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Report

The Stomatal Response to Reduced Relative Humidity Requires Guard Cell-Autonomous ABA Synthesis

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Summary

Stomata are pores on the leaf surface, bounded by two guard cells, which control the uptake of CO_2 for photosynthesis and the concomitant loss of water vapor. In 1898, Francis Darwin [1] showed that stomata close in response to reduced atmospheric relative humidity (rh); however, our understanding of the signaling pathway responsible for coupling changes in rh to alterations in stomatal aperture is fragmentary. The results presented here highlight the primacy of abscisic acid (ABA) in the stomatal response to drying air. We show that guard cells possess the entire ABA biosynthesis pathway and that it appears upregulated by positive feedback by ABA. When wild-type *Arabidopsis* and the ABA-deficient mutant *aba3-1* were exposed to reductions in rh, the *aba3-1* mutant wilted, whereas the wild-type did not. However, when *aba3-1* plants, in which ABA synthesis had been specifically rescued in guard cells, were challenged with dry air, they did not wilt. These data indicate that guard cell-autonomous ABA synthesis is required for and is sufficient for stomatal closure in response to low rh. Guard cell-autonomous ABA synthesis allows the plant to tailor leaf gas exchange exquisitely to suit the prevailing environmental conditions.

Results and Discussion

The response of stomata to a reduction in atmospheric relative humidity (rh) is made up of rapid, transitory opening followed by closure (Figure 1A). EDXA analysis showed that exposure of stomata to dry air results in a 30% reduction in guard cell-K^+ content (Figure 1B). To investigate the role of ABA, which builds up during drought and causes stomatal closure [3], we monitored the stomatal rh response in the *Arabidopsis aba3-1* mutant, which lacks a gene involved in ABA biosynthesis and is unable to increase its ABA level under drought. Unlike wild-type, when *aba3-1* stomata were exposed to dry air, they failed to close. Although the K^+ content of *aba3-1* guard cells decreased, it only dropped to the level of control plants before stimulation (Figure 1B). These data strongly support the suggestion that ABA is involved in the guard cell response to a drop in rh [4].

Transcriptome Analysis with Low-Humidity-Sensitive Guard Cells

To investigate stomatal responses to reduced rh, we used a transcriptomic approach based on samples enriched in intact guard cells [5, 6] that had been prepared mechanically to avoid the possible side effects of protoplasting [7]. Vital staining showed 90%–95% intact guard cells in the blended preparations, and contamination by other cell types was below 5%–10%. We tested the quality of our preparations by assessing the abundance of guard cell marker transcripts relative to those expressed in the leaf's vasculature (Table S1A). Except for *SUC2*, which is likely to be expressed in guard cells, no phloem marker genes were found enhanced in our preparations, suggesting that we have isolated fractions highly enriched in guard cells.

Our microarray studies identified 1,671 genes whose expression appears at least 2-fold enhanced in guard cells (Table S1B). Among these, we found 18 genes previously reported in a guard cell protoplast protein database [8] (Table S1C). Because transport is a major activity in guard cells [9], we classified the population of genes encoding transporters enriched in this motor cell type (Table S1D). In addition to *KAT1* (all genes are listed with the appropriate references and AGI codes in Table S1Appendix) *KAT2*, *GORK*, *SLAC1*, *SLAH3*, and others, we found ALMT12 (renamed *QUAC1*), recently identified as a component of the R-type anion channel [10].

The Response of the Guard Cell Transcriptome to Low Humidity

To identify guard cell-expressed genes, which respond to reductions in rh, we challenged leaves with dry air (20% rh) and monitored stomatal closure by the leaf turgor probe [2] (Figure 1A). The expression profiles of plants challenged by dry air and characterized by long-term stomatal closure revealed 588 differentially regulated genes (Table S1E). Within this cluster, 131 genes belong to the class of guard cell-enriched genes (Table S1F).

