Part II

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***STOMATAL CARPENTER 1* is expressed in leaf primordia and controls stomata specification by promoting SPEECHLESS protein accumulation**

Title

STOMATAL CARPENTER 1 is expressed in leaf primordia and controls stomata specification by promoting SPEECHLESS protein accumulation

Authors

Giulia Castorina, Samantha Fox, Chiara Tonelli, Massimo Galbiati and Lucio Conti.

Affiliations

GC, CT, MG and LC Dipartimento di Bioscienze, Università degli studi di Milano,

Via Celoria 26, 20133 Milano, Italy

SF, Cell and Developmental Biology Department, John Innes Centre, Norwich, NR4 7UH, UK

Correspondence

Massimo Galbiati Massimo.Galbiati@unimi.it and Lucio Conti Lucio.Conti@unimi.it

Ph +39.02.50315010, fax +39.02.50315044

Other contacts

Giulia.Castorina@unimi.it

samantha.fox@jic.ac.uk

Chiara.Tonelli@unimi.it

Running Title

Role of SCAP1in the control of GCs patterning

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SUMMARY

Guard cells (GCs) are specialised epidermal cells which form stomata pores, through which gas exchange can occur. The first committed state of the stomatal lineage are small triangular cells called meristemoids that originate after an asymmetric division of a meristemoid mother cell (MMC). *SPEECHLESS* (*SPCH*) is a master regulator of GC identity and its expression can be detected in protodermal and MMCs and is subsequently restricted to the meristemoids. Little is known about the transcriptional events which precede GC specification and are required for correct GC patterning. The DOF transcription factor *STOMATAL CARPENTER 1* (*SCAP1*) was previously shown to be involved in GC function, by activating a set of GC–specific genes required for GC maturation and activity. Here we show that *SCAP1* expression can also be observed in young leaf primordia, before any GC differentiation occurs. The study of transgenic plants carrying a *proSCAP1:GUS-GFP* transcriptional fusion coupled with qPCR analyses indicate that *SCAP1* expression is maximal in a stomatal lineage competence domain, coincident with *SPCH* expression. Independent *scap1* loss-of-function mutants have a reduced number of GCs whilst *SCAP1* over expression lines have an increased number of GCs in addition to altered GC distribution and spacing patterns. Confocal imaging of SPCH-GFP protein in a background carrying inducible *SCAP1* shows that SCAP1 activation results in an increased number of nuclei expressing SPCH-GFP. Our results suggest an early role for *SCAP1* in GC differentiation through SPCH protein stabilization. *SCAP1* may thus link different aspects of GC biology including specification, maturation and function.

INTRODUCTION

Guard cells (GCs) are specialised epidermal cells which form stomata pores, through which gas exchange can occur. Since transpiration is linked to plant growth and survival, control of GC number, distribution and activity is tightly regulated. Mature GC pairs form in the epidermal cell layer and originate from a single undifferentiated protodermal cells (PDC), which undergoes a series of cell divisions and successive cell-state transitions. These transitional states are characterized by changes in cell morphology, and associated with alterations in transcriptomic signature (Dong *et al.*, 2009; Pillitteri and Dong, 2013; Geisler *et al.*, 2000). A subset of PDCs (termed meristemoid mother cells – MMCs) becomes competent to initiate the stomatal cell lineage. The first specialized cells of the stomatal cell lineage are small triangular cell called meristemoids that originate after an asymmetric division of MMCs. These divisions produce a larger cell called a stomatal-lineage ground cell (SLGC) that has the potential to divide asymmetrically to produce satellite meristemoids or differentiate into a lobed pavement cell. New meristemoids are oriented at least one cell away from existing stomatal precursor according to a one-cell-spacing rule (Geisler *et al.*, 2000; Shpak *et al.*, 2005; Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt and Gray, 2009). After some rounds of amplifying divisions meristemoids mature into a guard mother cell (GMC) acquiring the distinct rounded shape A GMC divides symmetrically to generate two paired guard cells, which form the stomata pore. The genes responsible for GCs specification and development have been characterised. The bHLH-type transcription factors (TFs) *SPEECHLESS* (*SPCH*), *MUTE*, and *FAMA* act sequentially to regulate formation of meristemoids, GMCs and GCs, respectively (Ohashi-Ito and Bergmann, 2006; MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007). Alongside the afore-mentioned genes, another class of bHLH–type TFs, *SCREAM/ICE1* and *SCREAM2* redundantly affect the activities of *SPCH*, *MUTE* and FAMA through heterodimerization (Kanaoka *et al.*, 2008). *SPCH* is a master regulator of GCs identity (by inducing an asymmetric division of a MMC) and GCs distribution across the epidermis. *SPCH* activity is required for the transition from

