments, the remaining intact tissue fragments are predominantly epidermal peels containing some intact epidermal cells and intact GCs (Fig. 2a). These epidermal peels are sonicated at a power level sufficient to disrupt the cell wall of epidermal cells but not the stronger cell wall of GCs (Cornish & Zeevaart, 1986). Microscopic examination for the presence of chloroplasts serves as an indication of cell integrity and showed that only GCs were intact after this sonication procedure (Fig. 2a). Both the blending and sonication steps are conducted at 4°C and in the presence of transcription inhibitors (Leonhardt et al., 2004) to minimize the production of new transcripts during these steps. The intact GC preparations are treated with RNase A to digest any RNA released from the disrupted cells. The GCs are then pulverized by bead beating in the presence of TRIzol for RNA isolation (see the Materials and Methods section).

The cellular specificity of the RNA isolated from the GC preparations was then estimated by measuring the levels of GC1, a GC-specific transcript (Yang et al., 2008), and β-carbonic anhydrase 1 (βCA1), which is expressed in GCs and mesophyll cells, but is c. 4-fold more abundant in mesophyll cells (Hu et al., 2010). As expected, the RNA isolated from GCs had a high ratio of GC1 compared with βCA1 (Fig. 2b). By contrast, in RNA isolated from total leaf tissue, βCA1 RNA levels were higher, and GC1 was 931-fold less abundant than in GCs (Fig. 2b). These results indicate that this blender/sonication protocol rapidly isolates RNAs that are predominantly from GCs.
The transcripational memory response in GCs

We next evaluated transcripational memory responses of specific genes in GCs using the above RNA isolation protocol and compared these responses with those obtained in leaf tissues. We first analyzed four genes, *ras-related small GTPase homologue B18* (*RAB18*), *lipid transfer protein 4* (*LTP4*), responsive to desiccation 29A (*RD29A*) and responsive to desiccation 29B (*RD29B*), that are known to be expressed and/or induced by ABA or dehydration stress in GCs (Leonhardt *et al.*, 2004; Wang *et al.*, 2011). Importantly, we previously found that *RAB18*, *LTP4*, and *RD29B* displayed S3/S1 transcripational memory in leaf tissues as they were induced to higher levels in a third S3 dehydration stress relative to a first S1 dehydration stress (Ding *et al.*, 2012). By contrast, *RD29A* did not display S3/S1 transcripational memory in leaf tissues as it was induced to about the same or lower levels during repetitive stress cycles (Ding *et al.*, 2012). The results of our analysis of *RAB18*, *LTP4*, *RD29A* and *RD29B* transcript profiles in whole leaves were in agreement with our prior pubication (Ding *et al.*, 2012), as *RAB18*, *LTP4* and *RD29B* displayed significant S2/S1 transcripational memory as they were induced at higher levels during S2 relative to S1, while *RD29A* was not (Fig. 3). In GCs, these genes generally had transcripational response patterns that were similar to their memory response in leaf tissues (Fig. 3). The higher basal levels of *RAB18* and *LTP4* in the W state appear to be a GC-specific feature of these genes that is consistent with previous reports of their transcript levels (Leonhardt *et al.*, 2004; Wang *et al.*, 2011). These results demonstrate that S2/S1 transcripational memory occurs in GCs as well as mesophyll cells, and suggest that the dehydration stress transcripational responses are added to the high basal levels of expression observed for *RAB18* and *LTP4* in GCs.

ABA biosynthesis and degradation gene transcripts in GCs

A puzzling observation was that stomatal apertures remained partially closed at the end of the 22-h R1 interval, indicating that low levels of ABA signaling were being perceived. To help resolve this contradiction, we measured transcript levels of two genes involved in ABA biosynthesis, *NCED3* and *AAO3* (Iuchi *et al.*, 2001; Tan *et al.*, 2003; Wan & Li, 2006; Melhorn *et al.*, 2008), as well as two genes involved in ABA degradation, cytochrome P450, family 707, subfamily A, polypeptide 1 and 3: *CYP707A1* and *CYP707A3* (Kushiro *et al.*, 2004).
Transcript levels of these four genes were measured in GC and leaf RNA (Fig. 4). In leaves, NCED3 was induced to similar levels during S1 and S2 dehydration stresses and restored to basal watered levels at the end of the watered R1 recovery (Fig. 4). By contrast, in GCs NCED3 was induced to much higher levels in S1, remained at fairly high levels at the end R1, and then was induced to significantly higher levels in S2 than in S1 (Fig. 4). Therefore, NCED3 displayed an S2/S1 and R1/W transcriptional memory pattern in GCs that was different from its response in leaves. The transcript level of AAO3 was higher in R1 than in S1 in GCs, while transcript levels were back to watered levels in R1 in leaves (Fig. 4). Therefore, AAO3 also displayed GC-specific S2/S1 and R1/W transcriptional memory patterns.