ABA-Regulated GC Genes

To identify guard cell genes sensitive to ABA, we sprayed plants with the drought hormone. Within the pool of guard cell-expressed genes, 1,550 appeared sensitive to ABA, with 1,080 being upregulated and 470 being downregulated (Table S1G). The latter group also includes genes found to be induced by ABA in *Arabidopsis* seedlings ([11] and

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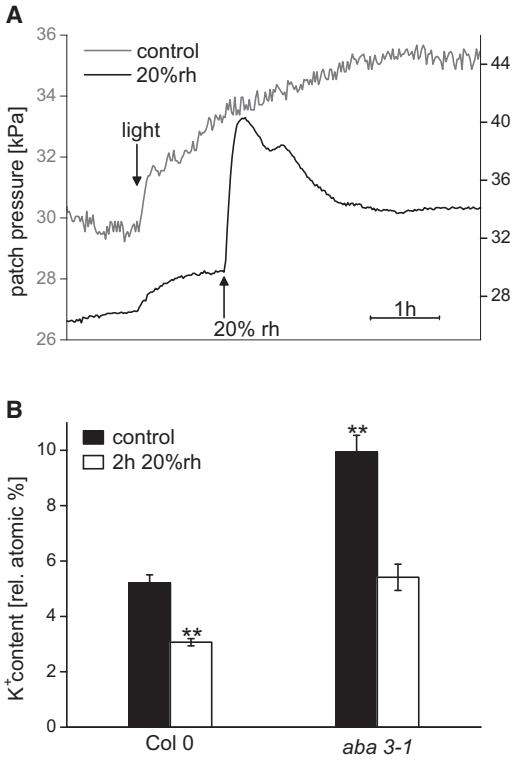

Figure 1. Stomatal Closure in Response to Dry Air

(A) Leaf turgor changes measured by a noninvasive pressure probe [2]. Stomatal opening upon illumination at 80% relative humidity (rh) is followed by stomatal closure on exposure to 20% rh.

(B) Guard cell K^+ content is dependent on rh. After experiencing a shift in rh from 85% to 35%, Col 0 and *aba3-1* exhibit a loss of K^+; *aba3-1* guard cell K^+ content was higher under control and stress conditions than WT ($n \geq 6 \pm$ SEM).

references therein). Among these, *HVA22*; the dehydrins *ERD10*, *ERD14*, *RAB18*, and *LEA*; and the protein phosphatases *HAI1*, *HAI2*, and *PP2CA* were highly induced.

The expression of ABA-regulated genes was validated by qPCR and confirmed ABA-dependent features seen initially with the microarrays (Table S1H). To monitor the time-dependent regulation of this ABA gene set, we analyzed samples 1, 2, 4, and 8 hr after stimulus onset. Following this protocol, the "ABA genes" were categorized as follows: an ABA-induced subset (Figure S1A), an ABA-repressed one (Figure S1B), and one exhibiting a more transient response to the stress hormone (Figure S1C).

Comparison with Other Guard-Cell Array Experiments

We used epidermal samples containing mature, intact guard cells together with mechanically disrupted epidermal pavement cells [5]. This approach, rather than one involving protoplasting, was chosen to avoid the expression of transcripts induced by enzymatic removal of cell walls and loss of turgor. Compared with processes requiring hours of treatment with lytic enzymes derived from fungal cell-wall preparations that very likely contain fungal elicitors [7], our automechanical approach is relatively fast (<8 min) and thus less likely to affect the guard cell transcriptome than procedures involving protoplasting. Based on meta-analysis using the guard cell protoplast versus the mesophyll protoplast transcriptome produced by Yang et al. [12], we identified 1,363 transcripts enriched at least 2-fold in the motor cell (a pool of 352 of these genes is shared by our guard cells in epidermal fragments and guard cell protoplasts derived from Yang et al. [Table S1I]). Interestingly, another guard cell data set produced by Wang et al. from guard and mesophyll cell protoplasts [13] shared only 359 guard cell-enriched transcripts with Yang et al. [12] (Table S1J). In our data set, 305 genes overlapped with those of Wang et al. [13] (Table S1K). A comparison of all three guard cell databases (this report, [12], and [13]) pointed to 194 transcripts that might be considered as guard cell-specifically expressed (Figure S1D and Table S1L). This common gene pool contains *GORK*, *SLAC1*, *MYB60*, *OST1*, *KAT1*, *KAT2*, *CER2*, *BOR5*, *RAB18*, and *QUAC1*, with all of these transcript species having been previously reported to be highly expressed in guard cells.

In previous studies, ABA treatment of guard cell protoplasts resulted in 1,363 [12] and 1,162 [13] differentially regulated genes. Interestingly, given the small differences between the two protoplast isolation procedures, comparison of the protoplast data revealed an overlap of only 189 ABA-regulated genes (Figure S1E). This could reflect differences in material preparation or transcriptomic approaches. When comparing the 1,550 (Table S1G) ABA-regulated genes identified in our "fungal-enzyme-free" and "cell-wall-free" guard cell isolation approach, 144 genes overlapped with Yang et al. [12] and 263 with Wang et al. [13]. All three data sets share 52 ABA-regulated genes (Table S1M). Taking only genes into account that were >2-fold increased in guard cells compared to the whole leaf, a common set of 29 genes could be identified (Table S1N). Within this cluster of "robust" ABA-induced genes, we found candidates that also respond to rh.

