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A Guard-Cell-Specific MYB Transcription Factor Regulates Stomatal Movements and Plant Drought Tolerance

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Summary

Stomatal pores located on the plant epidermis regulate CO_2 uptake for photosynthesis and the loss of water by transpiration. The opening and closing of the pore is mediated by turgor-driven volume changes of two surrounding guard cells [1]. These highly specialized cells integrate internal signals and environmental stimuli to modulate stomatal aperture for plant survival under diverse conditions [2]. Modulation of transcription and mRNA processing play important roles in controlling guard-cell activity, although the details of these levels of regulation remain mostly unknown [3–5]. Here we report the characterization of *AtMYB60*, a *R2R3-MYB* gene of *Arabidopsis*, as the first transcription factor involved in the regulation of stomatal movements. *AtMYB60* is specifically expressed in guard cells, and its expression is negatively modulated during drought. A null mutation in *AtMYB60* results in the constitutive reduction of stomatal opening and in decreased wilting under water stress conditions. Transcript levels of a limited number of genes are altered in the mutant, and many of these genes are involved in the plant response to stress. Our data indicate that AtMYB60 is a transcriptional modulator of physiological responses in guard cells and open new possibilities to engineering stomatal activity to help plants survive desiccation.

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Results and Discussion

During drought, plants accumulate the stress-response hormone abscisic acid (ABA), which induces the rapid closing of stomata, to prevent water loss by transpiration. In guard cells, ABA triggers a signaling cascade that reduces cellular turgor by causing the efflux of K^+ and Cl^- and the removal of organic osmolytes [1]. Recently, it has been shown that, in guard cells, all the known ABA signal transducers are modulated by the hormone at the transcript level [5]. This parallel ABA regulation of stomatal activity implies the existence of a guard-cell-specific transcriptional regulatory network that helps to modulate physiological responses in stomata.

We have characterized the *Arabidopsis* transcription factor AtMYB60, whose expression is rapidly downregulated by ABA and dehydration stress (Figures 1A and 1B). RT-PCR analysis showed that *AtMYB60* is expressed in most plant organs, with the exception of roots (Figure 1C). To gain more insight into its function, we produced transgenic lines harboring a construct in which 999 base pairs (bp) of the *AtMYB60* promoter were fused to the reporter *green fluorescence protein* (GFP). GFP signals were detected only in guard cells in ten independent lines analyzed (Figures 1D–1F). We generated a second set of transgenic plants containing the complete 5′ and 3′ *AtMYB60* intergenic genomic regions, cloned upstream and downstream of the *β-glucoronidase* (*GUS*) reporter gene. A strong GUS activity was visible in stomata of seedlings and adult plants in all the lines tested (n = 10) (Figures 1G–1I). No GUS signals were detected in any other cell type or in tissues devoid of stomata, confirming the guard-cell-specific expression of *AtMYB60*. These results are in accordance with data from a microarray survey of transcript abundance in stomata *versus* mesophyll cells, in which *AtMYB60* was found to be preferentially expressed in guard cells [5].

We next tested whether, in addition to drought, other environmental stimuli that are known to modulate stomatal activity affected the expression of *AtMYB60*. Elevated CO_2 concentrations and darkness induce stomatal closure, whereas blue light promotes stomatal opening [2]. High CO_2 concentrations did not alter the relative abundance of *AtMYB60* transcripts (Figure 1J). *AtMYB60* transcript levels drastically decreased in dark-adapted plants. Exposure to white light resulted in a slow induction of *AtMYB60* expression, whereas we observed a more rapid transcript accumulation in plants exposed to blue light (Figure 1K). Blue-light responses in guard cells are regulated by the two photoreceptors PHOT1 and PHOT2 [6]. Interestingly, a micro-