PDC to MMC since *spch* mutants fail to initiate a GC lineage. *SPCH* is also necessary to promote amplifying divisions of the meristemoids (MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007; Robinson *et al.*, 2011). Experiments utilising *SPCH* promoter-reporter transcriptional fusions revealed that *SPCH* is expressed in the developing leaf epidermis and persists in the stomatal lineage (GMC and GCs). The SPCH protein has only been detected in undifferentiated PDCs and MMCs, suggesting that *SPCH* is regulated at the post-transcriptional level (MacAlister *et al.*, 2007). The activity of SPCH protein is negatively regulated by a signalling cascade, which includes secreted peptides EPIDERMAL PATTERNING FACTORS 1 and 2 (EPF1/2), leucine-rich repeat (LRR) receptor-like kinases ERECTA and TOO MANY MOUTHS (Hara *et al.*, 2007; Hunt and Gray, 2009; Hunt *et al.*, 2010; Geisler *et al.*, 2000; Shpak *et al.*, 2005; Lee *et al.*, 2012). The *MITOGEN ACTIVATED KINASE* (*MAPK*) genes act downstream of the LRR receptors and include *YODA*, *MKK4*/*MKK5* and *MPK3*/*MPK6* (Lampard *et al.*, 2008; Lampard *et al.*, 2009; Bergmann *et al.*, 2004). Stimulation of MAPK results in SPCH phosphorylation and inactivation by proteasomal degradation (Jewaria *et al.*, 2013; Lampard *et al.*, 2008).

Besides the EPFs, LRR receptors and the MAPKs cascade other pathways play a role in SPCH activity and accumulation. An increase in brassinosteroid (BRs) signalling leads to SPCH phosphorylation independent of the MAPKs, a process involving the kinase BR INSENSITIVE 2 (Gudesblat *et al.*, 2012). Therefore, modulation of SPCH activity translates multiple environmental and endogenous developmental signals into different GCs patterns (Wang *et al.*, 2007).

Besides bHLHs, other transcription factors may play an important role in GCs specification. The DNA BINDING WITH ONE FINGER (DOF) proteins are an important class of transcriptional regulator in *Arabidopsis thaliana* comprising 37 members (Riechmann *et al.*, 2000). These proteins have been shown to be involved in several aspects of plant development including growth, germination and abiotic stress response (Yanagisawa, 2002). Also, DOF-type factors are implicated in cell cycle control (Skirycz *et al.*, 2008). In stomata development DOFs have been hypothesized

to play a role in GCs maturation (Galbiati *et al.*, 2008; Cominelli *et al.*, 2011). Recently the DOF transcription factor *SCAP1* has been shown to directly regulate essential processes related to guard cell maturation and function. Mutants of *scap1* show transcript alterations in multiple genes directly involved in stomatal movement and furthermore are defective in some cell wall mechanical properties of GCs (Negi *et al.*, 2013). The potential role of *SCAP1* in stomata patterning has not previously been investigated. In this work we provide evidence that *SCAP1* plays a key role in GC patterning, and that this is temporally and spatially distinct from its role in GCs maturation. At early developmental stages *SCAP1* is expressed when undifferentiated protodermal cells acquire the ability to initiate GCs lineage as a result of *SPCH* activation. The *SCAP1* promoter is active both in the epidermis and the mesophyll, although SCAP1 protein is quickly turned over in the epidermis. At later developmental stages, *SCAP1* is observed only in GCs. Mutants of *scap1* have significantly reduced stomatal density and stomatal index compared with wild type. Conversely, over expression of *SCAP1* results in increased stomatal parameters coupled with repression of the SPCH negative regulator *EPF2*, but not *EPF1* genes. Detailed confocal images of *proSPCH:SPCH-GFP* provide evidence that that *SCAP1* promotes SPCH protein accumulation. Our work thus extends previous data to demonstrate a novel role for *SCAP1* in GC patterning by affecting SPCH activity.

RESULTS

SCAP1 is activated in leaf primordia before GCs specification

To further elucidate the role of *SCAP1* in stomata development we characterised a *scap1* transposon insertion mutant publicly available in the Cold Spring Harbour collection. This allele (dubbed *scap1-2*) carries a gene trap construct, which allows identifying endogenous patterns of expression of the trapped gene. The *scap1-2* allele was likely a null since it did not produce any detectable full-length *SCAP1* transcript. Previously, a *proSCAP1:GUS* transcriptional fusion was used to show that *SCAP1* is preferentially expressed in GCs although a diffuse GUS staining was detected in young