Guard Cells Are Equipped for Autonomous ABA Synthesis

One of the most striking features to emerge from our analysis of low-humidity genes was the presence of *ABA2* [4], *NCED3*, and *AAO3*, genes associated with ABA biosynthesis and ABA-induced stomatal closure [14]. We therefore asked whether guard cells are equipped to synthesize ABA autonomously. Our guard cell microarray data set included the entire ABA biosynthesis pathway (Figure 2A), suggesting that this was indeed the case. This pathway originates in the chloroplast with hydroxylation of β-carotene to zeaxanthin by BCH2 (β-carotene hydroxylase 2). *BCH2* was 3.2-fold upregulated by ABA and 2.2-fold by dry air. Zeaxanthin is converted to antheraxanthin and then to violaxanthin by ABA1 (zeaxanthin-epoxidase, ZEP) and ABA4 (neoxanthin synthase), and violaxanthin is converted to xanthoxin by NCED (9-*cis*-epoxycarotenoid-dioxygenase). In *Arabidopsis*, there are five characterized members of the NCED family (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*) [15, 16], and *AtNCED3* plays a crucial role in ABA accumulation during dehydration [17]. In our investigations, *NCED3* transcripts increased 2.3-fold in response to the drop in rh and 1.8-fold after ABA treatment. Xanthoxin is oxidized to abscisic aldehyde in the cytosol by ABA2. The last step in the pathway involves the aldehyde oxidase AAO3, known to be expressed in guard cells [18] and activated by the molybdenum cofactor sulfurase ABA3 [19, 20]. AAO3 catalyzes the conversion of abscisic aldehyde to ABA [21, 22]. In our guard cell samples, *AAO3* appeared 1.8-fold upregulated upon ABA treatment. The prestress history of plant cells is reflected in the ABA-glucoside content. Following stress, ABA is conserved by ester coupling to a sugar moiety and storage of this inactive ABA pool in the vacuole. Upon demand, the esterase BG1 cleaves the ABA

glucoside and provides active ABA [23]. The level of BG1 transcripts is low in ABA-producing Col 0 plants but is elevated in the *aba3-1* mutant, where the transcript abundance increases in response to low humidity (Figure 2B). Our results suggest that guard cells express the entire repertoire of ABA biosynthesis genes and that the abundance of their transcripts increases after exposure to ABA. This latter result suggests the existence of a positive feedback loop.

Guard Cell-Specific Expression of the *ABA3* Gene in the *aba3-1* Mutant Restores Guard Cell ABA Synthesis

Knowing that guard cells are able to produce ABA autonomously, we engineered *aba3-1* mutants with guard cell-specific ABA synthesis. We introduced the *ABA3* gene under the control of the guard cell-specific promoter of the MYB60 transcription factor [24, 25] into the *aba3-1* background (referred to as *myb60::aba3*). Using this mutant, we tested for guard cell-specific ABA production. Because ABA detection methods are not sufficiently sensitive to measure ABA content of our guard cell preparations, we monitored expression of ABA-sensitive genes. Plants of WT, *aba3-1*, and *myb60::aba3* were subjected to a drop in rh (Figure 3). We quantified the expression of the strongly guard cell-enriched and ABA-regulated gene, *HVA22*, using qPCR. Transcripts were upregulated by 20% in rh-treated guard cells of *myb60::aba3* and WT, suggesting that guard cell levels of ABA had been increased in these plants (Figure 3A). In contrast, they were not upregulated in the *aba3-1* mutant. As a marker for leaf enriched genes, we quantified the expression of *EXP8*, a gene that is strongly downregulated by ABA. The data in Figure 3B show that when WT plants were exposed to reduced rh, there was a decrease in *EXP8* transcript abundance. In contrast, there was no significant decline in *EXP8* expression in either *aba3-1* or *myb60::aba3* plants after the same treatment. These data suggest that ABA synthesis is only restored in the guard cells, rather than in cells of the leaf blade in the *myb60::aba3* plants. To further investigate whether ABA synthesis is restored in the *myb60::aba3* background, we monitored light-induced stomatal opening by

