A Dof Transcription Factor, SCAP1, Is Essential for the Development of Functional Stomata in Arabidopsis

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Summary

Stomata are highly specialized organs that consist of pairs of guard cells and regulate gas and water vapor exchange in plants [1–3]. Although early stages of guard cell differentiation have been described [4–10] and were interpreted in analogy to processes of cell type differentiation in animals [11], the downstream development of functional stomatal guard cells remains poorly understood. We have isolated an Arabidopsis mutant, stomatal carpenter 1 (scap1), that develops irregularly shaped guard cells and lacks the ability to control stomatal aperture, including CO2-induced stomatal closing and light-induced stomatal opening. SCAP1 was identified as a plant-specific Dof-type transcription factor expressed in maturing guard cells, but not in guard mother cells. SCAP1 regulates the expression of genes encoding key elements of stomatal functioning and morphogenesis, such as K+ channel protein, MYB60 transcription factor, and pectin methylesterase. Consequently, ion homeostasis was disturbed in scap1 guard cells, and esterification of extracellular pectins was impaired so that the cell walls lining the pores did not mature normally. We conclude that SCAP1 regulates essential processes of stomatal guard cell maturation and functions as a key transcription factor regulating the final stages of guard cell differentiation.

Results and Discussion

We isolated stomatal carpenter 1 (scap1) as a mutant impaired in CO2-dependent leaf temperature change from an M2 population of ethyl methanesulfonate-mutagenized Arabidopsis plants, using thermography [12]. In this mutant, the typical changes of stomatal conductance that occur in wild-type (WT) plants in response to CO2 (Figure 1A) and light (Figure 1B) were inhibited. The mutant was defective also in the regulation of transpiration in response to drought stress (Figure 1C). A subset of stomata in this mutant appeared morphologically abnormal (Figure 1D), indicating a disruption in pore morphogenesis. In particular, the ventral cell walls, which form the inner surface of the pore, appeared floppy and seemed to remain adhered in mature stomata. This phenomenon was observed in approximately 50% of the stomata examined. Although the remaining 50% of total stomata appeared normal morphologically, all stomata of the scap1 mutant probably lack the ability to control stomatal aperture, because the scap1 mutant was completely insensitive to changes in CO2 concentration and light intensity (Figures 1A and 1B). To clarify the timing of morphological defects occurring during stomatal development, we investigated the morphology of stomatal lineage cells from meristemoids to mature guard cells. The morphological defects occurred after guard mother cells were divided to form young stomata (Figure 1E), suggesting that SCAP1 is a late-acting gene in guard cell differentiation.

By map-based cloning, we identified the SCAP1 gene as At5g65590, which encodes an uncharacterized DNA binding with one finger (Dof) transcription factor (Figure 2A; see also Figure S1A available online). The scap1 mutation possesses a single C-to-T nucleotide substitution, causing an R65-to-C exchange in the Dof domain (Figure 2A) that is required for DNA binding [13]. Thus, scap1 probably is a loss-of-function allele. Introduction of the SCAP1 open reading frame with its native promoter into scap1 plants fully restored the WT phenotype, confirming that At5g65590 is SCAP1 (Figure S1B). We also confirmed that SCAP1 RNAi plants exhibited similar phenotypes to the scap1 mutant (Figures S1C–S1F). To examine promoter activity and the localization of the gene product, we used the native SCAP1 promoter to drive expression of the GUS reporter and the translational fusion of a full-length SCAP1 protein and GFP (SCAP1-GFP). The latter construct complemented the scap1 phenotype, indicating that the SCAP1-GFP fusion protein was functional (Figure S1B). GUS expression driven by the SCAP1 promoter was highest in guard cells (Figure 2B). The SCAP1-GFP fusion protein was localized in the nuclei of guard cells (Figure 2C). Arabidopsis guard cells develop via three stages of asymmetric and symmetric cell divisions [9, 10]. Passage from one stage to the next is promoted by SPCH (asymmetric entry division) [5], MUTE (meristemoid to guard mother cell) [6], and FAMA/FLP (guard mother cell to guard cells) [7, 8]. No GFP signal was detected in meristemoids, guard mother cells, or recently divided guard cells (Figure 2D; Figure S2), indicating that SCAP1 is not involved in the early stages of guard cell differentiation. The timing of SCAP1 expression paralleled that of SLAC1, an S-type anion channel that plays an essential role in the regulation of stomatal closure [12, 14]. These findings suggest that SCAP1 acts as a transcription factor that controls guard cell maturation and the achievement of full functionality.