A simple interpretation of the elevated NCED3 and AAO3 transcript levels in GCs during R1 is that the rate of ABA biosynthesis was higher during R1 than in the W condition. However, this presumed increase in the rate of ABA biosynthesis was accompanied by an increase in GCs in the transcript levels of CYP707A1 and CYP707A3, which encode ABA degradation enzymes (Fig. 4). Both CYP707A1 and CYP707A3 transcripts showed S2/S1 and R1/W GC-specific transcriptional memory responses that were distinct from their much lower transcript levels found in total leaf cells (Fig. 4). In summary, all four ABA metabolism genes displayed GC-specific S2/S1 and R1/W transcriptional memory patterns that were different from those observed in leaves, suggesting that increased ABA biosynthesis and degradation occurred concurrently during the R1 watered recovery period.

The ABA signaling pathway regulates the GC stomatal memory response

The size of stomatal apertures in WT plants (Fig. 1) affects the rate of water loss from plants during repetitive dehydration stress. Measurement of the rate of water loss from plants experiencing their first dehydration stress (S1) or second dehydration stress (S2) demonstrates this difference (e.g. see the S1 and S2 water loss curves for WT plants in Fig. 5a). This difference in the rates of water loss provides a convenient assay for measuring S2/S1 GC stomatal memory in WT and mutant plants. We refer to this assay as a S2/S1 GC stomatal memory assay because it measures water loss during the S1 and S2 dehydration stresses. Note that the rate of water loss during S2 is affected by the R1 stomatal aperture being smaller at the beginning of S2 than the W stomatal aperture size at the beginning of S1 (Fig. 1). Thus, the S2/S1 GC stomatal memory assay is affected by R1 changes.

To gain further insight into the role of ABA in S2/S1 GC stomatal memory, the rate of water loss of various mutants involved in ABA-mediated stomatal responses was investigated during S1 and S2 dehydration stress. We found that the aba2 mutant, which is deficient in ABA biosynthesis (Nambara et al., 1998), had rapid rates of water loss that were not significantly different in S1 and S2 (Fig. 5a). This suggests that very little S2/S1 GC stomatal memory can occur at the very low or absent levels of ABA in aba2 plants. This ABA signaling for S2/S1 GC stomatal memory was mediated predominantly by SnRK2.2, SnRK2.3 and SnRK2.6, as a snrk2.2/2.3/2.6 triple mutant had rapid rates of water loss that were not significantly different in S1 and S2 (Fig. 5b). The snrk2.6 mutant displayed a rapid rate of water loss in S1, but had a significantly slower rate during S2, demonstrating that snrk2.6 retains S2/S1 GC stomatal memory (Fig. 5c). This result contrasts with the result for the snrk2.2/2.3 double mutant, which had rates of water loss in S1 and S2 that were not significantly different (Fig. 5d).

A snrk2.1/2.4/2.5/2.7/2.8/2.9/2.10 septuple mutant displayed S2/S1 GC stomatal memory, as the slower rate of water loss in S2 was significantly different from the faster rate in S1 (Fig. 5e).