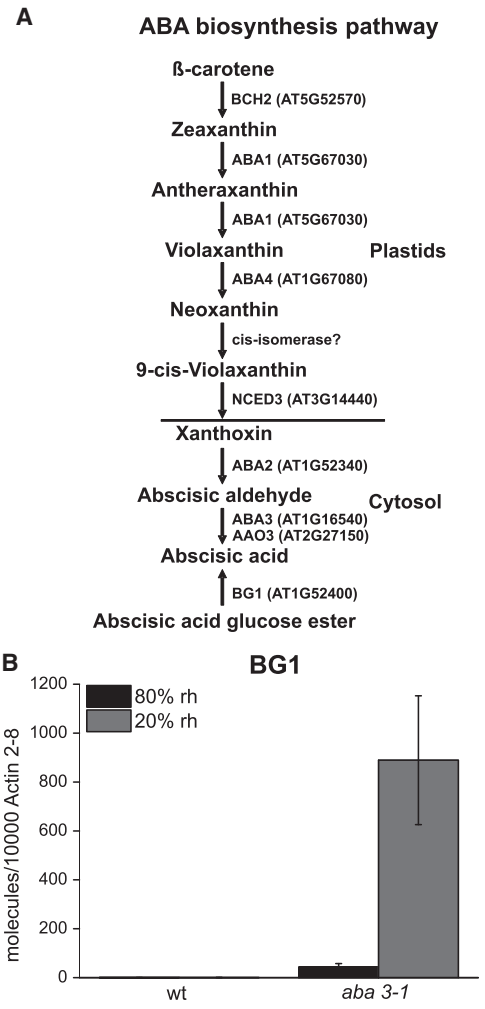

Figure 2. ABA Production in Plants

(A) ABA biosynthesis pathway. The required enzymes and the respective AGI codes are listed. The *cis*-isomerase that converts neoxathin into 9-*cis*-violaxanthin has not yet been characterized. The esterase BG1 releases ABA on demand by cleavage of ABA glucoside.

(B) Stomatal response to dry air requires ABA. Upon a drop in air humidity, WT plants close their stomata in an ABA-dependent manner (see Figure 1). *aba3-1* mutants, however, are unable to produce low-humidity-induced ABA. BG1, responsible for releasing ABA from its conjugated form, has low levels in WT guard cells but is elevated in the mutants, even under control conditions (80% ± 5% rh), and strongly increased upon a drop to 20% ± 5% rh ($n \geq 3$ ± SEM). This route might be in operation to partially compensate for lack of ABA biosynthesis. See also Figure S1.

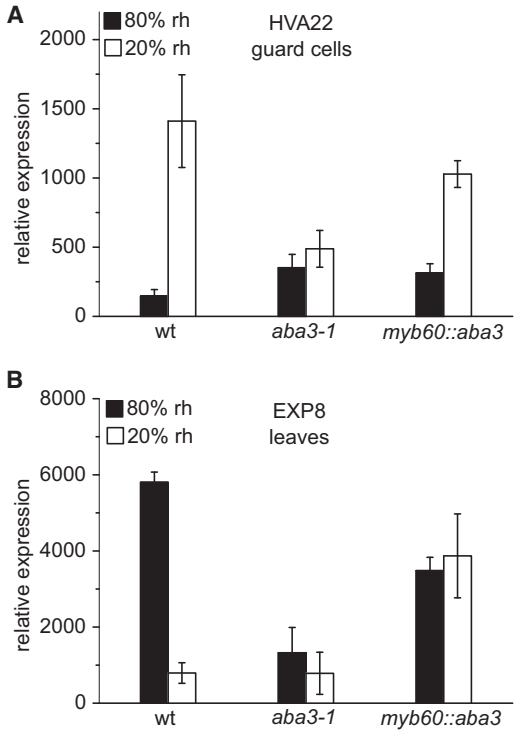

Figure 3. qPCR of ABA-Regulated Genes in Guard Cells and Whole Leaves in Response to a Drop in Air Humidity from 80% to 20%

(A) *HVA22* is enriched in guard cells and upregulated by ABA. In WT, *HVA22* transcripts increased in guard cells after a drop in rh. They did not increase in *aba3-1* guard cells but did increase in the guard cells of the *myb60::aba3-1* line.

(B) *EXP8* is enriched in leaves and downregulated by ABA. A drop in rh is followed by a strong decrease of *EXP8* expression in WT. This response was lacking in *aba3-1* as well as in *myb60::aba3* leaves. Thus, *myb60::aba3* mutant elevated ABA levels are restricted to guard cells ($n \geq 3$ ± SEM). See also Figure S2.