leaves (Negi *et al.*, 2013). To confirm these observations we looked at the localisation of GUS staining the *scap1-2* background at different developmental stages (Figure 1). We visualised two distinct expression profiles during leaf development. At early developmental stages GUS staining was present throughout the emerging leaf primordia (Figure 1a), when a leaf consists of few undifferentiated cells and no GCs. At later developmental stages, levels of GUS were highest at the flanks of the lamina – similarly to the pattern of expression of *SPCH* (Pillitteri *et al.*, 2007) – and much reduced in the midvein region (Figure 1b). In mature organs (i.e. leaves and cotyledons) the diffuse GUS staining disappeared and GUS signal was mainly confined to maturing GCs (Figure 1c,d). However GCs–specific *SCAP1* expression was very faint in *scap1-2* mutants and appeared later in development compared with transgenic *proSCAP1:GUS-GFP* lines (Figure 1d) (see below). The *scap1-2* mutant carries a *GUS* reporter gene in antisense orientation with respect to the *SCAP1* open reading frame (Figure S1). To verify that the GUS pattern observed in the *scap1-2* allele reflects endogenous *SCAP1* promoter activity we fused a 2977 bp genomic region upstream of the *SCAP1* coding sequence to *GUS* and *GFP* and generated independent Arabidopsis stable transformants. These transgenic plants (*proSCAP1:GUS-GFP*) displayed GUS activity in young leaf primordia which was similar to that observed in *scap1-2* plants (Figure 1e). At later stages of development, the pattern of GUS accumulation in the *proSCAP1:GUS-GFP* lines was broadly similar, with the one observed in the *scap1-2* line. Coincident with the expansion of leaf primordia GUS staining gradually disappeared in the midvein region (Figure 1f, g), but in comparison to *scap1-2*, GUS staining decreased more quickly at the periphery of the leaf lamina. In young leaf primordia *SCAP1* promoter activity was stronger in the part of the leaf where GCs are differentiating and much reduced in the maturation zone of GCs. This observation was confirmed by analysing transversal sections of GUS stained *proSCAP1:GUS-GFP* plants. At early stages of primordia differentiation, the *SCAP1* promoter was uniformly active in the mesophyll and the epidermis of leaf primordia (Figure 1i). Subsequently we observed a sharp proximal to distal

gradient of GUS accumulation, with increased promoter activity in the proximal part of the leaf primordia (Figure 1j). At this stage, GUS expression in GCs was strong although GCs–specific expression tended to decrease in a distal to proximal gradient, resembling the pattern of stomata differentiation (Figure 1l).

SCAP1 regulates GCs development

To further investigate the role of *SCAP1* in stomata development we compared the number of GCs in adult leaves of *scap1-2* mutants with that wild type. In *scap1-2* stomatal density is reduced (Figure 2a) but this was not reflected in a reduction of stomata index since *scap1-2* plants also have a significant reduction in pavement cells compared to wild type (Figure 2a, S2). To confirm these observations we generated two independent artificial microRNA (amiRNA1 and 2) constructs specifically targeting *SCAP1* in a wild type Columbia background (Figure S1). We isolated sixteen and fourteen independent T1 lines for *amiRNA1-SCAP1* and *amiRNA2-SCAP1*, respectively. Expression studies showed that T1 lines had reduced levels of *SCAP1* transcript abundance compared with wild type (Figure S2). Downregulation of *SCAP1* did not produce any obvious phenotypic effects on plant development, similar to *scap1-2* plants. Closer observations revealed that leaves of T2 and T3 *amiRNA-SCAP1* knock-down mutants produced significantly fewer GCs than wild type, analogous to the result observed in *scap1-2* (Figure 2b and S2). Interestingly, in the Col-0 background we also observed a reduction in both stomata density and index, suggesting that *SCAP1* plays a role in GCs specification alongside its general role in cell division (Figure 2b,f). A closely related gene to *SCAP1* is the floral organ abscission regulator *AtDOF4.7* (Wei *et al.*, 2010). To determine whether *AtDOF4.7* could act redundantly with *SCAP1* in GC formation we made double mutants of *amiRNA2-SCAP1* and *dof4.7* and analysed the stomatal phenotype. We found no alteration in GC density and index in the double mutant compared to wild type, indicating that *AtDOF4.7* does not act redundantly with *SCAP1* in stomata development (Figure S2).

We next wanted to ascertain whether *SCAP1* function was also sufficient in altering GC development. Several *SCAP1* overexpressing lines (n = 15) were obtained by fusing the *SCAP1* coding sequence to the *YELLOW FLUORESCENT PROTEIN* (*YFP*) gene under the control of the *CaMV35S* promoter. Different phenotypic classes were apparent in the T1 generation (Figure 2c). Plants classified as strong over-expressors of *SCAP1* (60%) exhibited numerous developmental defects including reduced germination, slow and stunted growth, upward-curling leaves and sterility. A second phenotypic class (40%) displayed a milder phenotype, exhibiting reduced growth compared with wild type at the seedling stage. These defects were milder at later stages of development so that these lines were eventually comparable in size to wild type. Given the strong phenotypic abnormalities in strong *SCAP1-YFP* overexpressing lines we analysed intermediate lines, more comparable to wild type in terms of final size and leaf area. Lines with intermediate levels of *pro35S:SCAP1-YFP* have an increased number of both GCs and pavement cells compared to wild type and this is accompanied by an overall increase in stomatal index (Figure 2d,f and S2). The epidermal phenotype of *pro35S:SCAP1-YFP* plants was characterised in more detail by crossing with a *proAtMYB60:GUS* reporter line, a GC specific marker (Cominelli *et al.*, 2005). This background allowed us to easily detect subtler GC patterning defects. The cotyledons of *pro35S:SCAP1-YFP* showed gross alterations in both stomata distribution and spacing between GCs which were clustered mainly at the edges of the cotyledon, especially on the adaxial surface (Figure 2e). However, no GC clusters were detectable in true leaves of *pro35S:SCAP1-YFP* plants (Figure 2e). Thus, *SCAP1* also plays an important role in determining GC positioning, most likely through the control of asymmetric cell divisions.