The correlation of the rate of water loss with stomatal apertures was analyzed by measuring stomatal apertures of snrk2.6, snrk2.2/2.3, and snrk2.2/2.3/2.6 mutants during repetitive dehydration stress (Fig. 6). The snrk2.2/2.3/2.6 triple mutant stomatal apertures did not change under repetitive dehydration stress (Fig. 6a), in agreement with its similar rates of water loss in S1 and S2 (Fig. 5b). Stomatal apertures of snrk2.6 were similar in the watered W, initial S1, and R1 recovery conditions, indicating very little response to these conditions. However, snrk2.6 stomata were significantly smaller during S2 conditions (Fig. 6b), in agreement with the slower rate of water loss in S2 (Fig. 5c). Stomatal apertures of the snrk2.2/2.3 mutant displayed R1/W GC stomatal memory, as R1 apertures were significantly smaller than W apertures (Fig. 6c). Most importantly, a comparison of the lack of stomatal control in the snrk2.2/2.3/2.6 mutant (Fig. 6a) with the partial stomatal closure during S2 for the snrk2.6 mutant (Fig. 6b) revealed that SnRK2.2 and SnRK2.3 are particularly important for GC stomatal closure during S2.
ABF2, ABF3 and ABF4 are SnRK2-regulated transcription factors that activate many ABA-responsive genes (Fujita et al., 2005; Yoshida et al., 2010). We found that the abf2/3/4 triple mutant had a higher rate of water loss in S1 and S2 dehydration stresses, relative to the WT control plants (Fig. 5f). However, abf2/3/4 plants lost water at a significantly slower rate in S2 than in S1 (Fig. 5f). These results demonstrate that ABF2, ABF3 and ABF4 can affect the degree of stomatal closure during dehydration stress, in agreement with earlier reports (Yoshida et al., 2010), and that abf2/3/4 mutant plants retain S2/S1 GC stomatal memory.

Transcriptional memory of membrane proteins and transcription factors in GCs

We next measured transcript levels of seven key ion channel and transporter proteins (reviewed in Kim et al., 2010), namely H⁺-ATPase 1 (AHA1), nitrate transporter 1 (NRT1.1), potassium channel in Arabidopsis thaliana 1 (KAT1), potassium channel in Arabidopsis thaliana 2 (KAT2), slow anion channel associated 1 (SLAC1), quick anion channel associated 1 (QUAC1) and gated outwardly-rectifying K⁺ channel (GORK), to gain further insight into transcriptional memory in GCs. AHA1 is a member of the H⁺-ATPase family of P-type ATPases, which are all expressed in the GCs and mediate the efflux of H⁺ from the GCs during the opening of the stomata (Ueno et al., 2005). NRT1.1, a nitrate transporter, also functions in stomatal opening (Guo et al., 2003). KAT1 and KAT2 encode potassium channels which mediate K⁺ uptake during stomatal opening (Schachtman et al., 1992; Pilot et al., 2001), whereas GORK encodes the K⁺out channel involved in stomatal closure (Hosy et al., 2003). SLAC1 and QUAC1 encode slow (S-type) and rapid (R-type) ion channels, respectively, and mediate anion efflux during the closure of the GCs.
resulted in higher levels of QUAC1 in leaf cells (Fig. 7). The R1/W transcriptional memory that thereby displaying R1/W transcriptional memory in GCs but not script levels were significantly higher in R1 relative to W levels, QUAC1 and S2. This R1/W transcriptional memory displayed by transcription levels did not significantly change in GCs during dehydration stress. The three genes involved in stomatal opening, AHA1, NRT1.1, KAT1 and KAT2, were all expressed at significantly lower levels during S1 relative to their W state, consistent with closing of the stomatal aperture during S1. Their transcript levels during R1 were not significantly different from their levels during the W state. However, NRT1.1 and KAT2 displayed S2/S1 transcriptional memory as their S1 and S2 transcript levels were significantly different, while those of AHA1 and KAT1 were not (Fig. 7). Note that, while stomatal apertures remained partially closed in R1 (Fig. 1), the GC transcript levels of these four genes returned to levels not significantly different from their levels in the W state (Fig. 7).

The three genes involved in stomatal closure, SLAC1, QUAC1, and GORK, had different responses (Fig. 7). SLAC1 transcript levels did not significantly change in GCs during dehydration stress. QUAC1 transcripts were higher in R1 in GCs relative to the W control, but were not significantly different in S1 and S2. This R1/W transcriptional memory displayed by QUAC1 was similar in GCs and leaf cells (Fig. 7). GORK transcript levels were significantly higher in R1 relative to W levels, thereby displaying R1/W transcriptional memory in GCs but not in leaf cells (Fig. 7). The R1/W transcriptional memory that resulted in higher levels of QUAC1 and GORK transcripts in R1 suggests a potential involvement of the R-type ion and K
out channels, respectively, in the partial closure of the stomatal aperture during the recovery period.