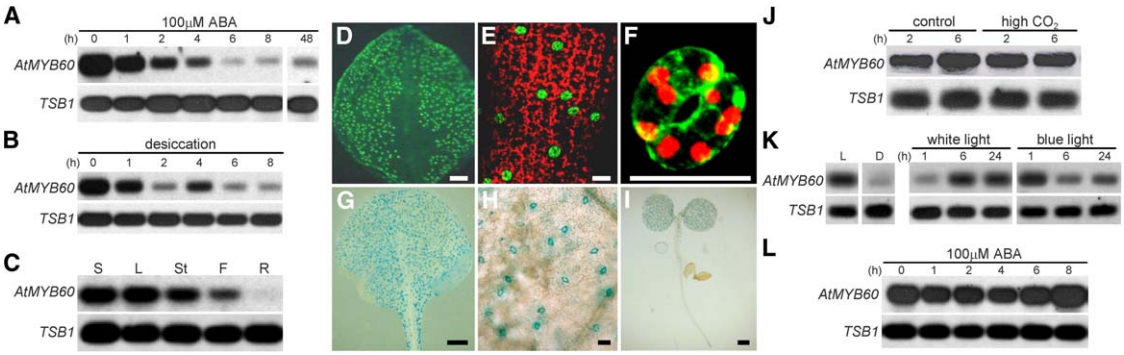

Figure 1. Analysis of *AtMYB60* Expression

(A–C) RT-PCR analysis of *AtMYB60* expression in response to ABA (A) and desiccation (B) and in different organs of plants grown under standard conditions (C). The following abbreviations are used: S, seedlings; L, leaves; St, stems; F, open flowers; and R, roots.

(D–F) Optical and confocal analyses of GFP expression in guard cells. Chlorophyll autofluorescence is shown in red.

(G–I) Histochemical localization of GUS activity in guard cells.

Scale bars in (D), (G), and (I) represent 1 mm; those in (E), (F), and (H) represent 20 μm.

(J and K) RT-PCR analysis of *AtMYB60* expression in response to high CO_2 concentration (700 mmol mol^{-1}) (J) and to white and blue light (K). The following abbreviations are used: L, plants grown under long-day conditions; D, plants adapted for 2 days in the dark.

(L) RT-PCR analysis of *AtMYB60* expression in response to ABA in the *abi1-1* mutant.

Total RNA samples were extracted at indicated time intervals, expressed in hours (h), and the *tryptophan synthase1-b subunit* (*TSB1*) gene was used as a control.

array analysis of blue-light regulation of *Arabidopsis* transcription-factor expression, in wild-type and *phot1-phot2* mutant seedlings, indicates that the blue-light-dependent upregulation of *AtMYB60* expression is not mediated by PHOT1 and PHOT2 [7]. We also analyzed the expression of *AtMYB60* in *abi1-1* plants, after the application of exogenous ABA. Guard-cell activity is impaired in the ABA-insensitive *abi1-1* mutant, and as a consequence, stomata are constitutively open, even during drought [8]. Interestingly, the ABA-induced down-regulation of *AtMYB60* expression, observed in the wild-type, was completely abolished in the *abi1-1* background (compare Figures 1A and 1L). As a whole, expression analyses show that the steady-state levels of *AtMYB60* transcript increase under conditions that promote stomatal opening (white and blue light, *abi1-1* mutation) and decrease under conditions that trigger stomatal closure, with the exception of elevated CO_2 concentrations (ABA, desiccation and dark).

We identified a mutant line (*atmyb60-1*) that harbored a T-DNA in *AtMYB60* and was inserted 3 bp from the translational start codon of the gene (Figure 2A) [9]. Segregation analysis on selective medium and Southern-blot experiments revealed the presence of a single insertion locus (not shown). No *AtMYB60* transcripts were detected in homozygous *atmyb60-1* plants, indicating complete loss of the gene function (Figure 2B). Under standard growth conditions, mutant plants did not disclose any morphological or developmental abnormalities.

Given the guard-cell-specific expression of *AtMYB60*, we tested whether stomatal responses were impaired in the mutant. Stomatal aperture measurements showed that stomata from wild-type and *atmyb60-1* plants closed to the same extent in the dark (Figure 2C). After exposure to light, mutant leaves displayed a significant reduction in the opening of stomatal pores ($p < 0.001$), indicating that a functional *AtMYB60* gene is required for light-induced opening (Figure 2C). Conversely, stomatal opening in CO_2-free air was not impaired in the mutant, suggesting that AtMYB60 does not mediate responses to CO_2 in guard cells (Figure 2D). It is important to note that the reduced stomatal aperture observed in the mutant could result from an increased sensitivity of guard cells to endogenous ABA. We performed an ABA-induced stomatal closing assay to test this hypothesis. After 3 hr of treatment with 0.5 or 10 μM ABA, wild-type and *atmyb60-1* stomatal pores displayed the same degree of reduction in their aperture, indicating that the loss of *AtMYB60* gene function does not result in ABA-hypersensitive stomatal regulation (Figure 2E). Similarly, other well-characterized responses to ABA, such as the inhibition of seed germination and the reduction of vegetative growth, were not altered in the *atmyb60-1* mutant (see Figures S1A and S1B in the Supplemental Data available with this article on line).