SCAP1 protein is differentially stabilised in different plant tissues

In the *pro35S:SCAP1-YFP* lines we found the accumulation of SCAP1-YFP protein was not ectopic in all plant tissues, despite the constitutive promoter regulation. When whole seedlings were

analysed a SCAP1-YFP protein–derived signal was noticeably absent in roots whereas control plants overexpressing soluble YFP showed an ectopic signal in all tissues (Figure 3a,f). We observed SCAP1-YFP accumulation in nuclei of mesophyll cells in developing leaves (Figure 3b,c), while very little, if any SCAP1-YFP signal was observed in the epidermis (Figure 3d). At later stages a strong signal was apparent in GCs, consistent with *SCAP1* playing a role in GC maturation (Figure 3e). Detailed analysis of *pro35S:SCAP1-YFP* cotyledons revealed that low levels of nuclear SCAP1-YFP protein were sometimes present in dividing cells, in close proximity with differentiated GCs (Figure 3 l, m).

Accumulation of SCAP1-YFP protein in the mesophyll was highest at the base of leaf primordia (Figure 3b). *SCAP1* promoter is active in the mesophyll and protodermal cells and its expression is more abundant in coincidence with the GC differentiation zone (Figure 1 b, g, j). These data suggest that SCAP1 protein is subject to a tissue specific turn over, so that, even if ectopically expressed, the pattern of SCAP1-YFP protein accumulation in leaves ultimately resembles the pattern of *SCAP1* gene expression.

SCAP1 **affects SPCH protein but not** ***SPCH*** **transcript accumulations**

The early activation of *SCAP1* in leaf primordia coupled with its role in stomata development led us to hypothesise a genetic interaction between *SCAP1* and stomata patterning genes. *SPCH* is an early stomata patterning gene expressed in the protodermal cells of leaf primordia. To determine the timing of *SCAP1* activation with respect to *SPCH* we sampled the first two leaf primordia from seedlings at different time points, representative of different stages of leaf development. Both *SCAP1* and *SPCH* transcript abundance peaked 7 days after sowing and decreased during the following 3 days (Figure 4a). After this time (at around 12 days after sowing), *SCAP1* expression levels subsequently reactivated, presumably in relation to GC formation in the maturing leaf (Figure 4a). Despite this temporal overlap we did not detect significant changes in *SPCH* transcript levels in

scap1 loss-of-function alleles, indicating that *SCAP1* does not regulate *SPCH* at the transcriptional level (Figure S3). In a complementary experiment, we crossed *scap1-2* (Ler) with *spch-4* (Col) mutants to obtain homozygous *spch* mutants carrying a transposon tagged version of *SCAP1*. Of 26 *spch* homozygous plants 2 displayed GUS staining that was similar in terms of pattern of expression to wild-type *SPCH* plants. The reduced frequency of this genotype could be due to genetic linkage since *SPCH* and *SCAP1* are physically close on chromosome 5. Nevertheless, *SPCH* is not required for the early *SCAP1* activation (Figure 4b).

If *SPCH* and *SCAP1* genetically interact we would predict that increased GC production in *pro35S:SCAP1-YFP* plants to be reflected in increased *SPCH* levels or activity. We did not observe alterations in *SPCH* transcript level in *pro35S:SCAP1-YFP* over expression lines, but did observe a general downregulation of *SPCH* when analysing a *SCAP1*-inducible allele (see below). No obvious changes in other stomata lineage genes such as *MUTE* or *FAMA* could be detected in *pro35S:SCAP1-YFP* lines (Figure S3). Among the gene products known to negatively regulate SPCH activity, only *EPF2* was consistently downregulated in two independent gain-of-function alleles of *SCAP1* (Figure S3). Downregulation of *EPF2* is usually associated with increased SPCH protein accumulation, which prompted us to test whether *SCAP1* could affect *SPCH* at the post-transcriptional levels (Engineer *et al.*, 2014).

An ectopic expression of *SCAP1* resulted in several developmental abnormalities, which could indirectly alter GC development. To avoid this potential problem, we generated a set of transgenic lines constitutively expressing *SCAP1* fused to the GLUCOCORTICOID RECEPTOR (*pro35S:SCAP1-GR*) (Aoyama and Chua, 1997). In this inducible system the fusion protein is normally localised in the cytosol but can shuttle to the nucleus upon application of DEXAMETASONE (DEX) to trigger a rapid SCAP1-dependent transcriptional activation. Also, unlike *pro35S:SCAP1-YFP*, *pro35S:SCAP1-GR* plants were phenotypically indistinguishable from wild type, despite accumulating high levels of *SCAP1* transcript (Figure S3).

We tested the ability of SCAP1:GR protein to activate expression of its target gene *AtMYB60* (Negi *et al.*, 2013). Indeed, *scap1* loss of function mutants displayed reduced levels of *AtMYB60* accumulation compared with wild type (Figure S4). Conversely, following a 1 h DEX treatment, *pro35S:SCAP1-GR proAtMYB60:GUS* plants showed increased GCs–specific GUS staining, indicating that the SCAP1-GR fusion protein is functional (Figure S4).