We also measured the transcript levels of two transcription factors, namely nuclear factor Y subunit A5 (NFYA5) and MYB60, both of which are involved in regulating GC apertures. Nuclear factor Y (NFY) is a ubiquitous transcription factor composed of three distinct subunits (NFYA, NFYB and NFYC). The Arabidopsis NFYA5 transcript is strongly induced by drought stress in an ABA-dependent manner (Li et al., 2008). Functionally, NFYA5 appears to facilitate stomatal closing as nfya5 mutants lose water at a faster rate, while plants overexpressing NFYA5 lose water at a slower rate (Li et al., 2008). In leaves, NFYA5 transcript levels increased in S1, decreased in R1 and then increased to levels in S2 similar to those in S1 (Fig. 7). However, in GCs, NFYA5 was expressed at increasingly higher levels in S1, R1 and S2, thus displaying S2/S1 and R1/W transcriptional memory in GCs (Fig. 7). The generally higher NFYA5 transcript levels in GCs in S1, R1 and S2, together with its apparent functional role in stomatal closure, suggest that NFYA5 is facilitating the closure of stomata that is occurring during S1, R1 and S2.

MYB60 is a transcription factor that functions in stomatal opening and which is down-regulated in response to ABA (Cominelli et al., 2005), which presumably facilitates stomatal closing. We found that its transcript levels were fairly low and unchanging in leaves but it was expressed at high levels in GCs in the W condition (Fig. 7), in agreement with MYB60 GC-specific promoter expression (Rusconi et al., 2013). In GCs, MYB60 expression was significantly lower in S1, R1 and S2 compared with its transcript levels in the W state. Its negative R1/W transcriptional memory, which means transcript levels were lower in the R1 recovery state than the W state, was consistent with attenuation of the role of MYB60 in stomatal opening during the watered R1 recovery interval when the stomatal aperture was partially closed.

Discussion

Physiological stress memory response improves tolerance to subsequent dehydration stress

Previous studies have observed that stomatal re-opening is incomplete during a watered recovery after drought stress conditions (Davies & Kozlowski, 1977; Ni & Pallardy, 1992; Loewenstein & Pallardy, 2002). Whereas leaf water potential had largely recovered within 6 h of rewatering in five species, Quercus alba, Quercus velutina (black oak), Juglans nigra, black willow (Salix nigra) and eastern cottonwood (Populus deltoides), stomatal conductance did not recover to control levels for at least one full day (Loewenstein & Pallardy, 2002). Our results indicate that Arabidopsis stomatal apertures also do not open to levels typical for watered conditions for at least 1 d. In our repetitive dehydration stress system, wherein dehydration stress was imposed on a 24-h cycle, the partially closed stomata at the end of the 22-h R1 recovery interval facilitated the slower rates of water loss. We propose that this is analogous to what happens during a diurnal cycle during drought stress. Plants experiencing...
dehydration stress during the day often recover turgor pressure at night, thereby temporarily alleviating dehydration stress conditions (Tardieu et al., 2010). The plant’s ability to use physiological and transcriptional stress memory to maintain its preparation for dehydration stress presumably improves its tolerance and reduces its water loss during the next day’s stress. Upon sustained water availability for several days, these memory processes are lost, allowing the plant to return to its nonstressed growth patterns (Ding et al., 2012).

ABA concentrations are likely to be partially elevated in GCs during recovery

Our results demonstrate that GC stomatal memory requires ABA, as the rates of water loss in S1 and S2 dehydration stresses were approximately the same in an aba2 mutant background. Elevated residual ABA concentrations and/or increased sensitivity to ABA have been proposed as potential mechanisms by which GC stomatal memory persists during watered conditions after a dehydration stress. For example, ABA concentrations in the xylem sap of recovering plants are two to three times above control concentrations 1 d after watering, although ABA returns to control concentrations before complete stomatal opening (Loewenstein & Pallardy, 2002). ABA concentrations in Arabidopsis plants subjected to our repetitive stress cycles have residual ABA concentrations two to three times above control concentrations during recovery periods of up to several days (Ding et al., 2012). ABA concentrations in GCs in dehydration-stressed Vicia faba leaves persist at elevated levels for 4–6 h, but not 8 h, in this detached leaf system (Harris et al., 1988; Harris & Outlaw, 1991). The higher transcript levels of the two key regulatory genes of the ABA biosynthetic pathway, NCED3 and AAO3, suggest that biosynthesis of ABA is active during the watered recovery in GCs. However, as we also observed an increase in the transcript levels of the ABA degradation genes CYP707A1 and CYP707A3, the net effect on ABA concentrations is unclear. Different subcellular sites of ABA biosynthesis, degradation, conjugation, export, and import may also be able to affect localized subcellular concentrations of ABA. In particular, different subcellular ABA concentrations could trigger plasma membrane stomatal responses and nuclear transcriptional responses differentially at low cellular ABA levels. (Nambara & Marion-Poll, 2005; Xu et al., 2013). As measuring ABA concentrations specifically in GCs is extremely difficult (Harris et al., 1988; Harris & Outlaw, 1991), we used an ABA-responsive gene in GCs as an indirect indicator of ABA concentrations.