Dof factors are plant-specific transcription factors with functions in a variety of physiological contexts [13], and guard cell-specific expression of a K+ channel protein gene was mediated by Dof-binding consensus sequences in its promoter region [15]. Consequently, an unidentified Dof factor, or factors, was proposed to be involved in guard cell-specific gene expression [16–19]. We therefore investigated the role of
SCAP1 in guard cell-specific gene expression by microarray experiments. We selected 1,540 genes that are expressed in guard cells, but not in mesophyll cells [16], and compared their expression levels in scap1 and WT guard cells (Figure 3A; Table S1). The scap1 mutation resulted in decreased expression of a number of genes, including genes for several factors directly involved in stomatal opening and closure: GORK, an outward K⁺ channel protein [20]; PYL2, a regulatory component of ABA receptor 2 [21, 22]; and MYB60, an essential transcriptional regulator for guard cell movements [23]. Thus, SCAP1 is not a mere transcription factor for guard cell-specific expression of a single gene but probably a key factor for guard cell function.

The results of a dual-luciferase transient reporter assay revealed that, in guard cell protoplasts (GCPs), SCAP1 activates the GORK and MYB60 promoters (Figure 3B), which have several potential Dof-binding sites (T/A-AAAG) (Figure 3C). Furthermore, in a chromatin immunoprecipitation experiment...
(ChIP) assay using a functional SCAP1-FLAG fusion protein expressed from a genomic fragment (Figure S1B), we observed robust enrichment of GORK and MYB60 promoter fragments, including Dof-binding sites (Figure 3D). These results indicated that SCAP1 directly binds and then activates the GORK and MYB60 promoters. We also showed that SCAP1 activated the GORK and MYB60 promoters not only in GCPs but also in mesophyll protoplasts (Figure S3A), suggesting that expression of SCAP1 alone may be sufficient to induce expression of its target genes during stomatal maturation. Consistent with the phenotype of the scap1 mutant, these results suggest that SCAP1 is a direct regulator for the genes essential for guard cell function.

Interestingly, the expression of genes controlling cell wall architecture was also altered by the scap1 mutation (Figure 3A). In scap1 guard cells, the expression of PME6, which encodes a pectin methylesterase (PME), was repressed particularly strongly, whereas expression of the pectin methylesterase inhibitor gene was enhanced. Cells secrete pectin as a fully methylesterified polymer that is demethylesterified extracellularly by PME (EC 3.1.1.11) [24]. The demethylesterified polymer can form Ca$^{2+}$ bridges between individual pectin molecules that tend to stiffen the wall [25, 26]. We investigated differential demethylesterification of pectins in the scap1 mutation using two monoclonal antibodies, JIM5 and JIM7, for the differential detection of methylesterified pectins. JIM7 binding was detected in ventral walls of scap1 guard cells, but not in the WT (Figure 4A). By contrast, JIM5 staining was similar in mutant and WT and was not restricted to the ventral walls (Figure 4A). Thus, the demethylesterification of
Figure 3. SCAP1 Is a Transcription Factor that Regulates Guard Cell-Specific Genes

(A) Relative expression levels of 1,540 stomatal genes that are induced or repressed by the scap1 mutation based on microarray data. By qRT-PCR analysis (Table S2), we confirmed that expression of GORK, MYB60, and PME6 was repressed strongly in scap1. Expression levels were normalized against the UBQ10 expression as an internal control. Values shown are means ± SE (n = 4).