To test whether the defective stomatal opening in the mutant is the result of *AtMYB60* gene disruption, we introduced the corresponding wild-type genomic region in transgenic mutant plants (*C60* lines). Three independent lines disclosed wild-type responses in a light-induced stomatal-opening assay, indicating full complementation of the null allele (Figure 2F).

Because stomatal opening is impaired in the *atmyb60-1* mutant, we investigated the effects of the mutation on water loss and transpiration rate during drought. First, we measured water loss from detached wild-type and mutant rosette leaves during incubation at 21°C in the light. Throughout the duration of the desiccation treatment, *atmyb60-1* leaves consistently lost less water than wild-type leaves ($p < 0.01$ at 2 and 8 hr, $p < 0.001$ at 4 hr) (Figure 3A). To estimate whole-plant transpiration under stress conditions, we determined the relative water content (RWC) [10] of wild-type and

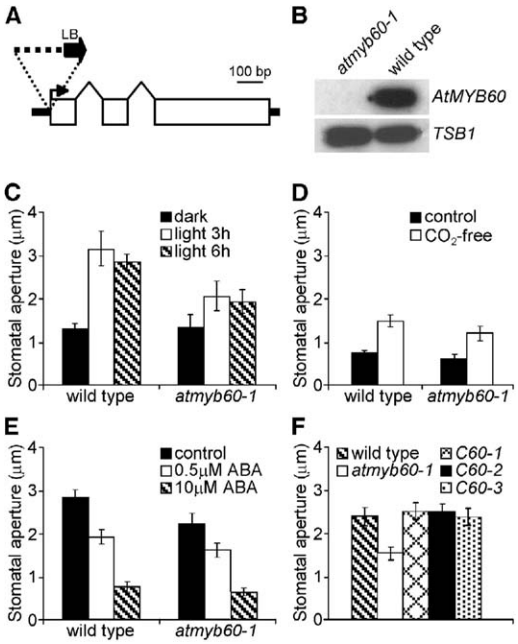

Figure 2. Light-Induced Stomatal Opening Is Impaired in the *atmyb60-1* Mutant

(A) Gene structure of the T-DNA-induced *atmyb60-1* mutation. The location of the ATG start codon is indicated (black arrow). The position and orientation of the T-DNA left border (LB) fragment, as determined by DNA sequencing, is shown.

(B) RT-PCR analysis confirmed the absence of the *AtMYB60* transcript in homozygous *atmyb60-1* plants. The *TSB1* gene was used as a control.

(C–E) Stomatal aperture measurements in wild-type and *atmyb60-1* epidermal strips determined after 3 and 6 hr of exposure to light (C), after 6 hr in a CO_2-free environment (D), or after 3 hr of treatment with 0.5 or 10 μM ABA (E).

(F) Comparison of stomatal aperture in leaves of wild-type, *atmyb60-1*, and complemented plants (*C60-1*, *-2*, *-3*), after 3 hr of exposure to light.

Each bar indicates the mean of three separate experiments (n = 80 stomata per bar), with standard errors.