The endogenous RD29B gene is highly responsive to ABA concentrations via activation of ABF2/3/4 by SnRK2.2/3/2.6 phosphorylation (Kang et al., 2002; Fujita et al., 2005, 2009; Fujii et al., 2009; Yoshida et al., 2010). The observation that RD29B was not induced at the end of an R1 watered recovery period indicates that it was not receiving an ABA signal. This result is puzzling as ABA signaling proceeds via a common SnRK2.2/3/2.6 step for both stomatal regulation and RD29B induction (Fujii & Zhu, 2009; Fujita et al., 2011) and both these processes are likely to have fully responded to ABA concentrations by the end of the 22-h recovery interval. A possible explanation is that the stomatal regulatory pathway responds at lower concentrations of ABA than the transcriptional regulatory pathway. Hypothetically, this could be a result of a higher affinity of SnRK2.2/3/2.6 for their substrates in the stomatal regulatory pathway and/or a slower rate of dephosphorylation of these substrates. Presumably, higher concentrations of ABA and SnRK2.2/3/2.6 activity are required to activate the ABF2/3/4 pathway that regulates RD29B (Fujita et al., 2009; Yoshida et al., 2010). Therefore, our data do not support the hypothesis that an overall increased sensitivity to ABA is occurring at the SnRK2.2/3/2.6 activation step that is shared in the stomatal and transcriptional ABA-response pathways.

A comparison of GC-specific and leaf expression analyses may resolve the puzzle as to why stomata continue to stay partially closed after ABA concentrations return to control values in leaves or xylem sap in many plants (Loewenstein & Pallardy, 2002). Total leaf NCED3 and AAO3 transcript levels return to control levels during the watered recovery period, while GC-specific transcripts of these genes remain elevated. This suggests that ABA measurements at the whole leaf level or in xylem sap are unlikely to detect ABA being specifically produced only in GCs during the watered recovery interval. Our results suggest that low concentrations of residual ABA and/or ABA newly synthesized in GCs are preferentially activating the stomatal control pathway at concentrations sufficient for partial closure of the stomata, but not at concentrations sufficient to activate the ABF2/3/4 transcriptional pathway in GCs during the watered recovery interval.

Some genes have different transcriptional memory patterns in GCs and mesophyll cells

Transcriptional memory in GCs displayed a variety of patterns relative to the behavior of the same genes in leaves, which are comprised predominantly of mesophyll cells. The RAB18, LTP4, RD29A, RD29B and RD29A structural genes had elevated levels of transcripts in S1 and higher levels in S2 in GCs and leaves, thereby displaying an S2/S1 transcriptional memory response in agreement with their previously reported S3/S1 transcriptional memory patterns in leaves (Ding et al., 2012). The main difference in GCs was that the basal levels of RAB18 and LTP4 were much higher in nonstressed GCs than in leaves, in agreement with prior studies of GCs (Wang et al., 2011).

By contrast, 10 genes with regulatory roles in GCs generally showed GC-specific patterns of transcriptional memory that were often distinct from their behavior in leaves. These included the ABA biosynthesis (NCED3 and AAO3) or degrading (CYP707A1 and CYP707A3) genes, which had higher levels of expression in GCs and displayed R1/W transcriptional memory in GCs but not in leaf cells. The ion channels, ion transporters, and membrane protein genes analyzed had transcript profiles in GCs that were also generally different from their leaf profiles. AHA1, NRT1.1 (CHL1), KAT1, and KAT2 are involved in stomatal opening and displayed decreased transcript levels in S1 in GCs, a result consistent with decreased stomatal opening during S1.
However, during the R1 recovery interval their transcript levels were restored to approximately their levels in W conditions, despite maintaining a partially closed stomatal aperture. Similarly, three genes involved in stomatal closure, SLAC1, QUAC1 and GORK, were expressed at higher levels in GCs than leaf cells, but differed in their GC stress responses. SLAC1 transcript levels did not change during dehydration stress, while QUAC1 and GORK displayed R1/W transcriptional memory in GCs, presumably facilitating partial stomatal closure during the R1 recovery period. These gene products are additionally regulated by post-translational mechanisms such as phosphorylation/dephosphorylation (Ho et al., 2009; Sato et al., 2009; Lebaudy et al., 2010; Caesar et al., 2011).