(B) SCAP1 regulates GORK and MYB60 promoter activity in a transient assay. The pGORK::LUC or pMYB60::LUC reporter plasmid and the 35S::SCAP1 or 35S::SCAP1(m) effector plasmid were cotransfected into guard cell protoplasts. SCAP1(m) represents mutated SCAP1 that has a scap1 mutation (R65S, Figure 2A). The empty vector (pBI221) served as a control. Firefly luciferase (luc) activity was normalized against the activity of Renilla (legend continued on next page).
pECTs seemed to be suppressed in the ventral walls of scap1 guard cells, suggesting that SCAP1 is involved in the control of guard cell wall mechanical properties. Increased highly methylesterified content in the ventral walls suggested a lower abundance of intermolecular crosslinking in the pectin fraction of the wall, possibly resulting in a flaccid, less sturdy wall. This interpretation, which is in line with the aberrant appearance of the unusual stomata in the scap1 mutant (Figure 3A; Figure S3B), could provide an explanation for the reduced efficiency of stomatal function observed in the mutant. In WT guard cells, ventral walls are less extensible than other cell wall portions, which forces them to bend outward when the cell expands reversibly under high turgor and results in the opening of the stomatal pore. An increased elastic extensibility of the scap1 ventral walls, which may be induced by an increased fraction of noncrosslinked pectins, could render this biomechanical machinery ineffective. Because we did not detect transactivation of the 2 kb PME6 promoter by SCAP1 in our transient reporter assays, we conclude that SCAP1 might regulate PME6 expression through interactions with motifs in the region outside of the promoter, such as the far upstream sequence and introns. Alternatively, SCAP1 might affect PME6 expression through regulating expression of an additional transcription factor or factors. In the pme6 mutant, we did not detect any notable stomatal morphological defects, but stomatal CO2 sensitivity was lower by 18% and light sensitivity was lower by 34% compared to WT (Figures S4C and S4D, p < 0.05). These phenotypes were weaker than the scap1 mutant. A possible explanation for this result is that SCAP1 affects the expression of multiple factors involved in stomatal functioning (Figure 3A; Figure S3B), so that scap1 mutant phenotype cannot be explained by a defect in a single component.

We examined whether the scap1 mutation also affects ion balance in guard cells. To avoid any possible effects of abnormal scap1 cell walls, we prepared GCPs. The WT GCPs showed the well-known swelling response when illuminated, but scap1 GCPs did not (Figure 4B). In accordance with this finding, the usual light-induced accumulation of inorganic and organic ions was not observed in scap1 GCPs (Figure 4C). These results indicated that SCAP1 is required for ion homeostasis in guard cells, a result consistent with the decreased expression of genes directly involved in ion homeostasis (data not shown), so a restricted expression pattern luciferase derived from an internal control plasmid. Values shown are means ± SE (n = 4). Asterisks indicate significant differences compared to the control at p < 0.05.

(C) Putative Dof-binding sites on the plus (top) and minus (bottom) strand of the upstream regions of the GORK and MYB60 genes. Thick lines indicate positions of fragments amplified in (D).

(B and C) Guard cell protoplasts (GCPs) were isolated from leaves of WT and scap1 plants. GCP volumes (B) and organic and inorganic ion levels (C) were quantified after incubation with or without white light (80 μmol m⁻² s⁻¹) for 1 hr. Values shown are means ± SE (n > 3). Asterisks indicate significant differences compared to the control values (–Ab) at p < 0.05.
for SCAP1 (Figures 2B–2D) may be important for proper SCAP1 functioning.

In summary, SCAP1 is a Dof-type transcription factor expressed during the late stage of guard cell differentiation (Figure 2). A mutation in SCAP1 impairs stomatal opening and closing (Figures 1A–1C) and represses the expression of genes involved in stomatal movement (Figure 3A; Figure S3B). SCAP1 also functions as a transcriptional activator that directly induces GORK and MYB60 expression (Figures 3B–3D). Furthermore, SCAP1 influences essential biomechanical parameters, as demonstrated by the modified cell wall structure (Figure 4A) and the disturbed ion homeostasis in scap1 guard cells (Figure 4C). Thus, our findings suggest that SCAP1 is a key transcription factor that controls the final stage of guard cell differentiation by regulating the expression of multiple genes responsible for stomatal maturation and function. Further study of SCAP1 will pave the way to a better understanding of processes essential for stomatal maturation and provide an opportunity to engineer stomatal function.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers in Table S1 or as follows: SCAP1, At5g65590; GORK, At5g37500; MYB60, At1g08810; PME6, At1g23200; SLAC1, At1g12480. The microarray data set is deposited in the Gene Expression Omnibus (GEO) with accession number GSE43964.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.001.

Acknowledgments

We thank L.G. Smith for critical reading of the manuscript. We also thank N. Kawahara and Y. Johno for technical assistance. This research was supported by Grants-in-Aid for Scientific Research on Innovative Areas 21114002 (K.I.), 21114004 (S.Y.), and 22380043 (S.Y.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry (K.I.); by the CREST program from the Japan Science and Technology Agency (S.Y.); by National Institutes of Health grant R01GM060396; by National Science Foundation grant MCB0916220 (J.J.S); and by the Mitsubishi Foundation (K.I.).

References