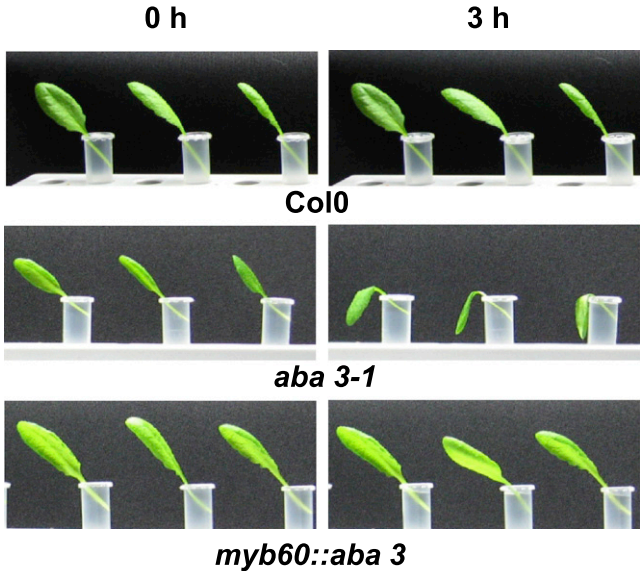

Figure 4. Complementation of the *aba3-1* Wilty Phenotype by Guard Cell-Specific ABA Synthesis

Excised leaves of *Arabidopsis* WT plants supplied with water were able to adjust their stomatal aperture when challenged for 3 hr with dry air and survived under these conditions (upper panel) as described previously [2]. *aba3-1* plants, lacking stress-induced ABA, were unable to close their stomata and wilted in consequence of excessive water loss (middle panel). ABA3 expression under the control of the guard cell-specific MYB60-promoter led to ABA synthesis in the *aba3-1* mutant background in a guard cell-specific manner and thus complemented the wilty phenotype of *aba3-1* mutants (lower panel).

infrared gas analysis (IRGA). Light-induced stomatal movement was identical to WT in the *myb60::aba3* plants (Figure S2). This indicates that, under nondrought stress conditions, *myb60::aba3* plants do not produce ABA levels sufficient to inhibit light-induced stomatal opening [26]. Interestingly, *aba3-1* plants revealed higher transpiration rates, and the IRGA measurements mirrored the K^+ levels of the *aba3-1* mutant shown in Figure 1.

The Stomatal Response to a Reduction in RH Uses ABA Synthesized in the Guard Cells

Our work suggests that guard cells have the capacity to synthesize their own ABA, so we decided to ask whether guard cell ABA is required for stomatal closure in response to dry air. To investigate this, we characterized the dry-air behavior of an *ABA3*-loss-of-function mutant and compared it with our *myb60::aba3* line, in which the capacity for guard cell-specific ABA synthesis had been restored. We subjected excised *Arabidopsis* Col 0 leaves and those of ABA synthesis mutants to low humidity (35% rh) (Figure 4), and this resulted in stomatal closure in the WT and the *myb60::aba3* line, whereas *aba3-1* wilted.

Conclusions

Understanding how stomata respond to changes in atmospheric rh has been a key challenge since the phenomenon was first described by Francis Darwin [1]. Our work and data from Okamoto et al. [27] reveal a significant role for ABA in the stomatal response to both reduced and elevated rh. We also show that stomata are capable of responding to a reduction in rh in a cell-autonomous way, and this is mediated through the highly localized production of ABA. The two ways in which this might be achieved are de novo synthesis and the release of previously conjugated ABA. Our finding that *aba3-1* mutants can be rescued by specifically expressing ABA3 in guard cells supports a role for de novo synthesis, whereas our identification of the ABA-glucose ester cleaving β-glucosidase, BG1 in guard cells of *aba3-1* and *aba3-1/myb60::aba3* mutants known to control stomatal movement in response to high humidity [23], means that it is tempting to speculate that *aba3-1* guard cells also have access to ABA via the glucose ester.

Guard cell-autonomous ABA synthesis not only allows an individual stoma to respond to changes in leaf hydration but also permits it to respond to changes in atmospheric rh and other stresses that use ABA as a signal. This in turn presents the plant with the possibility of exquisitely tuning leaf gas exchange to highly local environmental conditions. Furthermore, because our transcriptomic data are suggestive of a positive ABA-mediated feedback on ABA production, the possibilities for tightly controlled self-regulation at a highly local level over gas exchange are apparent. Our data add a new level of complexity to the overall understanding of how plants couple photosynthesis and water loss to changes in their environment.

Supplemental Information

Supplemental Information includes two figures, Supplemental Experimental Procedures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.11.022.

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