We used the *pro35S:SCAP1-GR* lines to investigate whether activation of SCAP1 could trigger an increase of SPCH protein accumulation by visualizing GREEN FLUORESCENT PROTEIN (GFP) fluorescence in a *proSPCH:SPCH-GFP* line after a DEX application. In control hemizygous *proSPCH:SPCH-GFP* plants no significant differences were found in the number of SPCH-GFP expressing cells following DEX treatment. Also, DEX did not alter the average nuclear SPCH-GFP fluorescent intensity, which ruled out a general effect of DEX on SPCH-GFP protein accumulation (Figure 4d, e). Similar observations could be made in mock treated *proSPCH:SPCH-GFP pro35S:SCAP1-GR* double hemizygous lines indicating that SCAP1-GR protein is highly inactive in the absence of an inducer (Figure 4c, d, e). However, after 6 hours following DEX treatment 50 to 80% of *proSPCH:SPCH-GFP pro35S:SCAP1-GR* plants (n = 6-8 independent 1st leaf primordia for each genotype/treatment combination analysed for each experiment) showed a significant increase in the proportion of nuclei expressing SPCH-GFP protein (Figure 4c,d). Also, this was accompanied with a general increase in the mean nuclear SPCH-GFP fluorescence intensity (n > 50 nuclei / 1st leaf primordia for each genotype/treatment combination) (Figure 4c,e). *SCAP1* may thus alter SPCH accumulation rapidly and affect stomatal density by promoting SPCH protein accumulation in undifferentiated PDCs.

DISCUSSION

Previously *SCAP1* was shown to control GC morphology and activity, a role coherent with its expression in developing and fully mature stomata (Negi *et al.*, 2013). Here we report an in-depth analysis of the spatio-temporal control of *SCAP1* expression throughout leaf development. Our results indicate an early activation of *SCAP1* expression in leaf primordia coinciding with the expression of the master stomatal regulator, *SPCH*. Furthermore, *SCAP1* gene expression is persistent in *spch* mutants demonstrating that *SCAP1* expression is independent of GC lineage specification. This is consistent with recent SPCH ChiPseq experiments which did not find *SCAP1* as a direct target of SPCH (Lau et al., 2014).

In addition to regulation at the transcriptional level, we provide strong evidence for a novel level of post-transcriptional regulation of *SCAP1*. Constitutively expressed *SCAP1-YFP* fusion did not accumulate in all plant tissues, despite high levels of ectopic expression (on average *SCAP1-YFP* was > 100 times more expressed than endogenous *SCAP1*). Interestingly, in leaf primordia SCAP1-YFP protein was mainly observed in the mesophyll and GCs and, at low levels, in dividing epidermal cells. The simplest explanation could be that SCAP1 protein is subject to a fast turn over in the epidermis. This observation may suggest that SCAP1 is not active in the epidermis and its role in GCs development is indirect (e.g. to promote signals from the mesophyll cells to the epidermis). Another possibility is that SCAP1 is only transiently acting in the epidermis and, more specifically, in dividing cells to orchestrate transcriptional events. The presence of SCAP1 protein in two neighbouring (and presumably dividing) cells or cells undergoing spacing divisions in close proximity to GCs favour a model of *SCAP1* promoting cell divisions. Similarly to SPCH, we could never observe SCAP1-YFP accumulation in the GCs lineage (i.e. meristemoid and GMC) (MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007). Overexpression of *SCAP1* results in a general increase in cell density and stomata index, reminiscent of *SPCH* overexpression (MacAlister *et al.*, 2007; Lampard *et al.*, 2008). In the context of GC lineage specification *SCAP1* may contribute to

SPCH action in promoting entry cell divisions. We propose that SCAP1 may either fulfil a single role of stabilising SPCH, which translates into an increased cell proliferation or have a dual role in both SPCH stabilization and cell cycle activation. Albeit at low levels, SCAP1-YFP protein was observed in dividing cells, which could suggest that SCAP1 protein stability is associated with cell division potential. Since *SPCH* plays a crucial role in maintaining cell self-renewing capacity it is tempting to speculate that SPCH could participate in controlling SCAP1 protein levels. In this view SCAP1 and SPCH may form a feed forward positive loop to fine tune cell division potential of protodermal cells. A key control of this loop could involve the *SCAP1*-mediated downregulation of *EPF2*. This model does not explain the presence of SCAP1 protein in mesophyll cells in emerging primordia, where *SPCH* is not expressed but where *SCAP1* promoter is transcriptionally active. Future experiments involving tissue/cell specific promoters may help elucidating the precise cell/tissue-specific pattern of SCAP1 stabilization and its physiological relevance.