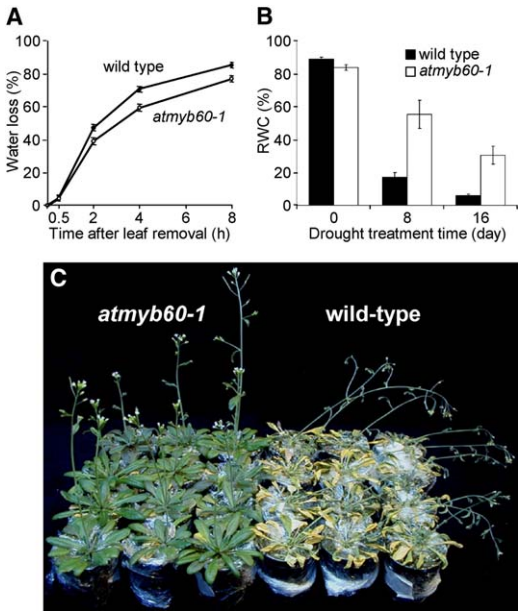

Figure 3. Increased Tolerance of *atmyb60-1* Plants to Desiccation

(A) Time course of water loss from excised leaves, expressed as a percentage of the initial fresh weight at indicated intervals. Each point indicates the mean of 12 measurements with standard errors.

(B) Changes in RWC of wild-type and *atmyb60-1* plants during drought. Plants grown under normal watering conditions for 24 days were drought stressed by complete termination of irrigation. Each bar indicates the mean of 20 measurements with standard errors.

(C) Representative wild-type and mutant plants 8 days after the cessation of irrigation.

atmyb60-1 plants after cessation of irrigation. RWC is a good indicator of the plant water status at any given time because it closely reflects the balance between water supply and transpiration rate [11]. Individual plants, grown in soil, were regularly watered for 24 days and then subjected to drought stress by complete termination of irrigation. Transpirational water loss, as determined by RWC measurements after 8 and 16 days from the start of the treatment, was greatly reduced in the mutant compared to the wild-type ($p < 0.001$) (Figure 3B). Consistent with RWC data, after 8 days of desiccation, wild-type plants showed severe wilting and chlorosis of rosette leaves, whereas *atmyb60-1* plants were turgid and their leaves remained green (Figure 3C). These results indicate that the reduced stomatal-pore aperture, induced by the *atmyb60-1* mutation, helps to limit water loss during drought and thus enhances plant tolerance.

Finally, we performed a microarray hybridization experiment to investigate the effects of the *AtMYB60* gene disruption on global gene expression. Total mRNAs, derived from *atmyb60-1* and wild-type rosette leaves were hybridized to a cDNA microarray [12]. Among the 6,863 genes represented on the array, only 36 disclosed significant differences in transcript accumulation in the mutant ($p < 0.0001$), suggesting specificity of AtMYB60 in regulating the transcription of a limited number of genes (Table 1). We confirmed the microarray expression data for 14 selected genes by RT-PCR analysis (Figure S2). The upregulation in *atmyb60-1* of the aquaporin *delta-tonoplast intrinsic protein* gene (*δ-TIP*) is of particular interest. Aquaporins modulate water transport across membranes and are highly expressed in cells associated with large fluxes of water, such as guard cells [13]. We found, among the transcripts downregulated in the mutant, genes involved in the plant response to dehydration (i.e., *erd10*, *erd13*) [14, 15] and to pathogen attack (i.e., *NHL3, Syp122,* and *Vpeγ*) [16–18], as well as activators and repressors of transcription known to modulate gene expression during both abiotic and biotic stress (i.e., *ERF* and *ZAT* genes) [19–22]. Evidence indicates a high degree of convergence of stomatal signaling pathways, which are induced by water stress, pathogenic elicitors, and hormonal responses [23]. Based on microarray expression data, it is intriguing to speculate that AtMYB60 could integrate multiple signal-transduction processes by modulating the expression of genes involved in different guard-cell responses.