The NFYA5 and MYB60 transcription factors have opposite functional roles in stomatal regulation (Cominelli et al., 2005; Li et al., 2008) and displayed different GC-specific transcriptional memory patterns. NFYA5 displayed a positive R1/W transcriptional memory while MYB60 displayed a negative R1/W transcriptional memory as its transcript levels were lower in R1 than in the W state. The directional increase in NFYA5 and decrease in MYB60 transcript levels in GCs in R1 are consistent with the partial stomatal closure during R1 and their functional roles in the induction of stomatal closure and stomatal opening, respectively.

SnRK2.2, SnRK2.3, and SnRK2.6 have functionally distinguishable roles in S2/S1 GC stomatal stress responses

The group III ABA-dependent SnRK2.2, SnRK2.3, and SnRK2.6 play important roles in stomatal regulation. SnRK2.6 phosphorylates ion channels, ion transporters, and other membrane proteins (Vahisalu et al., 2008; Sato et al., 2009; Sirichandra et al., 2009a,b). SnRK2.2 and SnRK2.3 have less well-defined roles but are known to activate SLAC1 (Geiger et al., 2009). Our results indicate that SnRK2.2 and SnRK2.3 are more important for regulation of stomatal apertures during S2 than during S1. This conclusion is based on several observations in the different snrk2 mutant backgrounds. First, the snrk2.2/2.3/2.6 triple mutants rapidly lose water at equal rates in S1 and S2, and essentially display little control of stomatal apertures (Fujii & Zhu, 2009). This result indicates that S2/S1 GC stomatal memory control is lost in this triple mutant. Secondly, the snrk2.2 mutant displays very little stomatal closing during S1 but does in S2, implicating SnRK2.2 and SnRK2.3 in S2 stomatal closure. Thirdly, snrk2.2/snrk2.3 plants display similar rates of water loss in S1 and S2. Thereby, SnRK2.2 and SnRK2.3 are identified as key regulators of S2/S1 GC stomatal stress memory, regulating stomatal apertures differentially in S2. These stomatal stress memory effects are not mediated by ABF2, ABF3, and ABF4 controlled transcriptional responses as the abf2/3/4 triple mutant still displays S2/S1 GC stomatal stress memory.

Taken together, our results demonstrate that GCs display S2/S1 and/or R1/W transcriptional stress memory that is often distinct from that in leaves and elucidate some of the mechanisms facilitating S2/S1 GC stomatal stress memory, which probably includes contributions from the R1 recovery period as well. These results support the hypothesis that ABA concentrations are partially elevated in GCs during watered recovery intervals via GC-specific biosynthesis of ABA, at concentrations sufficient for partial stomatal closure but not sufficient for inducing ABA-dependent transcriptional responses. This differential response to low concentrations of ABA might be enhanced by the subcellular specificities of ABA biosynthesis, degradation, conjugation, and compartmentalization (Nambara & Marion-Poll, 2005; Xu et al., 2013). We propose that the ABA-dependent mechanism of partial stomatal closure during watered recovery intervals facilitates plant adaptation to the diurnal cycle of dehydration stress that occurs during periods of low water potential such as drought stress conditions.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Comparison of stomatal apertures measured by microscopic analysis of fresh leaves or chemically fixed leaves and measurement of stomatal apertures in fresh unfixed leaves by guard cell autofluorescence visualized by confocal laser scanning microscopy.

**Table S1** ANOVA for the stomatal aperture measurements, the transcript levels in guard cells and total leaf cells and the water loss measurements

**Table S2** Primes used for the real-time PCR

**Method S1** Observation of the stomatal aperture of the leaves by confocal microscopy.

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