DOF type factors have been hypothesised to play an important role in GCs maturation and function based on an enrichment of a DOF binding motif in GCs specific genes (Cominelli *et al.*, 2011; Galbiati *et al.*, 2008). It would be interesting to test whether this observation can be also extended to genes involved in the early events of GCs lineage specification. In this sense, *SCAP1* may link GC patterning and function.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

In this study we used *Arabidopsis thaliana* ecotypes Columbia (Col) and Landsberg *erecta* (L*er*). Seeds were germinated and plants grown in a controlled- environment cabinet at a temperature of 20°C to 23°C, 65% relative humidity, under long day conditions (16 h of light/8 h of dark). Light was cool-white fluorescent tubes (Osram; Sylvania) at a fluency of 120 to 150 µE (photosynthetically active radiation). The *scap1-2* allele is a transposon insertion (line GT-23689, is

in L*er* background) obtained from the Cold Spring Harbour gene trap collection (http://genetrap.cshl.edu). The T-DNA insertion allele in *AtDof4.7* was obtained from NASC and derives from the GABI KAT collection (line GK-384G11) (Rosso *et al.*, 2003). The *spch-4* knock out allele and the *proSPCH:SPCH-GFP proPIN3:PIN3-GFP* line were previously detailed (Robinson *et al.*, 2011; MacAlister *et al.*, 2007). The *proAtMYB60:GUS* line (Col background) was previously described (Cominelli *et al.*, 2005). The *proSCAP1:GUS-GFP*, *pro35S:amiRNA-SCAP1*, *pro35S:SCAP1-YFP* and *pro35S:SCAP1-GR* lines were generated in this study in wild–type Col background except for the *proSCAP1:GUS-GFP* which was in the L*er* background. Transgenic lines were obtained using the floral dipping method (Clough and Bent, 1998). Transgenic seedlings were selected on Murashige and Skoog (MS) media with kanamycin (50 µg/ml) (*pro35S:SCAP1-GR*) or Basta (25 µM) (*proSCAP1:GUS-GFP*, *pro35S:amiRNA-SCAP1, pro35S:SCAP1-YFP* and *pro35S:SCAP1-GR*). For each construct several T1 independent lines were generated and single insertion transgenic plants were isolated based on the segregation of resistance genes. Glucocorticoid applications were done by administering a solution of DEX (13 µM DEX, 0.01% (v/v) Tween 20) either by spraying (for expression analysis) or by soaking seedlings in a MS medium containing DEX (for confocal and GUS experiments). Stratified *pro35S:SCAP1-GR* seedlings were germinated on MS plates for 5 to 7 days before spraying with DEX or mock treated.

Molecular cloning

To generate the *proSCAP1:GUS-GFP* construct a 2977 bp region upstream of the *SCAP1* start codon was amplified from genomic DNA by PCR with oligos attB1-SCAP1 and attB2-SCAP1 which contain the *AttB* adaptors for Gateway–mediated cloning. The PCR product was cloned into *pDONR207* and subsequently transferred to *pBGWFS7* destination vector (Karimi *et al.*, 2002) according to the guidelines detailed in the Gateway protocol (Life Tecnhnologies). The *pro35S:amiRNA-SCAP1* constructs were engineered as detailed in http://wmd3.weigelworld.org

(Ossowski *et al.*, 2008) with primes I, II, III and IV. The PCR products containing *SCAP1*–specific amiRNA were cloned in the pENTR-DTOPO vector (Life Tecnhnologies) and transferred to the destination vector pEarleyGate 100 (Earley *et al.*, 2006) via LR–mediated recombination. To generate the *pro35S:SCAP1-YFP* the *SCAP1* open reading frame (without stop codon) was amplified by PCR from Arabidopsis DNA, with primers SCAP1-Fw, SCAP1-Re2 and cloned into the pENTR-D TOPO vector (Life Tecnhnologies) and recombined with the Gateway destination vector pEarleyGate 101 (Earley *et al.*, 2006). The DEX-inducible *SCAP1* construct (*pro35S:SCAP1-GR*) was kindly provided by the RIKEN Plant Functional Genomic http://pfgweb.gsc.riken.jp. Sequences of the primers are detailed in Supplementary Figure 5.

Genotyping and transcript analysis

Sequences of the primers used for genotyping are provided in Supplementary Figure 5. Total RNA was extracted with TRIzol reagent following the manufacturer's instructions (Life Technologies). The first-strand cDNA was synthesized with 500 ng of total RNA using SuperScript VILO Reverse Transcriptase kit (Life Technologies). Quantitative real-time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems), and amplification was real-time monitored on a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Changes in gene expression were calculated relative to *ACT2* using the ΔΔCt method (Livak and Schmittgen, 2001). The qPCR primers to detect *SCAP1*, *AtMYB60*, *SPCH*, *EPFL9*, *EPF1,2*, *MUTE*, *FAMA* and *ACTIN* transcripts are detailed in Supplementary Figure 5.

β -glucuronidase (GUS) histochemical assay and Histological procedures

GUS staining was performed as previously described (Galbiati *et al.*, 2008). Depending on the experiment, incubation time was for 4 to 12 h at 37°C. For detection of GUS staining in thin resin sections, after staining, samples were dehydrated in 70% (v/v) ethanol, post-fixed over night at 4°C

in FAE (50% [v/v] ethanol, 5% [v/v] formaldehyde, 10% [v/v] acetic acid), and further dehydrated in a series of 85%, 95% and 100% (v/v) EtOH and embedded in Technovit 7100 resin according to the manufacture's instructions (Heraeus Kulzer). Samples were sectioned with a microtome fitted with a stainless steel blade to a 7 μM thickness.