Table 1. Microarray Gene Expression Analysis of Wild-Type and *atmyb60-1* Leaves

Clone ID	Gene ID	Description	Fold change	p value
205171	At2g20670	Expressed protein, unknown function	+ 2.55*	2.72 e-05
204478	At3g52780	Purple acid phosphatase (PAP20)	+ 2.47	8.59 e-09
202808	At3g16240	Delta tonoplast integral protein (δ-TIP)	+ 2.39*	3.87 e-07
207793	At1g33860	Hypothetical protein	+ 2.27	2.07 e-09
201818	At2g06950	Copia-like retrotransposon family	+ 2.06	4.82 e-09
207032	At5g19120	Expressed protein, unknown function	+ 2.00	3.50 e-05
200755	At4g22710	Cytochrome P450-like protein (CYP706A2)	−2.03	4.03 e-06
205228	At4g32020	Expressed protein, unknown function	−2.04	3.40 e-06
201462	At1g20450	Dehydrin ERD10	−2.12*	4.24 e-06
204765	At1g73600	Related to phosphoethanolamine N-methyltransferase	−2.14	3.77 e-06
201493	At4g38550	Putative phospholipase	−2.15	6.67 e-05
205791	At5g61600	AP2-domain transcription factor, similar to AtERF5	−2.16	6.59 e-08
200958	At5g59820	Putative zinc-finger protein (C2H2 type) ZAT12	−2.18*	2.20 e-05
201362	At1g14880	Expressed protein, unknown function	−2.21	1.72 e-06
204798	At4g31500	Cytochrome P450 83B1 (CYP83B1)	−2.33	6.47 e-05
205931	At1g21130	O-methyltransferase 1 putative	−2.33	1.74 e-08
200505	At2g30870	Glutathione S-transferase (ERD13)	−2.39	2.82 e-07
200131	At5g06320	NDR1/HIN1-like protein 3 (NHL3)	−2.41	6.20 e-07
202775	At1g27020	Expressed protein, unknown function	−2.43	1.52 e-07
204094	At1g73500	Mitogen-activated protein kinase kinase, putative MKK9	−2.46	1.96 e-09
205066	At2g26560	Similar to patatin-like latex allergen	−2.62	2.09 e-09
203761	At2g40100	Lhcb4:3 protein (light-harvesting chlorophyll-binding)	−2.68	3.03 e-10
203600	At3g46620	Similar to RING-H2 finger protein RHC2a	−2.68	5.52 e-12
240366	At3g15210	Ethylene-responsive element-binding factor 4 (AtERF-4)	−2.72	9.57 e-07
205714	At2g40000	Expressed protein, unknown function	−2.83	2.86 e-08
200912	At4g27280	Calcium-binding EF hand family protein	−2.83*	7.72 e-08
201356	At1g57990	Purine permease-related	−2.86*	6.77 e-05
206997	At4g17490	Ethylene-responsive element-binding factor 6 (AtERF-6)	−2.94*	2.75 e-05
206894	At4g02380	Late embryogenesis abundant protein homolog (SAG21)	−2.98*	8.21 e-05
208064	At4g24570	Mitochondrial substrate carrier family protein	−3.00*	3.94 e-07
205814	At4g32940	Putative vacuolar processing enzyme gamma-VPE	−3.21	2.24 e-06
202443	At5g47220	Ethylene-responsive element-binding factor 2 (AtERF-2)	−4.02*	9.72 e-05
204738	At3g52400	Syntaxin, putative (SYP122)	−4.29*	2.55 e-05
203517	At1g07135	Glycine-rich protein, unknown function	−4.47*	2.66 e-07
240892	At1g27730	TFIIIA-type zinc finger protein (ZAT10)	−5.10*	1.60 e-08
204291	At4g29780	Expressed protein, unknown function	−5.28*	6.59 e-06

Clone ID refers to the unique ID for the array feature; gene ID and description correspond to gene designation and annotation obtained from TAIR (www.arabidopsis.org). Asterisks indicate genes for which microarray expression data have been independently confirmed by RT-PCR (Figure S2). A complete dataset is available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/query/entry) under accession number E-MEXP-225.

The engineering of stomatal responses to reduce water loss is an attractive approach to enhancing drought tolerance in crops [24]. We have shown that light-induced stomatal opening is impaired in the *atmyb60-1* mutant and that mutant plants are more resistant to dehydration than are wild-type plants. Our hypothesis is that the lack of *AtMYB60* transcripts in the mutant is perceived by the guard cell as a signal that triggers a stress response producing long-term beneficial effects during drought. Importantly, the *atmyb60-1* mutation results in guard-cell-specific defects without affecting other developmental and physiological processes. Our findings indicate that the identification and modulation of a stomata-specific transcription factor opens new possibilities to improve crop survival and productivity during drought.

Supplemental Data

Supplemental Experimental Procedures as well as supplemental figures and tables are available with this article online at http://www.current-biology.com/cgi/content/full/15/13/1196/DC1/.

Acknowledgments

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