Microscopy and quantitative analysis of fluorescence emission

For analysis of the stomatal pattern, adult leaves of one-month-old plants were incubated in 70% ethanol. The epidermis of the abaxial side was peeled and examined under a transmission light microscope (DM2500, Leica). For determining the mean stomatal index and density, one square area (0,2 mm^2) of a leaf region was microphotographed and scored for cell parameters. Care was used to select a similar leaf region from the 6^{th} leaf from at least 12 independent plants for each genotype. For confocal laser scanning microscopy, the abaxial side of first leaf primordia of 5-days-old seedlings expressing GFP- or YFP-tagged proteins were analysed under a Leica TCS SP5 confocal microscope. Fluorochromes were excited using an Argon laser (488 nm and 514 nm excitation for GFP and YFP, respectively) and emission collected at a 500 – 570 nm and 525 – 600 nm for the GFP and YFP, respectively. When comparing independent samples, the acquisition parameters (including z step size) were maintained constant so to measure GFP intensity in different primordia images and different treatments. Image analysis was performed using the ImageJ software (http://imagej.nih.gov/ij/).

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1. Characterisation of the *scap1* and *Atdof4.1* mutant alleles

(a) Schematic representation of the *SCAP1* and *AtDof4.7* loci. Grey boxes represent amiRNAs target regions and triangles represent transposon or T-DNA genomic insertion points for *scap1-2* and *dof4.7*, respectively.

(b) Reverse Transcriptase-PCR analysis of *SCAP1* in wild type (Ler), *scap1-2,* and *AtDof4.7* in wild type (Col) and *dof4.7* plants. Total RNA was isolated from 2-week-old seedlings and PCR was conducted for 35 cycles. Actin was used as a positive control and amplified for 25 cycles.

Figure S2. *SCAP1* affects GCs development

(a) Representative abaxial epidermal phenotype of a mature leaf (the 6th leaf) of wild-type (Ler) and *scap1-2* mutants. Guard cells are false coloured in black. Scale bar = 50 μm.

(b) Number of Guard cells (GC), pavement cells (PC) and stomatal index in wild-type (Col), *dof4.7*, *pro35S:amiRNA2-SCAP1* (*amiRNA2-SCAP1*) and *dof4.7 pro35S:amiRNA2-SCAP1* double mutant plants. ** = P<0.01 two tails T Student test. Error bars = Standard Error.

(c) Pattern of *SCAP1* transcript accumulation determined by quantitative PCR in mature leaves in independent T1 *pro35S:amiRNA-SCAP1* (*amiRNA-SCAP1*) transgenic lines, compared with wild-type (Col). *ACTIN* (*ACT2*) was used for normalization. Values represent the mean of two technical replicates. Error bars = standard deviation.

(d) Number of Guard cells (GC), pavement cells (PC) and stomatal index in wild-type (Col) in wild-type (Col) or BASTA selected T2 *pro35S:amiRNA-SCAP1*(*amiRNA-SCAP1*) lines. A transgenic line transformed with empty vector (vector) was used as a further control to account for

BASTA treatment. Lines tested in this experiments are labelled in (c) with a filled arrowhead. Line #2, white arrowhead in (c), was not included in this particular experiment. All stomatal index values are significantly different from control ($P < 0.01$), except for line #11 which was not significant.

(e) Number of Guard cells (GC), pavement cells (PC) and stomatal index in wild-type (Col) or BASTA selected T2 *pro35S:SCAP1-YFP* (*35S:SCAP1*) lines. All cells density and stomatal index values are significantly different from control ($P<0.01$).

Figure S3. Role of *SCAP1* on stomatal genes transcript accumulations

Transcript accumulation of stomatal markers determined by quantitative RT-PCR.

(a, c, e-h) Transcript accumulation of *SPCH*, (a), *EPF2*, (c), *MUTE*, (e), *FAMA*, (f), *EPF1*, (g), and, *EPFL9*, (h) in wild-type (Col), *pro35S:amiRNA2-SCAP1 (amiRNA2-SCAP1)* and *pro35S:SCAP1-YFP (35:SCAP1)*. Total RNA was extracted from the first two-leaf primordia manually dissected at different developmental stages. Values represent the mean of three biological replicates (30 leaves / replica). Error bars = standard deviation.

(b, d) The mRNA levels of *SPCH*, (b) and *EPF2*, (d) in 10 days-old *pro35S:SCAP1-GR* or wild type plants treated by spraying with DEX or mock-treated and sampled at different time points. Values represent the mean of two biological replicates. Error bars = standard deviation.

Figure S4. *SCAP1* regulates *AtMYB60* expression

(a) *AtMYB60* accumulation determined by quantitative PCR in manually dissected first two-leaf primordia of wild-type or *scap1* seedlings at different time points. *ACTIN* (*ACT2*) was used for normalization. Values represent the mean of three biological replicates (30 leaves / replica). Error bars = standard deviation.

(b) *SCAP1* transcript accumulations determined by RT-PCR in *pro35S:SCAP1-GR* T1 lines. Total RNA was isolated from 2-week-old seedlings and PCR was conducted for 30 cycles. Actin was

used as a positive control and amplified for 25 cycles.

(c) GUS staining of double (*proAtMYB60:GUS pro35S:SCAP1-GR*) hemizygous lines. Shown are 2 weeks-old seedlings (inset, higher magnification). Bar = 1 mm (inset, 500μm).

Figure S5. Primers used in this study

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FIGURES LEGEND

Figure 1. *SCAP1* expression patterns in emerging leaves

(a to d) GUS staining of the *scap1-2* line and, (e to h), a representative *proSCAP1:GUS-GFP* transgenic line. Pictures were taken at different stages of leaf development from day 5 - 7 (a,b,e,f) to 10 (c,g,h) and 12 (d). (i,j) Transversal sections of leaf primordia of a young *proSCAP1:GUS-GFP* seedling at day 5, (i) and 7, (j). (l) GCs specific GUS staining at different maturation stages in

3-week-old 6th leaf. Bars = 50 μm (a,b,i,j); 500 μm (c,d) 100 μm (e,f,g,h) 1 mm (l).

Figure 2. *SCAP1* controls GCs development

(a) Number of Guard cells (GC), pavement cells (PC) and stomatal index in wild-type Ler, and *scap1-2* mutants and, (b), Col and a T3 homozygous *pro35S:amiRNA2-SCAP1* (*amiRNA2-SCAP1*, line #2).

(c) Morphological alterations observed in *pro35S:SCAP1-YFP* (*35S:SCAP1*) lines at different developmental stages (seedlings, rosette, bolting plants).

(d) Number of guard cells (GC), pavement cells (PC) and stomatal index in wild-type (Col) and a T3 homozygous *pro35S:SCAP1-YFP* (*35S:SCAP1*) intermediate line (line #7).

(e) GUS staining of double *proAtMYB60:GUS pro35S:SCAP1-YFP (35S:SCAP1)* or single *proAtMYB60:GUS* (WT Col) hemizygous lines. Shown are mature cotyledons (inset, higher magnification) and the first leaf of 10 days old seedlings. Bar = 200 μm (inset, 25 μm)

(f) Representative abaxial epidermal phenotype of a mature leaf (the 6th leaf) of wild-type (Col), *pro35S:amiRNA2-SCAP1* (*amiRNA2-SCAP1,* line #2) and *pro35S:SCAP1-YFP* (*35S:SCAP1,* line #7) mutants. Guard cells are false coloured in black. Bar = 50 μm.

In a,b,d ** = $P<0.01$ two tails T Student test. Error bars = Standard Error.

Figure 3. SCAP1 protein differentially accumulates in plant tissues

(a-e, l) Confocal images of *pro35S:SCAP1-YFP* (*35S:SCAP1-YFP*) and, (f-j), *pro35S:YFP* (*35S:YFP*) plants at different stages.

(a, f) Whole seedlings (5 dag). (b, g) Whole first leaf primordia (7 dag). (c, h) Mesophyll of the first leaf primordia (5 dag). (d,i) Epidermis of the first leaf primordia (5 dag). (e,j) GCs in a cotyledon (7 dag). (l) Epidermis of cotyledons (7 dag). Asterisks mark epidermal cells, arrows heads mark dividing cells. Images a, b, f and g are a montage of all the z stacks obtained across the entire

thickness of the sample. Images c, d, h and i are a montage of those z stacks corresponding to the mesophyll and the epidermis, respectively. Bars = 1mm (a,f), 200 μm (b,e,g), 50 μm (c-e, h-m). SCAP1-YFP/YFP protein signal is shown in yellow, autofluorescence (chlorophyll) in red.

Figure 4. *SCAP1* promotes SPCH protein but not transcript accumulations.

(a) Pattern of *SCAP1* and *SPCH* transcript accumulations determined by quantitative PCR in manually dissected first two leaf primordia of wild-type (Col) seedlings at different days after germination. *ACTIN* (*ACT2*) was used for normalization. Values represent the mean of three biological replicates (30 leaves / replica). Error bars = standard deviation.

(b) GUS staining of *scap1-2* in wild-type or *spch-4* mutant background in 5 day old seedlings. Bar = 100 μm

(c) Confocal images of hemizygous *proPIN3:PIN3-GFP proSPCH:SPCH-GFP pro35S:SCAP1-GR* transgenic plants. Shown is the first leaf of plants treated with DEX or mock-treated. Insets show a portion of leaf at higher magnification. Scale bar = 500 μm.

(d) Quantification of epidermal cells accumulating SPCH-GFP proteins in control (mock) or DEX-treated plants. Shown is the average total number of epidermal cells in 6-8 independent first leaf primordia (5 dag). This experiment was performed twice with similar results. Values were compared with one-way ANOVA. NS = not significant.

(e) Mean fluorescence intensity of SPCH-GFP protein nuclear accumulation in control (mock) or DEX treated plants described in (d).